Investigating the Genetic Causes of Primary Immunodeficiency and Autoimmunity

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ABSTRACT

Primary Immunodeficiencies (PIDs) are a heterogeneous collection of several hundred disorders, that have in common deficient or dysregulated immunity, leading to an increased susceptibility to infections, autoimmune disease, or to uncontrolled inflammation; in some PIDs there may be features of all three components. Understanding the pathogenesis of PIDs has led to insights into the immune system broadly, and this knowledge has aided improvements in the treatment of many more common infective and inflammatory diseases.

Many PIDs are monogenic, and advances in technologies that enable the interrogation of the genetic basis of these conditions, have led to a steep increase in the number of diseases now recognized as PIDs. In this project we aimed to establish a cohort of individuals with the most common form of PID, Predominantly Antibody Deficiency (PAD). We sought to characterize the diagnostic and clinical features of these individuals, and apply genomic sequencing methods for more precise diagnoses, as well as to identify new genetic etiologies of PAD.

One of the most striking findings regarding the clinical features of the patient cohort, was the morbidity associated with PADs. In particular we observed frequent complications of immune dysregulation, that manifest as autoimmune disease, particularly affecting the haematological system as cytopenias, or gastrointestinal tract as enteropathy, or malignancies. These complications are challenging to manage in the setting of PID, hence understanding the mechanism of disease is crucial to improving outcomes. In a group of PAD patients we identified monogenic causes of disease that led to some individuals receiving precision treatments.

In the realm of gene discovery, we identified several novel genetic variants, two in genes that had not previously been recognized as disease-causing in PAD. The first, *NFKB1*, is now recognized

as the most common genetic etiology of PAD. The importance of NF-κB signaling and its regulation was further highlighted by the discovery of other disease-causing variants in the NF-κB pathway. The effect of *NFKB2* mutations on the immune response was investigated in a kindred with two sisters affected by PAD, who demonstrated disparate clinical and immunological features. Further studies were performed in a mouse model of NF-κB2 deficiency, whereby B cell and $CD8^+$ T cell behaviours were studied in a quantitative manner, and revealed the cell-type specific effect of loss of this component on the adaptive immune response.

Finally, an entirely new monogenic PID was identified. In a young man with a clinical diagnosis of PAD, complicated by autoimmune disease, a homozygous, frameshift mutation in *NFKBID* was identified. The mutation was demonstrated to cause loss of expression of IκBNS, an inhibitor of the NF-κB pathway, and with that, dysregulated NF-κB signaling. The finding of a second patient with PAD and compound heterozygous variants in *NFKBID* has added further evidence that this is a bona-fide novel PID.

DECLARATION

This is to certify that:

i) this thesis contains only my original work towards the PhD, except where indicated in the preface,

ii) due acknowledgement has been made in the text to all the material used, and,

iii) the thesis is fewer than 100,000 words in length exclusive of tables, bibliographies and appendices.

PREFACE

The publication status of all chapters is as follows:

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Pitfalls of immunotherapy: lessons from a patient with CTLA-4 haploinsufficiency.
Watson LR, **Slade** CA, Ojaimi S, Barnes S, Fedele P, Smith P, Marum J, Lunke S, Stark Z, Hunter MF, Bryant VL, Low MSY. Allergy Asthma Clin Immunol. 2018 Oct 22; 14:65

Epistatic interactions between mutations of TACI (*TNFRSF13B*) and *TCF3* result in a severe primary immunodeficiency disorder and systemic lupus erythematosus.
Ameratunga R, Koopmans W, Woon ST, Leung E, Lehnert K, **Slade** CA, Tempany JC, Enders A, Steele R, Browett P, Hodgkin PD, Bryant VL. Clin Transl Immunology. 2017 Oct 20; 6(10):e159.

Review: Diagnosing Common Variable Immunodeficiency Disorder in the Era of Genome Sequencing. Ameratunga R, Lehnert K, Woon ST, Gillis D, Bryant VL, **Slade** CA, Steele R. Clin Rev Allergy Immunol. 2018 Apr; 54(2):261-268.

Chemokines, their receptors and human disease: the good, the bad and the itchy.
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some of the work presented in chapters 3 - 6 was collaborative, specifically:

Chapter 3: This publication was a collaboration across a number of sites. I estimate that I contributed more than 55% of the data collation, analysis and interpretation, and wrote the paper with significant contributions from Julian Bosco, and oversight from Vanessa Bryant and Menno van Zelm. All other authors identified patients and provided clinical information.

Chapter 4: Patients were recruited, and consent was obtained by genetic counsellors at each participating site. Sequencing was performed at AGRF, and bioinformatics and curation at VCGS. I was involved in the prioritisation and curation of genetic variants in all patients, and performed the analysis of the genomic findings. Overall, my contribution was 75% of this chapter.

Chapter 5:
Part 1: Immunophenotyping was performed by Drs S. Megaloudis and J. Tempany. Post-mortem analysis was performed by Drs C. McLean, and C. D'Arcy. My contribution was 80% of the publication.
Part 2: Experiments were planned, analysed and interpreted in collaboration with Philip D Hodgkin and Vanessa L. Bryant. Modeling was performed by HoChan Cheon. My contribution to this part of the Chapter was 90%.

Chapter 6: Dr S. Juneja performed histological studies. Dr M. Rashidi provided assistance with western blots. My contribution was 90% of the chapter.

LIST OF ABBREVIATIONS

ACMG – American College of Human Genetics
ACTH – adrenocorticotrophic hormone
Ag – antigen
AGRF – Australian Genomics Research Facility
ANCA – anti neutrophil cytoplasmic antibodies
APDS – activated PI3kinase delta syndrome
APECED – autoimmune polyendocrinopathy, enteropathy, candidiasis and ectodermal dystrophy
APRIL – A proliferation inducing ligand
ARD – ankyrin repeat domain
ASC – antigen secreting cell

BAFF – B cell activating factor
BAFF-R - B cell activating factor receptor
Bcl2l1l - B cell lymphoma-2 like protein 1 l
BCMA – B cell maturation antigen
BCR – B cell receptor
BrdU - bromodeoxyuridine

C_H- constant region of immunoglobulin heavy chain
C_L- constant region of immunoglobulin light chain
CADD – combined annotation dependent depletion
CC – colon cancer
CD – cluster of differentiation
CD – celiac disease
CD40L – CD40 ligand
CFSE – carboxyfluorescein succinimidyl ester
CID – combined immunodeficiency
CGD – chronic granulomatous disease
CHAI – CTLA4 haploinsufficency, autoimmune disease
CHARGE – coloboma, heart defects, choanal atresia, growth retardation, genital and ear abnormalities
ClinVar – clinically relevant variants
CMC – chronic mucocutaneous candidiasis
CMMRD - constitutional mismatch repair deficiency
CMV – cytomegalovirus
COX - cyclooxygenase
CRP – C-reactive protein
CS – Childhood Syndromes
CSIRO – Commonwealth
CTV – cell trace violet
CVID – common variable immunodeficiency

D – diversity region of immunoglobulin
DAVID – deficient anterior pituitary with variable immunodeficiency
DCM – dilated cardiomyopathy
DD – death domain
DD - division destiny
DN – dominant negative
DNA – deoxyribonucleic acid

EBER – Epstein Barr encoding region
EBV – Epstein Barr virus
ED – ectodermal dystrophy
EDA-ID - ectodermal dystrophy, anhydrosis, immunodeficiency
EDTA – ethylenediaminetetraacetic acid
ELISA – enzyme-linked immunosorbent assay
ENU - N-ethyl-N-nitrosourea

FA – frequency activated

FACS – fluorescence-activated cell sorter
Fc – crystallisable fragment
FCS – fetal calf serum
FITC – fluorescein isothiocyanate
FSC – forward scatter
FSC-A – forward scatter area
FSC-H – forward scatter height

GATK – genome analysis toolkit
GC – germinal centre
GOF – gain of function
GN - glomerulonephritis
gnomAD – genome aggregation database
GWAS – genome-wide association studies

HAE – hereditary angioedema
HC – healthy control
HEK293 – human embryonic kidney 293 cells
HIES – hyper-IgE syndrome
HLA – human lymphocyte antigen
HNPCC - hereditary non-polyposis colon cancer
HPV – human papilloma virus
HREC – human research ethics committee
HRP – horseradish peroxidase

IBD – Inflammatory Bowel Disease
ICOS – inducible co-stimulator
ICON – International Consensus
IEI – Inborn errors of immunity
IFN - interferon
Ig – immunoglobulin
IκBα – nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
IκBNS – nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, not specified
IKKα/ß – nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor kinase alpha/beta
IL – interleukin
IL21 – interleukin-21 receptor
IL-2Rα– interleukin-2 receptor alpha
IUIS –International Union of Immunological Societies

J – joining region of immunoglobulin

LATAIE – LRBA deficiency with autoantibodies, T reg cell defects, autoimmune infiltration, enteropathy
LOF – loss of function
LOVD – Leuven online variant database
LPS – lipopolysaccharide
LUBAC - linear ubiquitin chain assembly complex

MAF – mean allele frequency
MBL – mannose-binding lectin
MCRI – Murdoch Children's Research Institute
MDN – mean division number
MDS – myelodysplastic syndrome
MHC – major histocombatability complex
MMR – mismatch repair
MOPS – 3-(N-morpholino)propanesulfonic acid
MS – multiple sclerosis
MSH – MutS protein homolog

NaCl – sodium chloride
NATA – National Association of Testing Authorities
NEMO - NF-κB essential modulator

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
NLS – nuclear localisation sequence
NMD – nonsense-mediated decay
NZB – New Zealand Black
NZW – New Zealand White

PAD – predominant antibody deficiency
PAGID – Pan-American Group for Immunodeficiency
PASLI - p110δ-activating mutation causing senescent T cell, lymphadenopathy, and immunodeficiency
PEST – peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T)
PEX – plasma exchange
PHA – phytohaemagluttinin
PI – propidium iodide
PID – primary immunodeficiency
PBMC – peripheral blood mononuclear cell
PBS – phosphate buffered saline
PMA – phorbol 12-myristate 13-acetate
PMS2 – postmeiotic segregation increased 2
PolyPhen – polymorphism phenotyping

RHD – Rel homology domain
RPMI – Roswell Park Memorial Institute

SAID – systemic autoinflammatory disease
SAP – SLAM-associated peptide
SCID – severe combined immune deficiency
SDS – sodium dodecyl sulphate
SHM – somatic hypermutation
SIFT – sorting intolerant from tolerant
SLE – systemic lupus erythematosus
SNP - single nucleotide polymorphism
SNP-ID – single nucleotide polymorphism identification
STAT – signal transducer and activator of transcription

% v/v – percent volume/volume

TACI - transmembrane activating calcium modulator and cyclophylin ligand interactor
TMB – tetramethylbenzidine
TCR – T cell receptor
Th – T helper cell
Tfh – T follicular helper cell
Treg – regulatory T cell
T1DM – Type 1 Diabetes Mellitus
TLR – toll-like receptor
TNF – tumor necrosis factor
TNFRSF – tumor necrosis factor superfamily

V- variable (region of immunoglobulin)
VCF – variant call file

WEHI – Walter and Eliza Hall Institute of Medical Research
WES – whole exome sequencing
WHIM – warts, hypogammaglobulinemia, infections and myelokathexis

XIAP – X-linked inhibitor of apoptosis
XLA – X-linked agammaglobulinemia
XLP1 – X-linked lymphoproliferative syndrome, type 1
XLP2– X-linked lymphoproliferative syndrome, type 2

LIST OF GENES

AID – activation induced deaminase
AIRE – autoimmune regulator
BACH2 – BTB domain and CNC homolog 2
BLNK – B cell linker
BTK – Bruton tyrosine kinase
CTLA4 – cytotoxic T lymphocyte associated protein, 4
FANC – Fanconi anemia complementation group
FOXP3 - forkhead box protein 3
GATA2 – GATA-binding factor 2
IRF2BP2 – interferon regulatory factor 2 binding protein 2
IZKF1 – IKAROS
LRBA – LPS responsive beige anchor
NFKBID - nuclear factor kappa-light-chain-enhancer of activated B cells, inhibitor delta
PIK3CD – phosphoinositide-3-kinase delta catalytic subunit
PIK3CR – phosphoinositide-3-kinase delta regulatory subunit
PLCG2 – phospholipase gamma 2
PRKCD – protein kinase C, delta
RAG – recombinase activating gene
SEC61A – SEC61 complex, alpha subunit
TRNT1 – tRNA nucleotidyltransferase, CCA-adding 1
TCF3 – transcription factor 3
TNFRSF2 – tumor necrosis factor superfamily member 2 (TWEAK)
TNFRSF12 – BAFF-receptor
UNG – uracil-n-glycosylase

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Primary immunodeficiency diseases arise due to defects of the immune response

In a healthy individual the immune system serves to protect its host from threats of invading pathogens, and potentially dangerous 'self', such as infected or cancerous cells, whilst maintaining tolerance to normal 'self' tissues. It performs these functions by the tightly regulated development of many cell types and their subsequent coordinated activation, trafficking and interactions with each other, and their targets.

Primary immunodeficiency disease (PID) occurs when one or more components of this tightly regulated system becomes defective, leading to an increased susceptibility to infections, or the development of autoimmune, autoinflammatory and malignant complications, as a result of either inappropriate activation of the immune system, or a disordered immune response to pathogens, self, or both (Bousfiha, Jeddane et al. 2018). Since the first PID was described in 1957, there are now more than 350 PIDs reported by the International Union of Immunological Societies' Inborn Errors of Immunity Committee (IUIS, IEI), with a wide spectrum of clinical features and pathogenic mechanisms (Picard, Bobby Gaspar et al. 2017, Bousfiha, Jeddane et al. 2018). PIDs are individually rare, yet collectively common, with an overall population frequency estimated at approximately 1 in 1200 in the US (Boyle and Buckley 2007) but the prevalence varies depending on the population studied, and in many instances is likely to be under-diagnosed due to variability of clinical phenotypes and awareness amongst clinicians (Kobrynski, Powell et al. 2014). These are chronic conditions and require high cost health care resources; replacement intravenous immunoglobulin for antibody deficiency costs between AU$60, 000-$120, 000/patient/year (National Blood Authority, 2017). In many cases, PIDs are associated with reduced life expectancy, unless treatment is initiated early in the course of disease (Resnick, Moshier et al. 2012, Pai, Logan et al. 2014). Effective treatments are available for many of these conditions, for example,

haematopoietic stem cell transplant (HSCT), or targeted immunomodulatory therapies. However, selection of the appropriate therapy requires an accurate, and timely, diagnosis.

In vertebrates, the immune system is typically viewed as consisting of two major components: the innate and the adaptive immune systems. This distinction is a significant oversimplification, because the two arms do not function independently. The innate immune system, which is also common to invertebrates, and evolutionarily more ancient, characteristically provides the first line of immune defence, independent of any prior exposure or learning (reviewed in (Janeway and Medzhitov 2002)). Recognition of a potential pathogen occurs via receptors that sense molecular patterns common to bacterial or viral species, as well as other potential dangers, such as signs of cellular damage (Kumar, Kawai et al. 2011). Defects in these innate components may lead to susceptibility to specific infections or may manifest as inflammation in the absence of overt infection, known as Systemic Autoinflammatory Diseases (SAIDs) (reviewed in, (Moghaddas and Masters 2015)). Thus, SAIDs should also be considered PIDs, and increasingly the overlap between autoinflammation and immunodeficiency is being recognised, as many manifest both infectious susceptibility and autoinflammation.

In contrast to the innate immune system, the adaptive system characteristically has a slower initial onset of response, but the ability to retain memory of an exposure, and to adapt to create a more specific and rapid response on subsequent encounters. These hallmarks of the adaptive response, namely, specificity and memory, are due to the function of two major cell types, B and T lymphocytes, as well as the differentiation of B cells to antibody secreting cells (ASCs) under the influence of T cells (reviewed in (Bonilla and Oettgen 2010)). The major focus of this thesis is the adaptive immune response. In particular, defects of the humoral immune response, as manifest in patients with Predominantly Antibody Deficiencies (PADs), although the distinction between

innate and adaptive defects is not always clearly defined due to the interdependence of the two systems.

1.1.1 The discovery of humoral immunity and immune memory

Observations and theories about immunity had existed for hundreds of years, prior to experimental evidence provided by British physician, Edward Jenner, in the late 1700s. He observed that milkmaids with previous exposure to the cowpox infection, vaccinia, were resistant to smallpox infection, which at the time was a major cause of mortality. With the assistance of a young subject, James Phipps, he demonstrated that inoculation using an extract from cowpox pustules provided protection against infection from smallpox (Barquet and Domingo 1997). Almost a century later, Louis Pasteur developed specific vaccines against cholera, and rabies (Pasteur, Chamberland et al. 1881, Pasteur, Chamberland et al. 1881). Emil Von Behring and Baron Kitasato corroborated the theory that the protection derived from vaccines was due to a circulating substance in serum, as demonstrated by passive immunity, and von Behring was awarded the inaugural Nobel Prize in 1901 for this discovery (Grundbacher 1992).

The puzzle of how diverse antibody specificities are generated was further theorised and tested in the early to mid-1900s by eminent immunologists including Paul Ehrlich (Strebhardt and Ullrich 2008), and Niels Jerne. However, it was renowned Australian immunologist, Sir Francis MacFarlane Burnet, who came the closest to predicting the solution to this puzzle, with his Clonal Selection Theory (Burnet 1957). He correctly predicted that the generation of antibody specificity occurred by a process of random recombination of germline-encoded antigen receptor genes, thus accounting for the impressive diversity and adaptability of the response. Due to the number of potential specificities, the receptor of best fit would be ‘selected’ by its interaction with the antigen, leading to clonal proliferation and production of highly specific antibody. Evidence to support this

theory was subsequently provided experimentally by demonstrating that single cells responded specifically to a single antigen, in the case of immunisation against different species of *Salmonella* (Nossal and Lederberg 1958), confirming the 'one cell, one antibody' theory underpinning humoral immunity.

1.1.1.1 HUMORAL IMMUNITY DEPENDS ON THE PRESENCE OF TWO DISTINCT LYMPHOCYTE TYPES, B CELLS AND T CELLS, AND IS CHARACTERISED BY A STEREOTYPICAL RESPONSE TO ANTIGEN EXPOSURE

Although Nossal had demonstrated antigen specificity of individual lymphocytes, the nature of the antibody-secreting lymphocytes was not identified until a decade later. In 1968, Miller and Mitchell showed that, although the cells from murine thymus or thoracic duct (thus named, T cells) were required for a competent antibody response, they were not themselves responsible for producing antibody (Mitchell and Miller 1968). After the identification of the existence of the two populations of lymphocytes, they were also able to demonstrate that these distinct lymphocytes, namely B and T cells, interact in order to achieve a humoral immune response (Mitchell and Miller 1968). Interestingly, the first reported PID, X-linked agammaglobulinaemia (XLA), had already been described by Bruton a decade earlier (Bruton 1957). In a young boy with a history of multiple episodes of pneumococcal sepsis, Bruton demonstrated the lack of any antibody response to vaccination with multi-valent pneumococcal vaccines, and an absent response to diphtheria toxoid (Bruton 1957). Furthermore, the therapeutic benefit of immunoglobulin replacement was also first established in this case report.

Further extensive work has shown that the normal humoral response to a pathogenic insult, or vaccine, is characterised by a primary response of immunoglobulin M (IgM) secretion, within days of the exposure. Upon a second exposure, the production of Ig occurs with a shorter lag phase, and due to the process of class-switch recombination, is predominantly of the IgG isotype (Coffman

and Cohn 1977). This stereotypical response relies on the immune system's ability to recognise and present a massively diverse array of antigens to appropriately engage other effector cells. Specialised antigen presenting cells, such as dendritic cells and macrophages, play a critical role in initiating the innate immune response, but are also instrumental in guiding the adaptive immune response (reviewed in (Walsh and Mills 2013), however here I will focus largely on the role of B and T lymphocytes in the generation of antibody.

Immunoglobulins

Immunoglobulins (Ig) exist as either membrane bound molecules on the surface of B cells, where they form an integral component of the BCR, acting to recognise and respond to antigen; or are secreted by ASCs after those cells have differentiated from mature B cells. The basic structure of each Ig molecule is similar: 2 identical heavy chains are bound together by disulfide bonds, with a central hinge region, forming a Y-shaped structure. Adjacent to the 'upper limbs' of the Y are 2 identical light chains. Heavy chains are composed of 3 constant (C_H) and one variable region (V_H), whereas lights chains have one constant (C_L) and one variable region (V_L) (Milstein 1986). The variable regions are so-called because they are responsible for the generation of antigen specificity; rearrangement of these regions from minigene segments first occurs in developing B cells in the bone marrow to generate the enormous diversity of antigenic specificity. It also occurs during B cell clonal proliferation in secondary lymphoid tissue, via a process referred to as somatic hypermutation (Berek and Milstein 1988). Somatic hypermutation leads to the development of antibody of high affinity for its target, and is crucial for the development of affective adaptive immunity.

Immunoglobulin isotypes, so-named according to the use of the heavy chain (μ, γ, α, ε or δ), have differing structures and functional capabilities (reviewed in (Davies and Metzger 1983, Matthews, Zheng et al. 2014)). IgM, the first responder, typically circulates as a pentamer, thereby having

high avidity for its target, and is a powerful activator of the classical complement pathway. IgG, the most abundant isotype in human serum, circulates as monomers, typically with higher affinity for the cognate antigen. In humans, four IgG subclasses are secreted by ASCs, namely IgG1, IgG2, IgG3 and IgG4, and are induced by different signals (Matthews, Zheng et al. 2014). IgA is the isotype most abundant at mucosal surfaces, where it is secreted as a dimer, although in serum it circulates as a monomer. IgE is expressed on the surface of mast cells and basophils, but present only in trace amounts in serum and is the key protective response against parasitic infection. However, in non-endemic areas is of most relevance in allergic disease (Maurer, Ebner et al. 1995). IgD is the least abundant isotype in serum. Although it is normally expressed on the surface of naïve B cells, its role remains undefined and its deficiency appears to have no significant impact on B cell development in humans (Nechvatalova, Bartol et al. 2018).

The nature of the antigen also determines the outcome of the antibody response, as well as the involvement of other cells: protein antigens are processed and presented to cognate B cells by T cells, thereby generating a T-dependent immune response whereas polysaccharide antigens can stimulate a B cell directly, hence generating a T-independent response (Vos, Lees et al. 2000). These responses will be discussed in the next section in the context of Predominantly Antibody Deficiencies (PADs).

1.1.1.2 VACCINATION AND ANTIBIOTICS LED TO REDUCTION IN MORTALITY DUE TO INFECTIOUS DISEASE, AND REVEALED THE EXISTENCE OF PIDS

Vaccines have now led to the eradication of smallpox, and substantially reduced the incidence of many previously common infectious diseases (Lopez, Mathers et al. 2006, Lopez, Mathers et al. 2006). However, most individuals with PID do not mount sufficient antibody responses to vaccines, and are therefore not protected like healthy individuals. In fact, it is only as a result of the diminished overall burden of infectious disease, due to vaccines and antibiotic use, that the

existence of individuals with PIDs has been revealed: previously these individuals would likely have succumbed to infection without suspicion of an underlying defect, due to the high background prevalence of infection, and associated high overall mortality due to infectious disease. This is especially pertinent to the group of individuals with PADs.

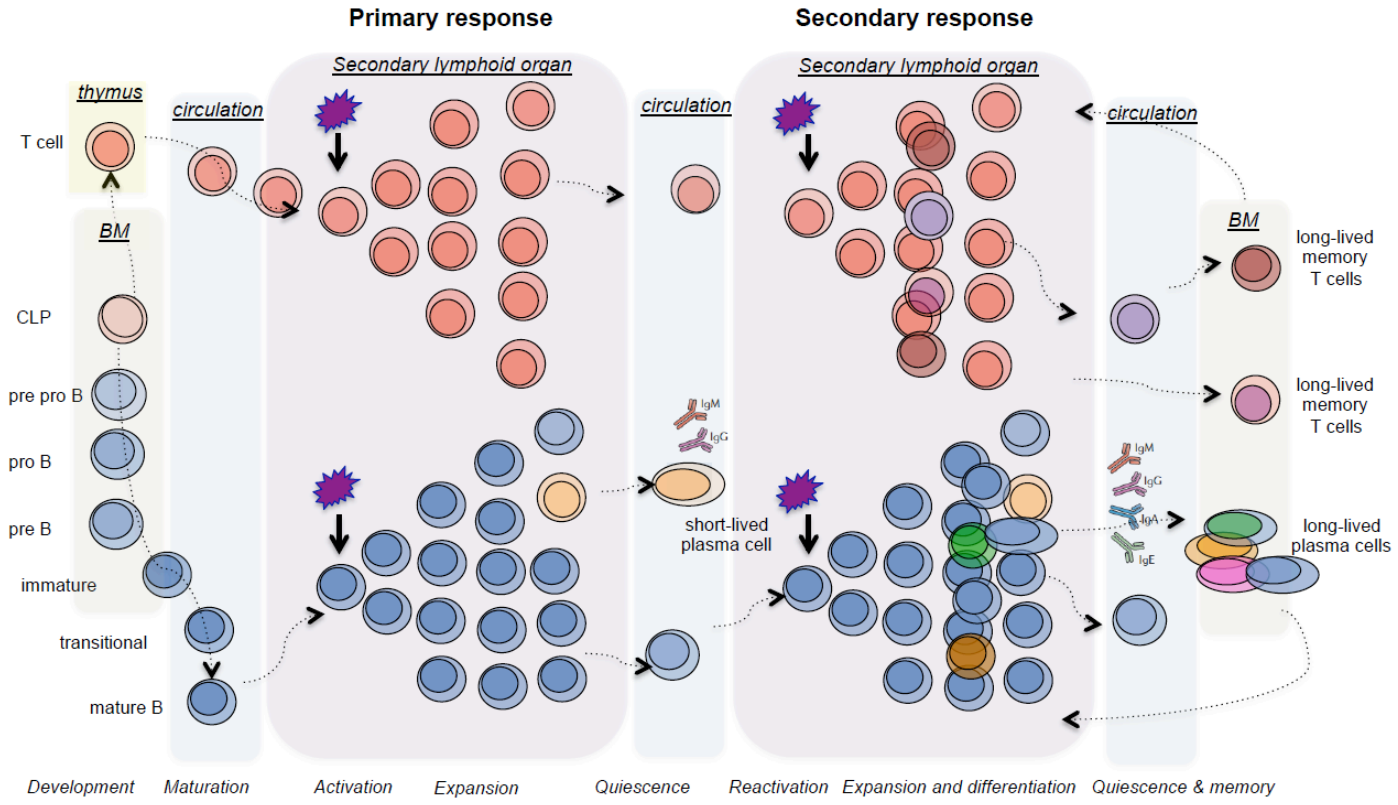
1.2 The spectrum of PIDs: from Predominantly Antibody Deficiency (PAD) to Severe Combined Immunodeficiency (SCID)

Diagnosis of PIDs is made by interpretation of the patient's clinical features together with results of laboratory investigations aimed at identifying the component/s of the immune system that is/are defective (Bousfiha, Jeddane et al. 2018). Infections are usually the major presenting feature: viral, fungal and other opportunistic infections are suggestive of T cell defects and collectively referred to as combined immunodeficiency (CID). The most severe form of PID, severe combined immunodeficiency (SCID), in the absence of a population screening, or predictive genetic diagnosis, typically presents in the first few months of life with failure to thrive, due to severe diarrhea; opportunistic infections, such as *Pneumocystis jiroveci* pneumonia, or overwhelming viral infections. Timely treatment for SCID has been shown to be crucial for reducing mortality. Typically the disorder is fatal if untreated, whereas haematopoietic stem cell transplantation (HSCT) in the first months of life, prior to serious infection, has a greater than 95% long-term survival (Pai, Logan et al. 2014).

By comparison, the more common PADs may present at any age, typically with recurrent or severe bacterial infections, in addition to other complications, such as autoimmune disease (Bousfiha, Jeddane et al. 2018). Patients with PAD are also at an increased risk of malignancy, particularly of gastrointestinal and lymphoid origin. Furthermore, whilst SCID may be now considered a curable disease, PADs are chronic, require life-long treatment, and cause significant morbidity and mortality. The increased mortality is primarily due to the organ damage of late effects of recurrent

infections during the period prior to diagnosis, as well as complications from immune dysregulation (Resnick, Moshier et al. 2012, Slade, Bosco et al. 2018).

A.


Primary response
Secondary response
Secondary lymphoid organ
Secondary lymphoid organ
thymus
circulation
circulation
circulation
T cell
BM
BM
CLP
pre pro B
pro B
pre B
immature
transitional
mature B
IgM
IgG
short-lived
plasma cell
IgM
IgG
IgA
IgE
long-lived
memory
T cells
long-lived
memory
T cells
long-lived
plasma cells
Development
Maturation
Activation
Expansion
Quiescence
Reactivation
Expansion and differentiation
Quiescence & memory

B.

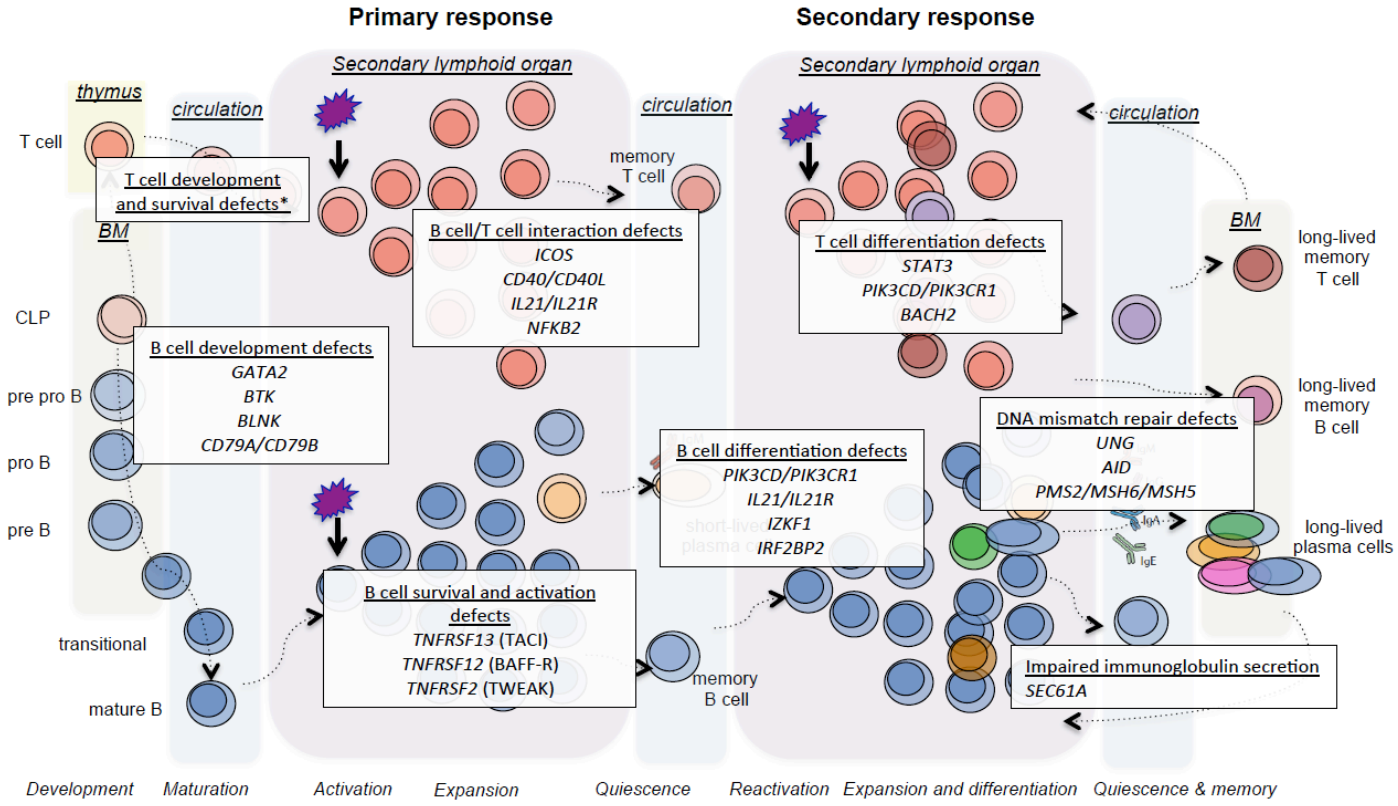

Primary response
Secondary response
Secondary lymphoid organ
Secondary lymphoid organ
thymus
circulation
circulation
circulation
T cell
T cell development
and survival defects*
memory
T cell
BM
BM
B cell/T cell interaction defects
ICOS
CD40/CD40L
IL21/IL21R
NFKB2
T cell differentiation defects
STAT3
PIK3CD/PIK3CR1
BACH2
long-lived
memory
T cell
CLP
B cell development defects
GATA2
BTK
BLNK
CD79A/CD79B
pre pro B
pro B
pre B
B cell differentiation defects
PIK3CD/PIK3CR1
IL21/IL21R
IZKF1
IRF2BP2
DNA mismatch repair defects
UNG
AID
PMS2/MSH6/MSH5
long-lived
memory
B cell
IgA
IgE
long-lived
plasma cells
B cell survival and activation
defects
TNFRSF13 (TACI)
TNFRSF12 (BAFF-R)
TNFRSF2 (TWEAK)
transitional
mature B
memory
B cell
Impaired immunoglobulin secretion
SEC61A
Development
Maturation
Activation
Expansion
Quiescence
Reactivation
Expansion and differentiation
Quiescence & memory

FIGURE 1.1 THE HUMORAL IMMUNE RESPONSE IS DEPENDENT ON MANY PROCESSES, AND GENETIC DEFECTS IN THESE PROCESSES CAN LEAD TO PREDOMINANTLY ANTIBODY DEFICIENCIES.

(A). Figure depicts a simplified view of B and T cell development, activation, and differentiation, during a primary and secondary immune response. Dashed arrows depict maturation sequence of B cells (blue) within the bone marrow, migration of T cell precursors (red) from bone marrow to thymus, and subsequent circulation of mature naïve T and B cells to the periphery. Magenta symbols depict an unspecified pathogen, leading to activation and clonal expansion of naïve B and T cells in a primary immune response, with subsequent contraction of the response. The secondary response is also characterised by clonal expansion and contraction of the response with additional characteristics of isotype switching and differentiation to memory cells and plasma cells that secrete immunoglobulin into circulation. Long-lived memory and long-lived plasma cells remain in BM niches. **(B).** Figure displays examples of pathogenic genes affecting each of the processes depicted in A. (*, mutations affecting T cell development and survival cause SCID, or CID, typically having a more severe clinical consequence than PAD; *BM, bone marrow; CLP, common lymphoid progenitor*).

1.2.1 The Pathogenesis of Predominantly Antibody Deficiencies is complex

PADs, which are characterised by an inability to generate a diverse, long-lived functional antibody response, may arise as a result of a single fault, or multiple defects in one of the following processes along the pathway of B cell development and differentiation (Figure 1.1), namely defects of:

- B cell development, or survival
- B cell activation (both B cell intrinsic and extrinsic defects).
- Differentiation to ASCs (both B cell intrinsic and extrinsic defects).
- Isotype switching and/or somatic hypermutation.
- Trafficking of B cells and ASCs

1.2.1.1 DEFECTS IN B CELL DEVELOPMENT, OR GENERATION OF THE B CELL RECEPTOR LEAD TO AGAMMAGLOBULINEMIA

B cell development is characterised by the sequential expression of proteins involved in development of a mature B cell receptor (BCR) via a process of somatic recombination of germline-encoded variable (V) region mini-genes (Milstein 1986). Early B cell development occurs in the bone marrow, from a common lymphoid progenitor (Figure 1.1). Defects in the development of these precursors may lead to hypogammaglobulinemia, but are accompanied by additional cytopenias, as is seen in GATA2 deficiency (Dickinson, Griffin et al. 2011, Hsu, Sampaio et al. 2011). An absence or reduction in circulating B cells results in a complete or near complete absence of Ig of any isotype, agammaglobulinemia, and can be identified with basic immunophenotyping of peripheral blood mononuclear cells (PBMCs) (Ma 2019).

B cells undergo a series of checkpoints to test for the development of a functional antigen receptor. Expression of the trans-membrane receptor, CD19, occurs at the earliest stage of B cell commitment, the pro-B cell, and is constitutively expressed throughout its development. CD19

deficiency does not typically lead to a complete absence of B cells, but does lead to hypogammaglobulinemia due to impaired B cell signaling and responses to antigen (van Zelm, Reisli et al. 2006).

Surface expression of the rearranged μ-heavy chain marks the transition from pro-B to pre-B cell, with expression of a surrogate light chain, ψ-LC prior to light chain rearrangement (Gathings, Lawton et al. 1977). Appropriate rearrangement of the μ-HC and effective co-expression with the ψ-LC, is the first checkpoint in B cell development. If functionality of the receptor is confirmed, the cell survives and undergoes further development to become an immature B cell, identifiable by surface expression of a κ or λ light chain.

In humans, mutations in genes encoding Ig proteins or downstream signaling molecules of the BCR that are essential for this checkpoint, lead to antibody deficiencies, that typically present as agammaglobulinemia and complete or partial B cell lymphopenia (Figure 1.1B) (Bernier, Gunderman et al. 1972, Stavnezer-Nordgren, Kekish et al. 1985, Rohrer, Parolini et al. 1994, Yel, Minegishi et al. 1996, Minegishi, Coustan-Smith et al. 1999, Minegishi, Rohrer et al. 1999, Boisson, Wang et al. 2013). For example, the protein tyrosine kinase, Bruton tyrosine kinase (Btk), and the scaffold protein BLNK are downstream signaling components essential for pre-B cell survival. Defects in *BTK* are the most common cause of agammaglobulinemia, resulting in an X-linked disease (OMIM #300755) (Tsukada, Saffran et al. 1993, Vetrie, Vorechovsky et al. 1993), whereas BLNK deficiency is a recessive condition (OMIM #613502) (Minegishi, Rohrer et al. 1999).

Expression of transcription factors, E2A, Ebf and Pax5, in pro-B cells regulate the expression of genes essential for BCR rearrangement and thus, B cell development (Nutt, Heavey et al. 1999, Kwon, Hutter et al. 2008). These genes include the recombinase-activating genes 1 and 2 (*RAG1*

and *RAG2*, respectively), which initiate recombination of Ig receptor genes. The process of somatic recombination of the 100s of germline-encoded variable (V), diversity (D) and joining (J) mini-genes, generates enormous possible combinatorial outcomes, and accounts for most of the extreme diversity of antibody responses (Milstein 1986). The recombination process also accounts for TCR diversity, but only B cells diversify rearranged V-region genes by somatic hypermutation. In humans, mutations in *TCF3,* encoding E2A (OMIM #616941) (Boisson, Wang et al. 2013), lead to a significant reduction, or absence of B cells, whereas *RAG1* and *RAG2* mutations also cause loss of T cells since they are involved in the generation of a functional TCR (OMIM #601457) (Schwarz, Gauss et al. 1996). Interestingly, the spectrum of RAG-associated disease now extends to later onset immune deficiency associated with autoimmunity and granulomatous inflammation (OMIM #233650) (Buchbinder, Baker et al. 2015, Walter, Rosen et al. 2015, Lawless, Geier et al. 2018).

Additional forms of SCID and CIDs occur due to an inability to rearrange the BCR and TCR, due to defects in DNA repair mechanisms, such as DNA ligase IV deficiency (OMIM #601837), Cernunnos (OMIM #611290) and Artemis deficiency (OMIM #605988) (Picard, Bobby Gaspar et al. 2017). These defects are typically associated with an increase in radiation sensitivity due to impaired DNA repair mechanisms, and therefore the genetic diagnosis has implications for patient care.

1.2.1.2 PADS MAY ARISE DUE TO DEFECTIVE B CELL MIGRATION, SURVIVAL AND ACTIVATION MECHANISMS

Immature B cells exit the bone marrow upon attenuation of the interaction between the chemokine receptor, CXCR4, expressed on the B cell surface, and its ligand CXCL12, expressed by stromal cells (Freitas, Wittner et al. 2017). A rare immunodeficiency, WHIM syndrome (OMIM#, is

characterised by impairment of B cell egress due to a gain-of-function (GOF) mutation in *CXCR4* (Hernandez, Gorlin et al. 2003, Bryant and Slade 2015). Normally, immature B cells exit into the circulation, and traffic to secondary lymphoid tissue (mostly to the spleen), where they undergo further maturation to the transitional, and subsequently mature stage (Loder, Mutschler et al. 1999) (Figure 1.1). It is worth noting the crucial role of BAFF (B cell activating factor) and TACI (transmembrane activating calcium modulator and cyclophylin ligand interactor) in maintenance and survival of mature B cells: BAFF, and its aptly named partner, APRIL, (a proliferation inducing ligand), are produced by stromal and hematopoietic cells. BAFF binds to three receptors: TACI, BCMA (B cell maturation antigen) and BAFF-R (BAFF receptor); APRIL binds to BCMA and TACI only, to promote the maturation and survival of B cells. To date there have been no reports of human APRIL or BCMA deficiency. The very few patients with BAFF-R deficiency (OMIM #613494) have a reduction in mature B cells (Lougaris, Baronio et al. 2016). In contrast to the rarity of BAFF-R deficiency, variants in *TNFRSF13B*, the gene encoding TACI, are identified in at least 10% of patients with PADs, but are also found in up to 2% clinically unaffected individuals (Castigli, Wilson et al. 2005, Salzer, Chapel et al. 2005, Salzer, Bacchelli et al. 2009). It is likely that *TNFRSF13B* variants act as disease modifiers, and will be implicated in cases of polygenic disease. Indeed, we recently reported the first instance of digenic inheritance of TACI and TCF3 gene mutations in a kindred with PAD and SLE, due to epistatic interactions between these two genes (Ameratunga, Koopmans et al. 2017).

The life of the B cell depends on its ability to bind antigen; its survival and subsequent proliferation depend on finding antigen of sufficiently high affinity, and becoming activated (Figure 1.1) (Reth and Wienands 1997, Batista and Neuberger 1998). The nature of the antigen influences the outcome of the response, and is typically viewed according to the requirement of help from T cells in responding, i.e. T-dependent or T-independent antigens.

1.2.1.2.1 T-CELL INDEPENDENT B-CELL ACTIVATION

In some instances, B cells may be stimulated directly by antigen cross-linking surface Ig, and intracellular signaling via cell membrane receptors. CD19, CD21 and CD81 deficiency (OMIM #613493, #614699, #613496) lead to clinical features of CVID (van Zelm, Reisli et al. 2006, van Zelm, Smet et al. 2010, Thiel, Kimmig et al. 2012) (Figure 1.2). Other microbial products, such as CpG derived from DNA viruses (or host cell damage), can stimulate the B cell via TLR9. Impaired TLR9 responsiveness of B cells has been observed in CVID patients (Cunningham-Rundles, J Imm 2006).

In the absence of T cell help, once triggered by a pathogen, a proportion of activated B cells will differentiate into ASCs that produce predominantly IgM (Figure 1.1). After the antigen is cleared by other components of the immune system activated by IgM (e.g. complement, phagocytes), the large majority of B cells will reach quiescence, and die. Typically, these T-independent responses do not generate long-lived memory responses, although in some circumstances, isotype switching may occur (Vos, Lees et al. 2000).

1.2.1.2.2 T-CELL DEPENDENT B-CELL ACTIVATION

A long-lived immune response requires signals received from T cells to help coordinate the immune response. T cells express a number of cell surface receptors that are able to directly modulate the outcomes of immune responses (Coffman, Seymour et al. 1988). For example, the interaction between CD40 ligand, expressed on the surface of a T-cell, with CD40 expressed by B cells was identified as one of the major co-stimulatory signals (Noelle, Ledbetter et al. 1992). CD40/CD40L interactions promote isotype-switching and generation of a germinal centre (GC) response. Evidence for this was originally gained from the human PID, X-linked Hyper IgM syndrome (OMIM #300386) (Aruffo, Farrington et al. 1993, Fuleihan, Ramesh et al. 1993), due to defects in *CD40LG*, and further studied in murine models of deficiency of CD40L, and its receptor

(Kawabe, Naka et al. 1994, Renshaw, Fanslow et al. 1994). Individuals with mutations in *CD40* (OMIM #606843), and *CD40LG* typically have very low levels of isotype switched IgG, IgA, and IgE, and are susceptible to opportunistic infections, due to the lack of T cell help. Although the GC was thought to be crucial for class switching, recent work has shown that this can also occur outside of GCs (Roco, Mesin et al. 2019). The more recently described autosomal recessive conditions, CD27 and CD70 deficiency, also lead to a defect in T cell activation, and reduction in isotype switching and differentiation, and will be discussed in the next section.

1.2.2 Defects in B-cell differentiation, isotype-switching and somatic hypermutation underlie a spectrum of PADs, and may be B-cell extrinsic

After B cells are activated by antigen, in the presence of additional signals, isotype switching occurs, and is accompanied by ongoing refinement of the response, due to somatic hypermutation of the BCR (reviewed in (Matthews, Zheng et al. 2014). These processes are dependent on NF-κB signaling, which is discussed in more detail in section 1.4.

1.2.2.1 DIFFERENTIATION DEFECTS

Long-lived immune responses arise from germinal centre reactions within spleen or lymph nodes, triggered by T-dependent antigens. The coordinated interaction of cells within GCs enhances the specificity of the immune response. In humans, IL-21 is a key cytokine involved in humoral immunity, secreted predominantly by the specialised subset of helper T cells known as T follicular helper cells (Tfh). IL-21 promotes the secretion of high affinity IgG and IgA (Bryant, Ma et al. 2007). Notably the first identified genetic form of CVID, ICOS deficiency (Figure 1.2), is characterised by impaired Tfh cell development and germinal centre formation (Grimbacher, Hutloff et al. 2003).

The development of high affinity Ig is also dependent on intracellular signaling via STAT3. Genetic defects leading to loss of IL-21 (OMIM#615767) (Salzer, 2014), or its receptor, IL-21R (OMIM#605383) (Kotlarz, 2013), have been identified to cause humoral immunodeficiency, in addition to susceptibility to opportunistic infections and colitis (Figure1.2), whereas deficiency of STAT3 leads to the autosomal dominant hyper-IgE syndrome (OMIM#147060) (Grimbacher, Holland et al, 1999). Subsequent studies have demonstrated an impaired differentiation to Th17 cells in STAT3 deficiency in addition to a Tfh defect (Milner, 2008, Ma, 2008). The humoral defects in this condition have been further elucidated to be due to an insensitivity of STAT3 deficient B cells to IL-2, leading to impaired plasmablast differentiation (Berglund, 2013), demonstrating the complex pathogenesis of PAD even when arising due to a monogenic defect.

1.2.2.2.DEFECTS IN CLASS SWITCH RECOMBINATION AND SOMATIC HYPERMUTATION

Impaired isotype switching, due to CD40L and CD40 deficiency, has been discussed in the previous section, however additional genetic etiologies also cause class switch recombination defects. Activation-induced deaminase, AID, and uracil-N-glycosylase, UNG, are involved in both isotype switching and somatic hypermutation, and their deficiency also leads to a PAD, frequently accompanied by high IgM levels (OMIM#605258, #608106) (Revy, Muto et al. 2000, Imai, Slupphaug et al. 2003).

Biallelic mutations affecting the DNA repair enzymes, *MSH6*, *PMS2*, *MLH1*, or *MSH2*, lead to a condition known as constitutional mismatch repair deficiency (CMMRD), which is characterised by early onset malignancy, as well as hypogammaglobulinemia, with elevated IgM due to impaired class switching (OMIM #276300) (Ostergaard, Sunde et al. 2005, Peron, Metin et al. 2008, Gardes, Forveille et al. 2012). Due to the expression of AID and UNG being largely restricted to immune cells, these patients do not typically experience the early onset malignancies of CMMRD. Whereas heterozygous mutations in *MLH1, MSH2, MSH6, and PMS2* lead to Lynch syndrome, or hereditary

non-polyposis colorectal cancer (HNPCC), they are not typically characterised by immunodeficiency, although an association between variants in *MSH5* and PAD is reported (Sekine, Ferreira et al. 2007).

1.2.3 Combined immunodeficiencies

1.2.3.1 COMBINED IMMUNODEFICIENCIES ARISE DUE TO DEFECTS IN T CELL MATURATION, ACTIVATION, AND DIFFERENTIATION, OR ABNORMAL THYMIC DEVELOPMENT

T-cell defects also lead to antibody deficiency, however the clinical features are typically more severe than isolated antibody deficiencies. In healthy individuals, immature T cells develop from hematopoietic stem cells in the bone marrow and circulate to the thymus where they undergo a series of tests of functionality (reviewed in, van Boehmer, 1992). Mature T cells are characterised by the expression of an antigen-specific TCR. T cells that are involved in coordinating adaptive immune responses utilise the alpha and beta subunits of the TCR and are thus referred to as αβ T cells. As described earlier, the α and β subunits undergo re-combination of their variable regions so as to generate diverse antigen specificities. In contrast, γδ T cells are so named due to the use of the gamma and delta TCR subunits, which are not variable, and involved in more innate-like responses. These cells will not be discussed further here.

Like B cells, during T cell development the αβ TCR is subject to a number of checkpoints, to test its functionality. Immature cells are referred to as double negative cells (DN), as they express neither CD4, nor CD8 on the cell surface. Initial signals via Notch and c-kit start the process of TCR generation (Ciofani, Schmitt et al. 2004, Schmitt, Ciofani et al. 2004). Cells progress further through development with expression of a surrogate alpha chain, analogous to the surrogate light chain on B cells, which is expressed together with a Vβ subunit, as the pre-TCR. When the pre-

TCR is complexed with CD3 molecules on the cell surface, signaling can occur, triggering proliferation, in the absence of antigen. At this stage the immature T cells express both CD4 and CD8 on their cell surface, and are referred to as double positive T cells. Subsequent steps involve selection of T cells determined by their responsiveness to antigen presentation by either major histocompatability complex- I (MHC-I), or MHC-II. For example, $CD8^+$ T cells are selected if they bind with moderate affinity to cognate antigen expressed by MHC I, and $CD4^+$ cells selected by MHCII binding. Defects in MHC I or MHC II expression and their related proteins thus lead to CID, due to impaired T cell development (Sullivan, Stobo et al. 1985). Defects in thymic function or development may also lead to combined immunodeficiencies as well as autoimmune disease. As an example, loss-of-function (LOF) of *TBX1*, either due to mutations in *TBX1* alone or due to the 22q11.2 deletion syndrome, lead to a CID due to thymic dysgenesis (Yagi, Furutani et al. 2003). Furthermore, thymic development may be affected by some of the genetic lesions that cause CVID, including, *NFKB2* mutations (Zhu, Chin et al. 2006), and this may account in part for some of the overlapping clinical features between PADs and CIDs.

Like naïve B cells, naïve T cells exit into the periphery, where they circulate through lymphoid tissue, seeking cognate antigen. The context of antigen presentation is crucial to the outcome of the immune response. When the TCR binds antigen it requires additional activating signals in order to be capable of proliferation. After extensive efforts to identify the so-called 'second signal', the trans-membrane receptor, CD28, was shown to be a major co-stimulatory molecule for inducing T cell proliferation (Linsley, Brady et al. 1991, Harding, McArthur et al. 1992). Further investigations have shown that CD28 signaling positively regulates T cell proliferation by driving production of IL-2, a key cytokine already recognized to promote T cell proliferation (Cantrell and Smith 1984, Sanchez-Lockhart, Marin et al. 2004). *CD28*$^{-/-}$ mice demonstrate reduced GC formation and reduced IgG responses to T-dependent antigens (Ferguson, Han et al. 1996). Interestingly, human disease due to loss of function mutations in CD28 has recently been identified and is characterised

by a surprisingly narrow susceptibility to cutaneous papilloma virus infections, leading to the clinical phenotype of 'tree-man syndrome' (Beziat et al, personal communication 2019).

The 'second signal' is an extreme oversimplification; there are many signals that may promote T cell activation and expansion, and the requirement for additional signals is likely to be context specific. Indeed, recent work demonstrated, in a murine model, that the combination of antigen affinity, additional co-stimulatory signals, and cytokine inputs, add in a linear fashion to the overall outcome of a $CD8^+$ T cell response (Marchingo, Kan et al. 2014). Defects in T-cell activation due to loss of CD40 or CD40L, and IL-21 or IL-21R are also classified as CID, and have been discussed in the previous section, as the impaired T-B cell interactions pertain to impaired Ig class switching and somatic hypermutation (Aruffo, Farrington et al. 1993, van Zelm, Bartol et al. 2014). Additional defects that may lead to altered T cell differentiation in the context of B and T cell interactions occur in X-linked lymphoproliferative syndrome type 1 (XLP-1), due to mutations in *SH2DIA*, encoding SLAM-associated peptide (SAP) (OMIM #308240)(Tangye, Nichols et al. 2003). This condition is associated with severe Epstein-Barr Virus (EBV) disease, hypogammaglobulinemia, and an increased risk of lymphoproliferative disease. Impaired function of some populations, in particular NKT cells, and T cell activation defects contributes to impaired production of antibody, due to impaired B cell activation, thereby causing a B-cell extrinsic antibody defect (Ma, Hare et al. 2005, Nichols, Ma et al. 2005). More recently autosomal recessive CD27 and CD70 deficiencies have been described, which have similar features to XLP1, with susceptibility to EBV-induced lymphoproliferation, although they may also present with a CVID-phenotype (Figure 1.2) (van Montfrans, Hoepelman et al. 2012, Abolhassani, Edwards et al. 2017, Izawa, Martin et al. 2017).

1.2.3.2 SEVERE COMBINED IMMUNODEFICIENCY ARISES DUE TO IMPAIRED DEVELOPMENT OF T CELLS, WITH VARIABLE DEFECTS IN DEVELOPMENT OF B AND NK CELLS

Although previously thought to occur less frequently than 1 in 100,000 births, the advent of newborn screening for SCID in the United States, has revealed it to be significantly under-diagnosed with actual incidence of 1 in 50,000 (Kwan, Abraham et al. 2014). SCID is characterised by a severe deficiency of T lymphocytes, with variable loss of B and NK cells (T-/B-/NK-; T-/B-/NK+; or T-/B+/NK+) depending on the genetic etiology (Cirillo, Giardino, Ann NY A S, 2015).

The most common cause of SCID is due to mutations in the gene encoding the common gamma chain of the interleukin 2 receptor (IL-2R) (Noguchi, Yi et al. 1993, Puck, Deschenes et al. 1993). Mutations in *IL2RG* lead to impaired responsiveness of T cells and NK cells to the cytokines crucial for their development, homeostasis, and proliferation, namely interleukins-7 (IL-7), -2 (IL-2), and -15 (IL-15), and hence cause a T-/B+/NK- SCID. Despite the presence of B cells, however, the lack of T cell help, significantly limits their functionality; manifesting as hypogammaglobulinaemia. Reflecting the differential dependence on certain cytokines for lymphocyte development, bi-allelic mutations affecting *IL7R*, the receptor for IL-7, leads to a T-, B+, NK+ SCID (Puel, A. et al. Nat Gen, 1998).

Initial signals via Notch and c-kit start the process of TCR generation (Ciofani, Schmitt et al. 2004, Schmitt, Ciofani et al. 2004). Cells progress further through development with expression of a surrogate alpha chain, analogous to the surrogate light chain on B cells, which is expressed together with a Vβ subunit, as the pre-TCR. Mutations in *RAG1* and *RAG2*, account for a significant proportion of T-/B-/NK+ SCID cases (Schwarz, Gauss et al. 1996).

Major advances have been made with regard to treatment and management of SCID over recent decades; from the first haematopoietic stem cell transplant (Horowitz, Bach, Lancet, 1975) to pre-emptive diagnosis with newborn screening programs (Kwan, Abraham et al. 2014), and excellent long term survival after early HSCT (Pai, Logan et al. 2014). Furthermore, gene therapy, despite

initial setbacks, also holds great promise for SCID and other CID, and is currently under investigation in clinical trials in Europe and the US (Aiuti, Cattaneo et al. 2009, Cicalese, Ferrua et al. 2016, Thrasher and Williams 2017).

1.2.4 Common Variable Immunodeficiency (CVID): the most common predominantly antibody deficiency

The majority of cases of PAD are not well-defined, and most patients are given a descriptive diagnosis of CVID, which is currently estimated to have a prevalence of at least 1 in 10000 in some populations (Selenius, Martelius et al. 2017). CVID was first reported in 1961 in 2 unrelated individuals, with recurrent infections and described as *dysgammaglobulinemia* (Rosen, Kevy et al. 1961). By 1970, the condition now referred to as CVID, was recognised as a the experts in the field as *"an immunodeficiency group [that] presumably lumps together a series of syndromes because of lack of information on definite patterns and/or cause"* (Fudenberg, Good et al. 1970). CVID may present at any age, although typically 2nd and 3rd decades of life and the condition may not only manifest as a susceptibility to infections, but a significant proportion of patients develop features of immune dysregulation; presenting with autoimmune or auto-inflammatory complications; or malignancy (Cunningham-Rundles and Bodian 1999).

Interestingly, there is significant geographical variation in the reported prevalence of CVID, which is likely to be multifactorial; due to a combination of genetic, and environmental factors, as well as underdiagnosis and reporting bias (Barton, Bertoli et al. 2014, Graziano, Pecoraro et al. 2017, Selenius, Martelius et al. 2017). For the most part, treatment involves regular Ig replacement therapy. Many groups have attempted to generate diagnostic criteria for this enigmatic disorder, in order to help physicians to make predictions about prognosis and therapeutic decisions (Conley, Notarangelo et al. 1999, Ameratunga, Brewerton et al. 2014, Immunodeficiencies. 2017) However

despite these criteria, the enigmatic nature of these frequently complex conditions remains difficult to diagnose PID, associated with long diagnostic delays.

1.2.4.1 DIAGNOSTIC MEASURES AND COMPARISON OF DIAGNOSTIC CRITERIA FOR CVID

In some ways the complexity of the diagnostic criteria for CVID reflect the complexity of the condition itself, and the challenges in attempts to understand its pathogenesis. From these efforts, it is clear that CVID is not a single disease, but rather a group of disorders, which manifest some common immunological features. In some cases, not all diagnostic criteria may be met, and a patient may be given an alternate label, such as '*specific antibody deficiency*', 'p*ossible CVID'* or '*CVID in evolution*'. The 3 major diagnostic criteria of CVID, are briefly outlined below.

ESID/PAGID diagnostic criteria of CVID

The European Society for Immunodeficiency and the Pan-American group (ESID/PAGID) criteria for CVID have evolved over two decades (Conley 1999). Originally, the diagnosis of CVID required *(i)* serum IgG and *(ii)* either IgA and/or IgM to be 2 SD below the age-adjusted reference range, *(iii)* in the absence of a secondary cause for low immunoglobulins. In addition, an impaired specific response to antigen, to either vaccines or iso-hemagglutinins, was required. The newer criteria, allow reduced switched memory B cells to be a criterion in place of impaired vaccine responses, avoiding some of the diagnostic challenges regarding measurement of specific antigen/vaccine responses, discussed in the section, '*Challenges of diagnostic criteria'*.

ICON diagnostic criteria of CVID

The International Consensus (ICON) was generated by several international professional groups (Bonilla, Barlan et al. 2016) to provide criteria for a *definite* diagnosis of CVID. According to ICON criteria, a patient must have *(i)* reduced IgG, and *(ii)* either low IgA and/or IgM, and *(iii)* other causes of hypogammaglobulinemia excluded, in order to be given a diagnosis of 'definite

CVID'. In addition, one of three major clinical features should also be present (infection, autoimmunity or lymphoproliferation), although patients meeting all the other criteria may be diagnosed without any clinical features. Exclusion criteria include monogenic etiology or an alternative PID, and secondary causes of hypogammaglobulinemia, such as medications; gastrointestinal or renal protein loss; and malignancy.

Ameratunga diagnostic criteria of CVID

Ameratunga criteria were published in 2014, included as an appendix ((Ameratunga, Brewerton et al. 2014); Appendix 1). These criteria are more comprehensive in nature than the ESID/PAGID and ICON criteria, and are more inclusive, yet their complexity makes them difficult to use in practice, as there are several components encompassing clinical features, laboratory evidence, as well as histological markers. The Ameratunga criteria define patients as either having '*probable'* or '*possible'* CVID,

Challenges of diagnostic criteria

Regardless of which criteria are used, it is clear that diagnostic methods for this condition are imperfect, and diagnosis of CVID remains challenging. An additional difficulty with incorporating these criteria into practice, is the role for measurement of vaccine responses. These are problematic for a number of reasons including a lack of standardisation of the vaccines to be administered and variability in vaccine and infectious history, and the challenges of measuring serial responses. This frequently leads to a delay in access to immunoglobulin therapies.

Exclusion criteria: monogenic causes and T cell dysfunction

Further issues arise with exclusion of monogenic causes. Whilst the term CVID is cumbersome and imprecise, it is useful for classification and therapeutic decision-making. For example, in circumstances where a monogenic defect is identified, but about which little is known, a more

recognisable ‘label’ of CVID may be useful for treating clinicians, and patients themselves, than the label of an exceedingly rare genetic condition.

The exclusion of patients with signs of T cell dysfunction is also problematic. In many cases diagnostic assays of T cell function would not be a routine diagnostic measure for CVID. However, there is frequently an evolution of the clinical phenotype over time, from one more typical of a PAD, to one more consistent with CID; and thus the distinction of CIDs from PADs, especially CVID, is not well-defined. It is likely that subtle lymphocyte defects are present in patients with PADs, and that they may be measurable by studying isolated cell populations treated with specific stimuli. Further to this, a variety of T cell defects have been previously demonstrated in CVID, including impaired responsiveness to specific antigens and altered cytokine secretion, including IL-2, IFNγ, and IL-10 (Fischer, Hauber et al. 1993, Holm, Aukrust et al. 2003, Giovannetti, Pierdominici et al. 2008). Other studies of clinical phenotypes have demonstrated an association between peripheral blood $CD8^+$ T cell numbers and the development of autoimmune disease (Chapel, Lucas et al. 2008). In addition, abnormalities of regulatory T cell number and function have also been described in CVID, and are often associated with the development of autoimmune complications (Horn, Manguiat et al. 2009, Arandi, Mirshafiey et al. 2013).

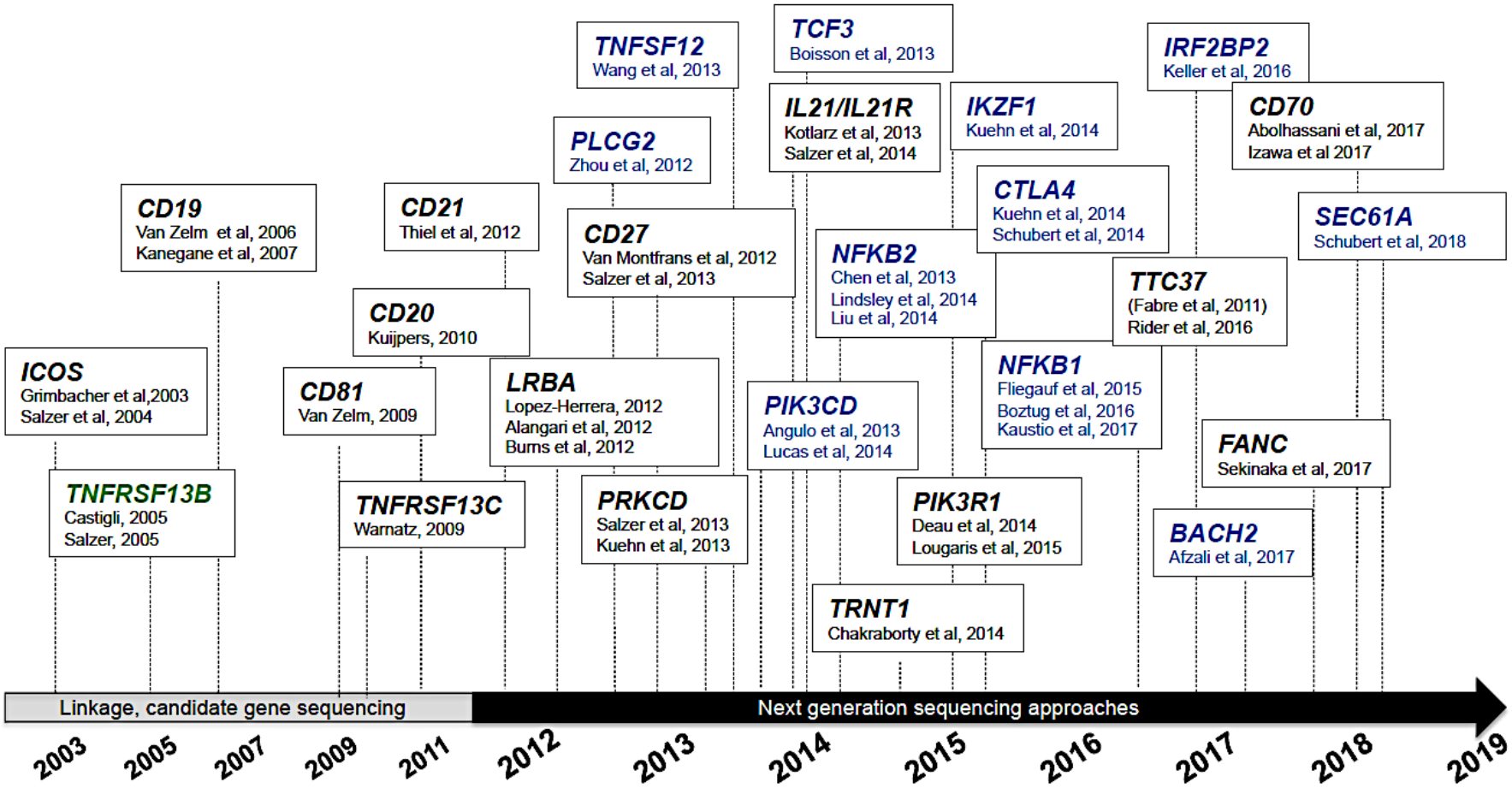

TNFSF12
Wang et al, 2013
TCF3
Boisson et al, 2013
IRF2BP2
Keller et al, 2016
IL21/IL21R
Kotlarz et al, 2013
Salzer et al, 2014
IKZF1
Kuehn et al, 2014
CD70
Abolhassani et al, 2017
Izawa et al 2017
PLCG2
Zhou et al, 2012
CD19
Van Zelm et al, 2006
Kanegane et al, 2007
CD21
Thiel et al, 2012
CD27
Van Montfrans et al, 2012
Salzer et al, 2013
CTLA4
Kuehn et al, 2014
Schubert et al, 2014
SEC61A
Schubert et al, 2018
NFKB2
Chen et al, 2013
Lindsley et al, 2014
Liu et al, 2014
TTC37
(Fabre et al, 2011)
Rider et al, 2016
CD20
Kuijpers, 2010
ICOS
Grimbacher et al,2003
Salzer et al, 2004
CD81
Van Zelm, 2009
LRBA
Lopez-Herrera, 2012
Alangari et al, 2012
Burns et al, 2012
PIK3CD
Angulo et al, 2013
Lucas et al, 2014
NFKB1
Fliegauf et al, 2015
Boztug et al, 2016
Kaustio et al, 2017
FANC
Sekinaka et al, 2017
TNFRSF13B
Castigli, 2005
Salzer, 2005
TNFRSF13C
Warnatz, 2009
PRKCD
Salzer et al, 2013
Kuehn et al, 2013
PIK3R1
Deau et al, 2014
Lougaris et al, 2015
BACH2
Afzali et al, 2017
TRNT1
Chakraborty et al, 2014
Linkage, candidate gene sequencing
Next generation sequencing approaches
2003
2005
2007
2009
2011
2012
2013
2014
2015
2016
2017
2018
2019

FIGURE 1.2 TIMELINE OF GENE DISCOVERY IN CVID.

Monogenic etiologies of CVID and year of discovery, genes in black text cause autosomal recessive disease; genes in blue cause autosomal dominant disease; susceptibility gene in green. Grey arrow represents the era of linkage and candidate genes Sanger sequencing; black arrow represents era of NGS technologies, such as exome and genome wide sequencing. (Note: genes listed are the most well recognised CVID-associated genes; other PID-associated genes may also lead to a CVID phenotypes; complete references in text).

1.3 The evolution of genetic studies of CVID etiology in the era of genomic sequencing

Some of the genetic causes of PIDs have been discussed in the introduction of B and T cell development, activation and differentiation (Section 1.2.1). Here, the tools by which the discoveries of monogenic causes of CVID and other PADs were made, are introduced.

1.3.1 Genetic causes of CVID were first identified in rare cases, on the basis of linkage analysis, and candidate gene approaches.

Initially genetic testing was used to establish diagnoses in a small number of cases of CVID/PAD. In the era prior to exome and genome sequencing, linkage analysis, and Sanger sequencing of candidate genes was applied to identify genetic causes of disease. In many of these instances, linkage studies relied on sequencing large kindreds with a number of affected and unaffected individuals, in order that areas of linkage disequilibrium, and/or homozygosity, could be identified. (refs) These tools identified isolated cases of mostly autosomal recessive (AR) causes of these conditions, most likely due to the selection of patients with the most striking clinical phenotypes, and in some instances due to prioritised selection of consanguineous families (refs). For the most part these were confined to rare cases of only one or two families, typically in geographically isolated regions (Figure 1.2). Inducible co-stimulator (ICOS) deficiency was the first monogenic etiology of CVID identified, and whilst several families were studied, all affected individuals shared a common homozygous deletion within *ICOS*, due to a founder effect in the isolated region of the Black Forest in Germany (Grimbacher, Hutloff et al. 2003).

1.3.2 GWAS made limited advances into revealing the genetic causes of CVID

Genome-wide association studies heralded a new era in the quest to understand genetic

susceptibility to common diseases. Comparing differences between large numbers of affected individuals, and healthy controls has identified a number of genes implicated in the pathogenesis of more common immune diseases, such as inflammatory bowel disease (IBD), multiple sclerosis (MS), type 1 diabetes mellitus (T1DM), celiac disease (CD) and systemic lupus erythematosus (SLE) (Duerr, Taylor et al. 2006, International Multiple Sclerosis Genetics, Hafler et al. 2007, Parkes, Barrett et al. 2007, Todd, Walker et al. 2007, Hom, Graham et al. 2008, Hunt, Zhernakova et al. 2008, Kozyrev, Abelson et al. 2008).

This approach has also been applied to a cohort of CVID patients (Orange, Glessner et al. 2011). Cases, numbered at 363, were further stratified into subgroups according to a number of clinical phenotypes. As is common with most immune disorders, a moderate association with disease risk was observed at the region of the MHC. Although several weak associations were identified, these have not since lead to any obvious common pathogenesis. Perhaps this study was limited by insufficient cases, and the study of too heterogeneous a disease (Orange, Glessner et al. 2011). One might consider the associations may offer clues to areas of the genome and pathways warranting further study in the investigation of CVID, as may be performed with genomic sequencing. Another study of 778 CVID patients and 10,999 healthy donors did identify some additional associations at loci, including most strongly with *CLEC16A,* as well as other previously recognised disease-associated genes, *CD21, ICOS, MSH5* and *TNFRSF13B,* and the HLA region (Li, Jorgensen et al. 2015).

1.3.3 Next generation sequencing has led to major advances in discovering the pathogenesis of PADs

The availability of NGS technologies has led to an explosion of new genetic causes of PADs in the last five years (Meyts, Bosch JACI 2016) (Figure 1.2). The role of these technologies has been

particularly evident in the diagnosis of disorders with complex, or severe phenotypes; the first large cohort of patients offered this testing for diagnostic purposes had mostly neurodevelopmental conditions, with a diagnostic rate of around 25% (Yang et al, NEJM 2013).

Even as recently as 2016 it was estimated that <10% of CVID diagnoses had a genetic basis (Bogaert, Dullaers et al. 2016). Other studies of CVID cohorts internationally have suggested this may be closer to 30% when patients are selected for additional features, including comorbid autoimmune disease, younger age of onset and/or a family history of disease (Maffucci, Filion et al. 2016). In populations where rates of consanguinity are high, the proportion of monogenic cases is even higher (Abolhassani, Aghamohammadi et al. 2018). It has become clear that genes involved in B- and T-cell signaling pathways are overrepresented as etiologies for PADs (Meyts, Bosch, 2016) with NF-κB a particularly pertinent example (Tuijnenburg, Lango Allen et al. 2018).

1.4 NF-κB-related Disorders

1.4.1 NF-κB is a major hub of immunity and inflammation

NF-κB was first identified over 30 years ago, and so-named due to its clear role in B cells, '*the nuclear factor binding near the kappa light chain in B cells*' (Sen, Cell, 1986; Lenardo, Nature, 1986). However, its role in immunity is much broader than in B cell activation alone. Over time, the importance of the NF-κB signaling pathway has been extensively investigated in animal models and, more recently, its relevance in the pathogenesis of human immune disease has become better understood (Zhang, Lenardo et al. 2017).

NF-κB, comprises a family of 5 proteins which function as transcription factors involved in the immune response. Two pathways, referred to as the canonical and non-canonical (or alternative) pathways, coordinate the transcription or repression of several hundred genes involved in generating an inflammatory or adaptive immune response. NF-κB1 (p105/p50), and NF-κB2 (p100/p52), are synthesised as precursor proteins that are phosphorylated upon receipt of a stimulatory signal, and proteolytically cleaved to their respective active subunits, p50 and p52 (Figure 1.3). There are five key active NF-κB subunits: p50, p52, Rel A, Rel B and c-rel (Hayden and Ghosh, 2008) all of which are broadly expressed across tissues (Figure 1.3). Each of these contains a N-terminal Rel-homology domain (RHD) that enables their homo- and hetero-dimerisation and DNA binding (Hayden and Ghosh 2008). In quiescent cells, NF-κB proteins remain in the cytoplasm bound to one of several inhibitory proteins, known as IκBs. The inhibitory proteins are characterised by the presence of a variable number of ankyrin repeat domains (ARD). The classical inhibitory proteins bind to NF-κB proteins in the cytoplasm, until a stimulus leads to their phosphorylation, and subsequent ubiquitination, freeing the NF-κB molecules to dimerise and access the nucleus, thereby activating or inhibiting transcription by binding to their target genes.

The balance of hetero- and homodimers of the Rel proteins leads to transcription or repression of genes involved in immune response, inflammatory cytokines, lymphoid organ development, antibody class-switching Thus, deficiency of any one of these proteins may have a significant impact on the immune response. However, due to the multitude of processes impacted, the end result may be challenging to predict.

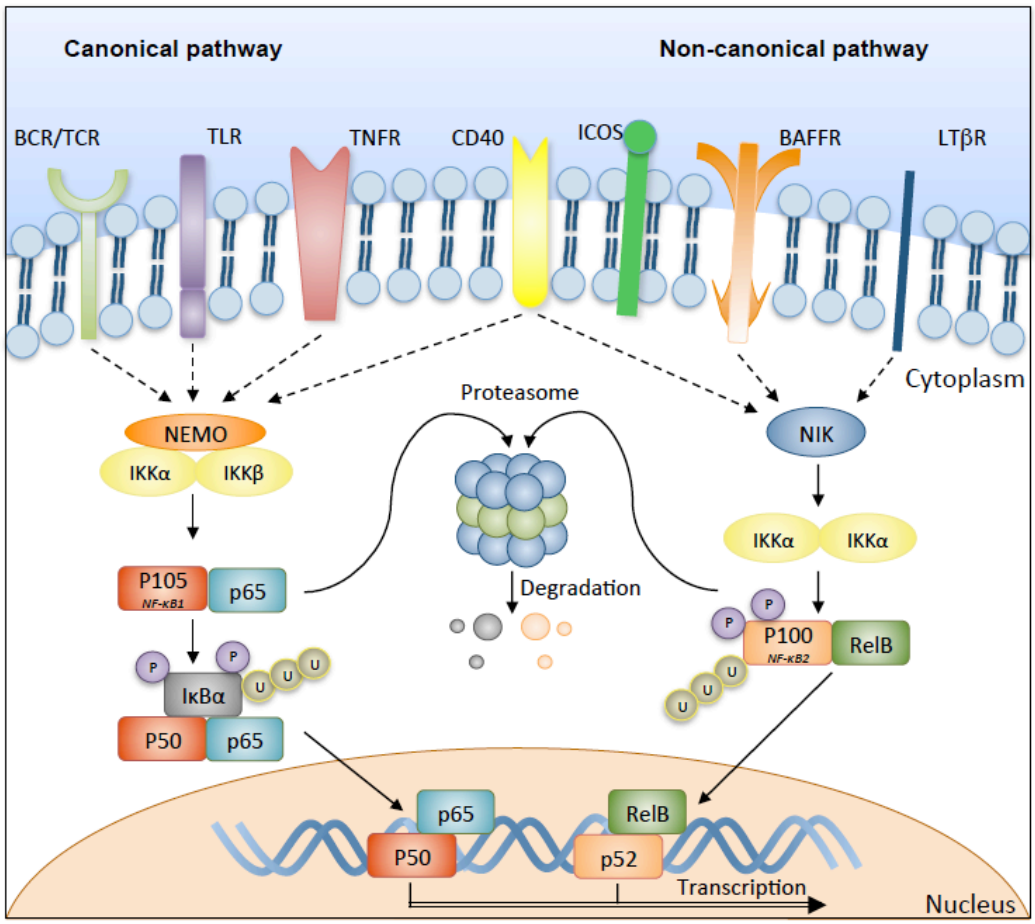


FIGURE 1.3 NF-κB SIGNALING INVOLVES TWO MAJOR PATHWAYS, REFERRED TO AS THE CANONICAL AND NON-CANONICAL PATHWAYS.

Schematic represents the NF-κB signaling pathways and their activating signals: upon binding of cell surface receptors with their ligands, a cascade of phosphorylation events leads to ubiquitination of inhibitors of NF-κB (IκBs), whereupon NF-κB dimers are free to enter the nucleus and modulate the transcription of >500 genes involved in immunity and inflammation. (*BCR, B cell receptor; TCR, T cell receptor; TNF-R, tumor necrosis factor receptor; ICOS, inducible T cell co-stimulator; BAFF-R, B cell activating factor receptor; LTβR, lymphotoxin beta receptor; TLR, Toll like receptor; NEMO, NF-κB essential modulator; IKK, IκB kinase; NIK, NF-κB inducing kinase; P, phosphorylation sites; U, ubiquitination sites*) (Figure adapted from an original by Dr Alex Strathmore.)

1.4.2 Defects of NF-κB signaling are the dominant monogenic cause of CVID

Of the monogenic causes of CVID-like disorders, heterozygous mutations in *NFKB1* and *NFKB2* are now known to contribute to the majority of cases (Stray-Pedersen, Sorte et al. 2017, Tuijnenburg, Lango Allen et al. 2018). These mutations lead to autosomal dominant disease, and in the case of *NFKB1,* mutations may be associated with late onset presentation (Fliegauf, Bryant et al. 2015) (Lorenzini and Grimbacher, personal communication). In addition, they lead to strikingly variable clinical phenotypes, ranging from manifestations of auto-inflammation (Kaustio, Haapaniemi et al. 2017), mild recurrent sinopulmonary infections (Fliegauf, Bryant et al. 2015), to an absence of any clinical features. In fact, the penetrance is estimated to be approximately 70%, although this is dependent on age (Lorenzini and Grimbacher, personal communication). Thus, understanding the impact of these mutations is of major interest and relevance in CVID.

NFKB2 mutations were first reported with the prominent feature of comorbid central adrenal insufficiency (Chen, Coonrod et al. 2013). This complication may be rapidly fatal yet is readily treatable with cortisol replacement. Since the first report, the spectrum of disease is clearly broader than originally described, and includes more extensive ectodermal abnormalities in addition to autoimmune complications, such as alopecia, as well as a complete absence of mature B cells in some, but not all, patients (Lee, Fulcher et al. 2014, Shi, Wang et al. 2016). Early onset disease is a key feature of NF-κB2 deficiency, unlike the later onset disease observed in NF-κB1 deficiency (Klemann, Camacho-Ordonez et al. 2019).

More recently a report of a patient with NF-κB2 deficiency with deep-seated pyogenic infections in association with anti-cytokine antibodies, broadened the phenotype even further (Ramakrishnan, Rae et al. 2017). The presence of anti-cytokine antibodies adds to the phenotypic overlap with

APECED (*A*utoimmune *P*olyendocrinopathy, *E*nteropathy, *C*andidiasis and *E*ctodermal *D*ystrophy). APECED, or Polyglandular Syndrome 1, arises due to biallelic mutations in the autoimmune regulator gene, *AIRE,* and in that condition anti-cytokine antibodies are well described (Bjorses, Aaltonen et al. 1998) Indeed NF-κB2 is known to regulate the expression of AIRE (Zhu, Chin et al. 2006).

Some mutations in *NFKB2* appear to lead to a GOF by enhanced signaling; perhaps somewhat paradoxically these 'GOF' mutations seem to be associated with a more significant infectious susceptibility (Kuehn, Niemela et al. 2017). This is consistent with the more severe phenotypes observed in conditions due to loss of regulation of NF-κB signaling, which will be discussed below.

1.4.3 Loss of regulation of NF-κB signaling underlies immunodeficiency syndromes with additional non-immune manifestations

NEMO deficiency

Mutations affecting regulation of canonical NF-κB1 signaling were first identified in humans in the gene *IKBKG,* which encodes the NF-κB-essential modifier (NEMO) (Zonana, Elder et al. 2000). NEMO deficiency was found to cause an X-linked, early onset PID, characterised by susceptibility to mycobacterial, viral bacterial infections associated with ectodermal abnormalities in some cases (Zonana, Elder et al. 2000, Doffinger, Smahi et al. 2001). Complete loss of functional NEMO is embryonic lethal however some residual function remains in patients with X-linked anhidrotic ectodermal dystrophy and immunodeficiency (EDA-ID). NEMO performs its modulatory action be sequestering the kinases IKKα and IKKβ, encoded by *IKBKA* and *IKBKB*, respectively. Upon receipt of a stimulatory signal, NEMO is degraded, enabling the kinases to phosphorylate IκBα, leading to release of active NF-κB dimers and activation of NF-κB signaling

(Figure 1.3). In the absence of functional NEMO, the pathway is poorly responsive to certain stimuli, including IL-1β, IL-18, LPS and TNF (Doffinger, Smahi et al. 2001).

IκBα and IKKβ defects

Heterozygous mutations in *NFKBIA* that disrupt the phosphorylation sites of IκBα, lead to a gain of inhibitory activity and cause an immunodeficiency phenocopying NEMO deficiency (Courtois, Smahi et al. 2003). Further demonstration of the required stringent balance between NF-κB activation and inhibition has come about from the recent discovery of heterozygous variants in *IKBKB*, leading to a gain of function in IKKβ, and resulting in a combined immunodeficiency, whereas biallelic loss of function mutations lead to a SCID phenotype (Pannicke, Baumann et al. 2013, Mousallem, Yang et al. 2014, Cardinez, Miraghazadeh et al. 2018).

Other defects in upstream regulators of NF-κB signaling

Further defects in processes controlling regulation of NF-κB have also been identified. Deficiency of components of LUBAC (linear ubiquitin chain assembly complex), leads to the combination of immunodeficiency and autoinflammation in humans (Boisson, Laplantine et al. 2015). Again, demonstrating the importance of regulation of the pathway, is the phenotype observed in patients with deficiency of the deubiquitinase, otulin, that regulates LUBAC. Biallelic loss of function mutations in *FAM105B*, the gene encoding Otulin, lead to a syndrome of autoinflammation that is responsive to TNF inhibition, without signs of immunodeficiency (Damgaard, Walker et al. 2016). The variation observed in these monogenic disorders, as well as the large proportion of cases where a monogenic or even digenic cause is not found, suggests that non-genomic methods may also be useful in determining the etiology of the immune phenotype. One approach to this is the application of quantitative techniques to measure the immune response in immune cells from patients with PID, which will be discussed in the next section.

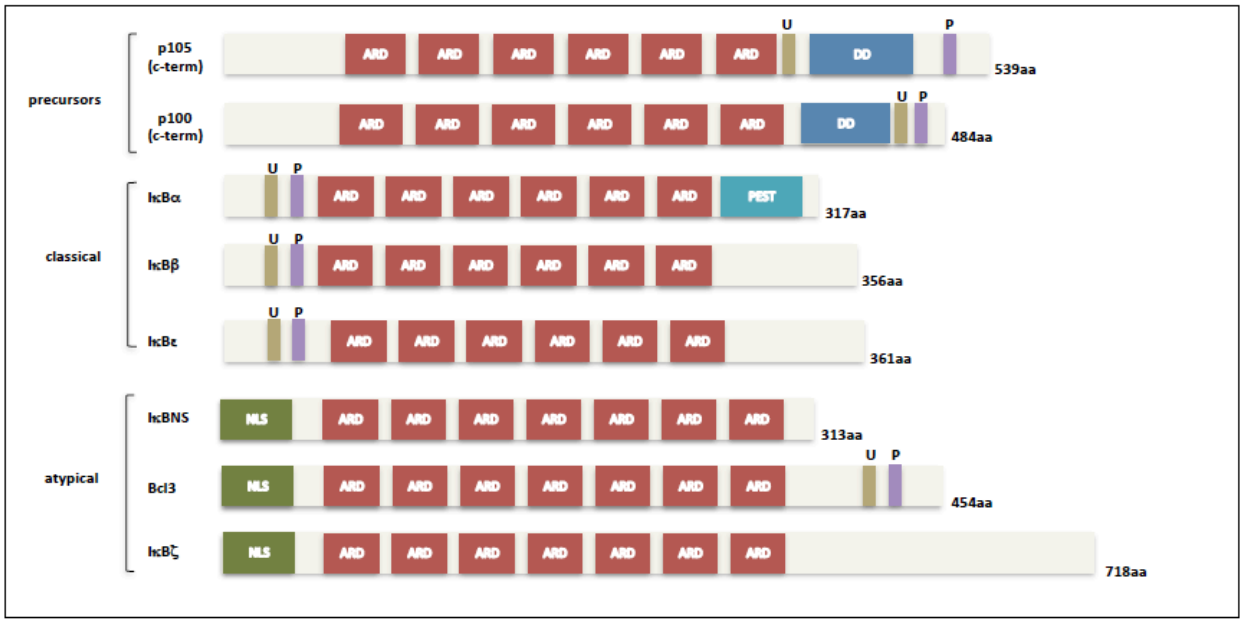


FIGURE 1.4. INHIBITORS OF NF-κB SIGNALING

The inhibitory members of the NF-κB pathway are depicted, together with the inhibitory components of the precursor components, p105 and p100, which comprise death domains (DD) at their C-terminal ends. The classical inhibitors, IκBα, IκBβ and IκBε, share similarities with p105 and p100 inhibitory components in the number of ankyrin repeat domains (ARD), and the presence of ubiquitination (U) and phosphorylation sites (P). IκBα also is comprised of a peptide sequence rich that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) domain. The non-classical/atypical inhibitors, IκBNS, Bcl3 and IκBζ, are characterized by nuclear localization sequences (NLS) at their N-terminal ends.

1.5 Quantitation and modeling of the immune response

There are many inputs required to generate an effective humoral immune response, as described in detail in sections 1.1 and 1.2. In contrast to quantification of the inputs, the output is relatively straightforward to measure directly. For example, total serum concentrations of each of the Ig isotypes, and pathogen and vaccine specific titres are routinely measured for clinical purposes in diagnostic laboratories. However, if these values fall outside of the normal range, it offers little insight into the pathophysiological cause that underlies impaired antibody production. In some situations, the cause of a humoral immune defect may be obvious, due to the absence or reduction in a particular cell population (see section 1.2). In other situations, an inappropriate antibody response arises by more complex mechanisms.

Measurement of the cellular immune response, by lymphocyte proliferation studies, is typically only performed in the clinical setting to confirm or exclude the diagnosis of a suspected CID (Bonilla, Barlan et al. 2016). Diagnostic measures of proliferation vary widely, not only with regard to the marker of proliferation (e.g. H3 thymidine incorporation, bromodeoxyuridine (BrdU), or cell division-tracking dyes), but also the cell population/s studied (e.g. total T cells, or PBMCs), the stimulation conditions (potent mitogens, or specific antigens) and the timing of stimulation and the output measured. Typically, total PBMCs are studied with a single time-point at which proliferation is measured, recorded as a proportion of the total population. Often this is compared to a single healthy control. Analysing responses of purified cell populations to a variety of signals will provide insight into and understanding of the complexities of the immune response. Furthermore, elucidation of the mechanism of the lymphocyte defect may provide rationales for novel therapeutic approaches.

1.5.1 Modeling the immune response based on quantitative assays of lymphocyte proliferation and death

Mathematical models are powerful tools for understanding complex systems such as the immune response, but rely on precise and quantitative measures of the system. The ability to measure the kinetics of an *in vitro* immune response, by performing time course analyses, has provided crucial insight regarding lymphocyte proliferation and death (De Boer, Ganusov et al. 2006). Sophisticated analysis of these data has enabled the development of robust models of the immune response (De Boer, Ganusov et al. 2006, Hawkins, Hommel et al. 2007, Hawkins, Turner et al. 2007).

Cell proliferation studies were limited until the mid-1990s to the use of tritiated thymidine or BrdU to indirectly estimate cell division by determining the release of radioactivity, or the incorporation of BrdU into DNA of dividing cells. A significant advancement in the study of cell proliferation was made with the development of cell tracking dyes. Due to the dyes' property of halving fluorescence with each division, precise measures of the cellular response could be made by use of flow cytometry (Lyons and Parish 1994). Since the first dye, CFSE, was introduced, there have been a number of other dyes developed with superior characteristics, including reduced toxicity and enhanced resolution (Quah and Parish 2012, Tempany, Zhou et al. 2018).

Remarkably, despite the immune response being dependent on a number of complex events and the coordination of multiple cell types, detailed testing of isolated cell populations has revealed the regulation of the immune response can be distilled into a set of simple rules, focused around the programming of a series of independent timers of cell proliferation and death. That is, when lymphocytes are stimulated *in vivo* or *in vitro* the characteristic immune response, of activation, cell division, and cell loss (depicted in Figure 1.1), can be explained by a series of just four timed events related to cell division and death:

1. The time to enter division (*time to first division, ttfd*)
2. The time between subsequent divisions (*division rate*)
3. The time to die *(death time)*
4. The time cells cease to divide and return to quiescent state (*time to division destiny, DD*)

While small changes in ttfd (Hasbold, Gett et al. 1999) and division rate can have a substantial impact on the strength of the immune responses (Gett and Hodgkin 2000), it is also clear that the number of times a population of cells divide, or its DD, and the time it takes to reach the DD, also has a major impact on the overall response magnitude and is therefore a sensitive regulator of the immune response (Marchingo, Kan et al. 2014). The proportion of cells responding to stimulation (frequency activated, FA) also contributes to the overall strength of the immune response (Jessica Tempany, PhD thesis 2019).

Comprehensive models, the Cyton models (Hawkins, Turner et al. 2007), were developed and incorporated probability distributions for populations of cells for competing times to divide and die in each generation and introduced the parameter of DD. The Cyton models implemented DD as a division-counted outcome and reset a death time in cells following each division. The model is well-suited to T cell proliferation responses that incorporate saturating levels of the growth factor IL-2 to sustain cell division. In recent studies, the pattern of T cell proliferation when endogenous IL-2 secretion is blocked, or the level is controlled to be sub-saturating exogenous IL-2, have led to an updated, modified model where activation programs T cells to effectively carry a time to divide, (i.e. DD), and a time to die, through each generation.

Specific information regarding the signals T and B cells receive, and the way these are integrated, has been gathered primarily from murine and healthy human cells. In murine $CD4^+$ T cells, co-stimulation with an anti-CD28 antibody, a generic TCR signal (anti-CD3), in the presence of

saturating IL-2, has been demonstrated to shorten the time to start dividing (*time to first division*) (Gett and Hodgkin 2000). At non-saturating IL-2 concentrations, increases in IL-2 enhance the proportion of cells entering division (Frequency activated, FA) in a dose-dependent manner, but do not affect the ttfd (Deenick, Gett et al. 2003). Further studies of murine $CD8^+$ T cells, using a transgenic TCR specific for the ovalbumin peptide sequence, SIINFEKL, demonstrated that antigens of lesser affinity, manipulated by altering the N residue in position 4 of the peptide, combine in a predictable, additive manner (Marchingo, Kan et al. 2014). Strikingly, and somewhat surprisingly, additional signals, either co-stimulation, or soluble cytokines, also added to division linearly, thus increasing cell number exponentially, demonstrating that the two-signal theory is an oversimplification of the processes involved in regulating a T cell response (Gett and Hodgkin 2000, Marchingo, Kan et al. 2014). In addition to division and death, differentiation has also been studied using these experimental systems. Murine and human B cell differentiation to ASC and Ig isotype switching is closely linked with the number of times a cell divides, and influenced by production of cytokines from T cells (Hodgkin, Lee et al. 1996, Tangye, Ferguson et al. 2002).

From these studies, it is clear that incorporating quantitative assays of lymphocyte behaviour with models of immunodeficiency, and autoimmune disease, could reveal novel insights into the pathogenesis of disease and to identification of targeted therapeutic strategies. Furthermore, knowledge of these parameters, and testing of the impact of therapeutic agents is likely to enable accurate predictions with regard to the impact of manipulating immune responses in a greater range of diseases and could offer the potential for individualised immunomodulation.

1.6 Treatment of PAD

1.6.1 'One-size fits all' approach is flawed in most cases of PAD

The main strategy of therapy for PADs has been to replace the 'missing piece' with immunoglobulin derived from pooled sera of 100s of blood donors. This is a resource intensive therapy, of significant cost, and is insufficient to treat many of the other non-infectious complications of disease (Chapel, Lucas et al. 2008). Morbidity and mortality of PADs is increased in individuals with immune dysregulation (i.e. autoimmune disease, inflammatory conditions and malignancy) (Resnick, Moshier et al. 2012, Slade, Bosco et al. 2018). Unfortunately, for the most part when these complications arise, individuals are treated with standard treatments for autoimmune disease and malignancy, yet they are particularly susceptible to their adverse effects due to immunosuppression. In addition, the pathogenesis of the underlying autoimmune condition is likely to be different for patients with PADs, than it is for individuals with common autoimmune diseases, yet they are treated the same.

Another complicating factor is that many patients are treated for disorders of immune dysregulation prior to the diagnosis of an immunodeficiency. This makes differentiating a PID from a secondary immunodeficiency difficult. Furthermore, understanding these complications of immune dysregulation, by determining the molecular mechanisms of disease pathogenesis, is likely to lead to improved diagnosis and therapies (Lee, Moon et al. 2016).

1.6.2 Precision diagnosis has led to advances in the treatment of some PIDs

Identifying the underlying genetic defect of PID may offer some benefit in and of itself in terms of genetic counselling, and fertility planning. However, the main goal is to improve overall morbidity and mortality of disease. The most significant impact of a genetic diagnosis would be an indication for HSCT in an individual with a CID where sufficient evidence exists to be certain the risk of HSCT outweighs the known long-term outcome of disease. In other instances, where HSCT is not appropriate, a therapeutic that specifically targets the underlying molecular defect may be

available. For example, in WHIM syndrome, which is due to a GOF mutation in *CXCR4,* a small molecule inhibitor of the chemokine receptor, first developed for use clinically to enhance mobilisation of HSCs for autologous transplantation (Bilgin and de Greef 2016), has been shown to be effective in treatment of the WHIM syndrome (McDermott, Liu et al. 2011, McDermott, Liu et al. 2014) (Table 1.1). A clinical trial of an oral CXCR4 antagonist is currently underway in Australia, and internationally, for WHIM syndrome.

Additional examples of precision therapies that have arisen from understanding genetic etiologies of more common PIDs and are summarised in Table 1.1. For example, the treatment options for patients with CTLA4 haplo-insufficiency (OMIM#123890) and LRBA deficiency (OMIM#606453) has been significantly improved with abatacept, a CTLA4-Ig fusion molecule (Lee, Moon et al. 2016), originally developed to treat more common autoimmune diseases, such as rheumatoid arthritis. Ideally, registered clinical trials will be carried out, in order to provide more robust long-term evidence for this therapy. Another PID with great potential for personalised therapeutics, is *A*ctivated *P*I3-kinase *D*elta *S*yndrome (APDS, OMIM #615513), arising as an autosomal dominant immunodeficiency due to *PIK3CD* mutations that lead to a gain-of-function (GOF) of the p110δ subunit of PI3kinase (Angulo, Vadas et al. 2013), or LOF in *PIK3R1*, the regulatory subunit of p110δ (Lucas, Zhang et al. 2014). These patients may benefit from targeted inhibitors, already used in clinical practice for lymphoproliferative disease, such as idealisalib (Dornan, Siempelkamp et al. 2017), or leniolisib, which has been studied in a small group of patients and found to be safe and effective (Rao, Webster et al. 2017). Interestingly, PI3kinase inhibitors may have potential benefit in other conditions due to impaired class-switching and somatic hypermutation, as idealisalib therapy has been shown to increase these processes by an AID-dependent mechanism (Compagno, Wang et al. 2017, Avery, Kane et al. 2018).

Dysregulated immune responses are a major cause of morbidity in PIDs, and for some conditions,

novel therapeutic strategies have arisen due to understanding the pathomechanism of disease complications. For example, modulating the effect of increased JAK-STAT signaling in a group of patients with *STAT1* or *STAT3* GOF mutations, by the use of ruxolitinib, a JAK1 and JAK2 inhibitor, or tofacitinib, a JAK1 and JAK3 inhibitor, was observed to improve features of immune dysregulation (Forbes, Vogel et al. 2018). Additional targeting of the downstream effects of enhanced STAT3 signaling by blocking the inflammatory cytokine, IL-6, was of additional benefit in some patients with disease refractory to JAK inhibition alone (Forbes, Vogel et al. 2018).

Table 1.1 Precision Therapies for monogenic PIDs (excluding gene therapies and HSCT*)*

PID	Major Clinical Features	Gene Defect	Precision Therapy (mechanism)	References
WHIM syndrome	Neutropenia, leukopenia, susceptibility to HPV infections, sinopulmonary infections	*CXCR4* GOF	Plerixafor (CXCR4 antagonist) Gardasil (HPV vaccine)	(McDermott, Liu et al. 2011, McDermott, Liu et al. 2014)
STAT1 GOF	Severe candidal infections, thyroid autoimmunity, risk of esophageal cancer	*STAT1* GOF	Ruxolitinib (JAK1/2 inhibition)	(Forbes, Vogel et al. 2018)
CHAI, LATAIE	Autoimmune cytopenias, enteropathy, infections, low Ig, low B cells	*CTLA4* or *LRBA*	Abatacept (CTLA4-Ig replacement)	(Lee, Moon et al. 2016, Schwab, Gabrysch et al. 2018)
IPEX	Enteropathy, severe autoimmunity, allergic disease	*FOXP3* LOF	Sirolimus (mTOR inhibition)	(Yong, Russo et al. 2008)
APDS, PASLI	Lymphadenopathy, autoimmunity, enteropathy, susceptibility to herpes virus	*PIK3D* GOF or *PIK3R1* LOF	Sirolimus (mTOR inhibition) Idealisilib, leniolisib (PI3kinase inhibition)	(Rao, Webster et al. 2017)
STAT3 GOF	Polyautoimmunity	*STAT3* GOF	Tofacitinib (JAK1/3 inhibition) Tocilizumab (anti-IL6R antagonism)	(Forbes, Vogel et al. 2018)

(WHIM, warts, hypogammaglobulinemia, infections and myelokathexis; CHAI/LATAIE, CTLA4 haploinsufficiency and autoimmune infiltrates/ LRBA deficiency with autoantibodies, regulatory T (Treg) cell defects, autoimmune infiltration, and enteropathy; IPEX, immunodeficiency, polyendocrinopathy, enteropathy, X-linked; APDS/PASLI, activated PI3-kinase delta syndrome/ p110δ-activating mutation causing senescent T cell, lymphadenopathy, and immunodeficiency; CXCR4, chemokine receptor 4; STAT, signal transducer and activator of transcription; FOXP3, forkhead box protein 3; HPV, human papilloma virus; JAK, janus kinase; mTOR, mammalian target of rapamycin)

1.7 Hypotheses and Aims

Since Bruton first described XLA in 1957, incremental advances have been made towards greater understanding of the pathogenesis of PIDs. However, at the start of this project, in 2014, it was evident that there were many more opportunities for discovering novel monogenic causes of disease, as well as a need for greater understanding of the drivers of disease complications. Within this thesis, I set out to investigate the most common group of disorders, PADs, firstly by establishing a clinically characterized cohort of patients, and then by detailed genomic and functional studies.

Hypotheses:

1. Early onset and familial PADs, or PADs affecting consanguineous individuals are largely monogenic disorders where candidate genes can be identified via genomic sequencing.
2. Functional studies of patients' primary cells will provide a relevant cellular phenotype that demonstrates a causal relationship between the candidate genotype and the clinical phenotype.
3. Small differences in lymphocyte behaviours, such as division, survival and differentiation combine to generate a dysfunctional immune system in human immune disorders and the relative contribution of each can be identified using quantitative methods of lymphocyte behaviour in animal models of disease.

Aims:

1. To establish a cohort of patients with PADs and characterise the diagnostic and clinical features of disease (Chapter 3)
2. To identify novel and pathogenic genetic variants, present in individuals with PADs, by genomic sequencing that are responsible for disease (Chapters 4, 5 and 6).
2. By performing functional studies of immune cells, demonstrate a relevant cellular phenotype that shows a causal relationship between the candidate genotype and the clinical phenotype (Chapters 5 and 6).
3. To study the activation and sensitivity of mutant T and B cells to various stimuli in order to understand how such gene defects influence the immune response (Chapter 5).

CHAPTER 2: MATERIALS AND METHODS

2.1 Introduction

Materials used for experimental work are listed in section 2.2, and where reagents differ for mouse and human experiments, this is specified. The methods are divided in 2 sections, the first for mouse experiments (data presented in Chapter 5) and human experiments (data presented in Chapters 4, 5 and 6). Additional methods are presented in the publication: "*Delayed diagnosis and complications of Predominantly Antibody Deficiencies in a cohort of Australian adults*", which constitutes Chapter 3.

2.2 Materials

Table 2.2.1 Buffers

Buffer/media	Reagents	Supplier
B/T cell medium	RPMI 1640	GIBCO/Invitrogen
	10% heat inactivated fetal calf serum	Invitrogen
	0.01 M HEPES	GIBCO/Invitrogen
	1 mM sodium pyruvate	GIBCO/Invitrogen
	0.1 mM non-essential amino acids	GIBCO/Invitrogen
	2 mM L-glutamine	Sigma
	50μM β-mercaptoethanol	Sigma
	2mM Glutamax	Sigma
	110μg/ml streptomycin; 100U/ml penicillin	Invitrogen
	100μg/ml normocin	Invitrogen
MACS buffer	Phosphate Buffered Saline	WEHI media
	2 mM EDTA	Sigma

	0.5% Bovine serum albumin	Sigma
FACS buffer	Phosphate Buffered Saline 0.1% Bovine serum albumin	WEHI media Sigma
Triton Lysis buffer	1mM HEPES 100 mM NaCl 1 mM EDTA 10% (v/v) glycerol 1% (v/v) Triton X-100	WEHI media Sigma Sigma Sigma Sigma
Laemmli Buffer	277.8mM Tris-HCL 4.4% sodiumdodecyl sulfate 44.4s (V/v) glycerol 0.02% bromophenol blue	Sigma Sigma Sigma Sigma
MOPS Buffer		

Table 2.2 Tissue culture stimuli

Reagent	**Clone**	**Concentration**	**Supplier**
Purified anti-CD3 mAb	145-2C11	10μM	WEHI antibody facility
Purified anti-CD28 mAb	37.51	as stated	WEHI
Recombinant human IL-2	N/A	as stated	PeproTech
Purified anti-mouse IL-2 mAb	S4B6	25μg/ml	WEHI
CpG 1668	N/A	as stated	GeneWorks
Recombinant human IL-4	N/A	as stated	WEHI
Purified anti-CD40 mAb	IC-10	as stated	WEHI

Table 2.3 Cell isolation reagents and cell division tracking dyes

Reagent	Supplier
EasySep Mouse CD8α$^+$ T cell isolation kit	Stem Cell Technologies
EasySep Mouse B220$^+$ B cell isolation kit	Stem Cell Technologies
EasySep Human CD14$^+$ Monocyte isolation kit	Stem Cell Technologies
EasySep Human CD3$^+$/CD4$^+$/CD45RO$^+$ T cell isolation kit	Stem Cell Technologies
CellTrace Violet proliferation kit	Invitrogen
Ficoll-Paque	GE Healthcare (Uppsala, Sweden)
Percoll	Sigma-Aldrich
Rainbow calibration beads	BD Biosciences
Propridium-iodide	BioLegend

Table 2.4 Antibodies for flow cytometry (mouse)

Antibody	Clone	Supplier	Concentration
FITC rat anti-CD8α	OX-8	BD Biosciences	1:20
PE rat anti-CD3	1F4	BD Biosciences	1:20
PE Cy7 anti-CD19	1D3	BD Biosciences	1:20
APC rat anti-B220	RA3-6B2	BD Biosciences	1:10
FITC anti-IgM	II/41	BD Biosciences	1:20

Table 2.5 Antibodies for flow cytometry (human*)*

Antibody	Clone	Supplier	Concentration
Pacific Blue anti-CD3	OKT3	BD Pharmingen	1:20
BV510 anti-HLA-DR	L243	BioLegend	1:5
BV605 anti-CD25	BC96	BioLegend	1:5
BV650 anti-CD127	AO19D5	BioLegend	1:5
BV711 anti-PD1	EH12.2H7	BioLegend	1:5
FITC anti-CXCR5	RF8B2	BD Horizon	1:10
Alexa-Fluor788 anti-CCR7	150503	BioLegend	1:20
PE anti-CD45RO	UCHL1	eBioscience	1:20
PECy7 anti-CD45RA	HI100	eBioscience	1:20
APC anti-CD38	LS198-4-3	BD Pharmingen	1:20
APC-EF780 anti-CD8	SK1	eBioscience	1:20
APC-H7 anti-CD8	SK1	eBioscience	1:20
BUV395 anti-CD20	2H7	BD Horizon	1:5
BV650 anti-CD19	B-ly4	BioLegend	1:5
EF450 anti-IgM	SADA4	eBioscience	1:20
BV510 anti-IgD	IA6-2	BioLegend	1:5
FITC anti-CD27	M-T271	Miltenyi Biotec	1:20
PerCP-Cy5.5 anti-CD38	HIT2	BD Pharmingen	1:10
PE anti-IgA	IS11-8E10	Miltenyi Biotec	1:20
PE-CF594 anti-CD10	HI10A	BD Horizon	1:10
PE-Cy7 anti-IgG	G18-145	BD Pharmingen	1:10
APC anti-CD21	B-ly4	BD Pharmingen	1:20

PerCP anti-CD14	23-3781-00	BD Biosciences	1:10
PE anti-CD123	7G3	BD Pharmingen	1:20
PE-Cy7 anti-CD56	NCAM16.2	BD Biosciences	1:10
APC anti-CD11c	B-ly6	BD Pharmingen	1:20
APC-H7 anti-CD16	368	BD Pharmingen	1:20

Table 2.6 Antibodies for Western blotting (human)

Protein target	**Species**	**Clone**	**Epitope**	**Size (kDa)**
Phospho-NF-κB1 p105	Rabbit	18E6	Ser933	120
Phospho-NF-κB2 p100	Rabbit	Q5U2Z4	Ser866/870	110
NF-κB1 p105/p50	Rabbit	D7H5M	Gly415	120/50
NF-κB1 p105/p50	Rabbit	D4P4D	N-term	120/50
NF-κB1 p105	Rabbit	polyclonal	C-term	120
NF-κB2 p100/p52	Rabbit	D7A9K	N-term	120/52
Phospho-NF-κB p65	Rabbit	93H1	Ser536	65
NF-κB p65	Rabbit	D14E12	Glu498	65
RelB	Rabbit	C1E4	Ser424	70
c-Rel	Rabbit	D3B8S	C-term	78
Phospho-IκBα	Rabbit	14D4	Ser32	40
IκBα	Rabbit	L35A5	N-term	39
Phospho-IKKα/β	Rabbit	16A6	Ser176/180	85, 87
IKKβ	Rabbit	D30C6	C-term	87
NF-κB p65	Mouse	L8F6	C-term	65
IκBNS	Rabbit	Polyclonal	N-term Rabbit	27 1:1000

Table 2.7 Immunoassays

Immunoassay Kit	Supplier
Ready-Set-Go ELISA	Bio-Rad
Bio-Plex Pro Human Cytokine Screening Panel 48-Plex	Bio-Rad

Table 2.8 Primers for Sanger Sequencing

Gene	Reverse Primer Sequence	Forward Primer Sequence	Supplier
NFKB2	3'-GTTGCTGTACCGTAAGTTGG-5'	3'-GGTGAAAGAAGACAGTGCCTAT-5'	GeneWorks
NFKBID	3'-AACCCCACTCTGGTTCAGC-5'	3'-GCTGCTCATTCTCCAGGTTG-5'	GeneWorks

2.3 Methods: Mouse Experiments

All animal experiments were carried out with ethics approval from the WEHI Animal Ethics Committee (2016.015). NZB/W mice were a gift from Dr Andrew Lew (WEHI). NZB and NZW mice were obtained from the WEHI animal facility, Kew. *Nfkb2*$^{-/-}$ and *Nfkb2*$^{+/-}$ mice were provided by Dr Lorraine O'Reilly (WEHI). All mice were maintained under pathogen-free conditions, and used between 7 – 14 weeks of age.

2.3.1 Murine lymphocyte preparation

Lymph nodes (except mesenteric) or spleen were harvested from age and gender matched mice. Murine CD8$^+$ T lymphocytes were isolated using EasySep Mouse CD8$^+$ T cell isolation kit (StemCell technologies) according to the manufacturer's instructions. Purity of the isolate was confirmed by staining for CD3 and CD8α and determining the percentage of the population expressing this marker by flow cytometry (typically >95%). Murine naïve B cells were isolated

using a percoll gradient and EasySep Mouse cell $B220^+$ isolation kit (StemCell technologies). Purity was confirmed by staining for CD19, B220 and IgD and IgM (Table 2.4), and was >95%.

2.3.2 Division-tracking dye labelling

Isolated murine cells were labelled with the cell tracking dye, CellTrace Violet (Invitrogen) at 5 μM for 20 minutes at 37°C (Figure 2.1A) at a density of $2x10^7$ cells/mL in PBS 0.1% BSA. Cells were washed twice in ice-cold media, and counted before being placed into culture.

2.3.3 Cell culture

Isolated, labelled, murine cells were cultured in RPMI containing non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, 2-mercaptoethanol, 2 mM L-glutamine and 10% FCS. For T cell experiments, murien CD4+ or CD8+ T cells were cultured with anti-mouse IL-2 blocking antibody (clone S4B6) at 25 μg/L, blocking any endogenous IL-2 production so that the only IL-2 stimulus received was that from the controlled concentration of the recombinant human IL-2 and or anti CD28, where stated. When anti-CD3 stimulation was performed, flat-bottomed plates were coated overnight at 4°C with 10 μg/L of the anti-CD3 antibody (clone 2C11) in PBS, and washed 3 times in PBS. Cells were incubated at 37°C in 5% CO2. Addition of other stimuli including anti-CD28, recombinant human IL-2 for T cell assays. B cells were cultured with CpG 1668, or IL-4 and anti-CD40 at concentrations as stated in results section.

2.3.4 Flow Cytometry

Samples were acquired for analysis on FACS Canto II (BD Biosciences) at uniform time-points, typically twice daily over 7 days, to measure the kinetics of the lymphocyte response. The data were analysed using FlowJo software (Treestar) (Figure 2.1A). Cell counts were performed by the addition of $1x10^5$ calibration beads (CaliBRITE, BD Biosciences) to each sample prior to acquisition. Propidium Iodide (PI) was added with the beads in order to distinguish dead from live cells. The ratio of live cells to beads was used to calculate the absolute cell number per well. The total live cell count (Figure 2.1B), and cell count per division was determined at each time-point, in order to determine mean division number over time (Figure 2.1C). Additional calculations were performed using Microsoft Excel 2011. The precursor cohort method was utilised to determine the number of starting cells that contributed to the total cell count in each division, in order to determine cell survival over time (Figure 2.1D). This method removes the effect of division by dividing the total live cell number/division by 2^i, where *i* is the number of divisions those cells have undergone as described. GraphPad PRISM was used for graphical presentation of the data and statistical calculations. Additional measures of division, namely time to first division, division destiny, destiny time could be determined (Figure 2.1 E, F).

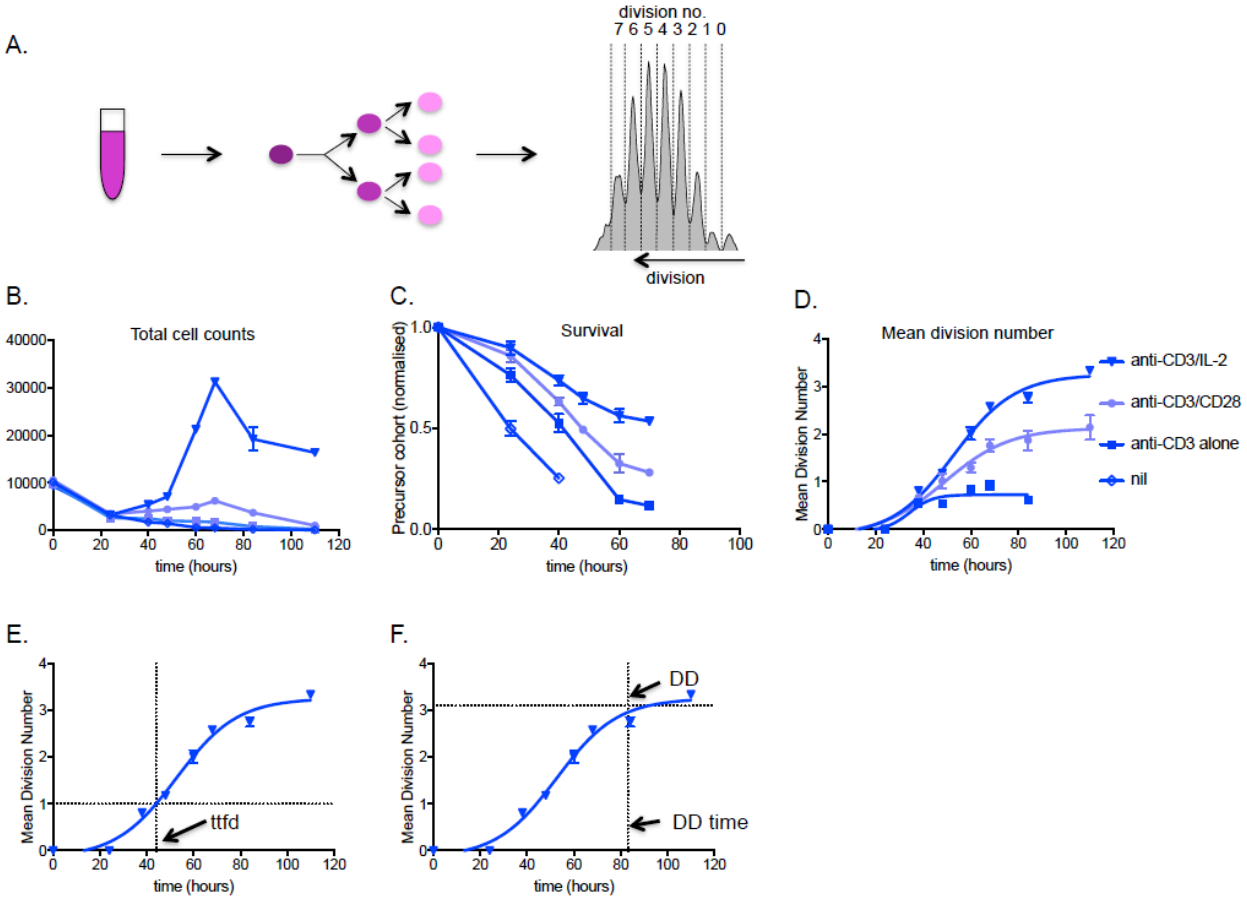


FIGURE 2.1. QUANTITATIVE ASSAYS OF LYMPHOCYTE BEHAVIOR.

Populations of purified lymphocytes can be studied to determine a number of parameters regarding cellular behavior. **(A)** After purification, cells were labeled with CTV and placed in culture as described. Cells were harvested at regular intervals, and analysed by flow cytometry to determine cell number, and division history. Representative plot of division peaks at right. **(B)** Total number of cells determined at each timepoint, (n = (no. beads added/ beads counted) x no. cells counted) for the duration of the experiment, for each stimulation condition tested; **(C)** Precursor cohort number represents survival over time, and is determined by counting the number of live cells per well and correcting for division using the formula: *Precursor cohort number = total cell count in generation i* $\div 2^i$ **(D)** The average number of cell divisions (mean division number) is determined by counting the number of cells in each generation over time and dividing by the cohort number (data for unstimulated cells not shown as there is no cell division); **(E)** Time to first division (*ttfd*) is determined by the point on the x axis, where y = 1 (indicated in dashed lines.) **(F)** Division destiny (*DD*), and destiny time (*DD time*) can be determined by the value of y, and x, respectively, at the plateau of the division curve (indicated in dashed lines).

2.4 Methods: Human experiments

2.4.1 Ethics approvals and consent

Two separate projects involving human subjects generated results presented in this thesis and both are described below, as a 'Novel gene discovery project', and a 'Clinical genomics project' respectively.

2.4.1.1 NOVEL GENE DISCOVERY PROJECT

The first was a research project referred to as 'Novel gene discovery project', approved by the Human Research and Ethics Committee (HREC) at RMH and WEHI (MH2009.162, WEHI 10/02). For this study, all participants were consented at the Royal Melbourne Hospital. Venesection was performed according to hospital protocols. Samples were processed within a minimum of 12 hours of collection. Healthy control blood samples were obtained from the Volunteer Blood Donor Registry (WEHI), following informed consent.

Inclusion criteria:

- individuals with a physician-determined diagnosis of any primary immunodeficiency
- capable of giving informed consent (or a guardian/parent capable of doing so)

Exclusion criteria:

- probable secondary immunodeficiency

Family members were also eligible for recruitment to the study, and detailed family histories and clinical histories were recorded in an electronic database (Excel, and RedCap).

2.4.1.2 CLINICAL GENOMICS PROJECT

The second project was part of the Melbourne Genomics Health Alliance study. Patients were predominantly identified retrospectively, from four major hospitals: RMH; RCH; The Austin Hospital and Monash Medical Centre (MMC) and were eligible for inclusion if a clinical diagnosis of a suspected or confirmed PID, Systemic Autoinflammatory Disease (SAID), or Hereditary Angioedema (HAE), had been made by a consultant physician (see below for clinical summaries). Individuals were ineligible if a previous genetic diagnosis to account for the immunological phenotype or other extensive genetic testing had already been performed. Only one patient per kindred was eligible in the case of a positive family history of disease. The project was ethically approved (MGHA; HREC/13/MH/326; WEHI 14/01) and carried out according to the principles of the Declaration of Helsinki.

Inclusion criteria

A suspected diagnosis of one of the following conditions (no. known pathogenic genes at commencement of study):

- PID – clinical diagnosis on the basis of susceptibility to infections (recurrent, severe, persistent or unusual), and/or immune dysregulation (>350 genes)
- SAID – clinical features of inflammatory disease in the absence of infective causes; characterised by periodic fevers; pustular, neutrophilic, erythroid or other skin manifestations; and systemic or organ/tissue-specific inflammation (>30 genes)
- HAE – episodic angioedema in the absence of urticaria (Types 1 and 2 with low levels and or function of C1 inhibitor; and HAE with normal C1 inhibitor) (2 genes)

Exclusion criteria:

- Probable secondary immunodeficiency
- Known genetic diagnosis
- Previous extensive genetic testing performed

2.4.2 Genomic DNA extraction

For the 'Novel gene discovery project', genomic DNA was extracted at WEHI from whole blood using Qiagen DNeasy blood and tissue extraction as per manufacturer's instructions, and quantified on a Nanodrop 1000 spectrophotometer (ThermoFisher). Typically yields of >20 ng/μL were obtained, with a purity of 240/260 nm considered suitable for sequencing. For the 'Clinical genomics project', genomic DNA was extracted from whole blood at the recruitment site, according to accredited diagnostic laboratory protocols.

2.4.3 Whole-exome sequencing

For both projects, sequencing of gDNA was performed at the Australian Genome Research Facility (AGRF) on either a HiSeq4000 or NextSeq500 (Illumina) using the Agilent SureSelect XT CREv2kit, with a targeted mean coverage of 100x. The minimum coverage was >90% of bases sequenced to at least 15x read depth. For the 'Clinical Genomics project', an additional 'SNP-ID' check was performed to confirm the identity of the sample.

2.4.4 Variant prioritisation and curation

2.4.4.1 NOVEL GENE DISCOVERY PROJECT PIPELINE

Raw WES data was aligned with Novoalign, read duplicates were removed with PicardTools, re-alignment performed with GATK and variants called with GATK's UnifiedGenotyper. LINKDATAGEN (LDG) was used to select SNPs from the WES data for a number of tests, including most likely ethnicity, signatures of inbreeding, and subsequently a linkage analysis. Variants were selected for further investigation based on a threshold for known minor allele frequency (MAF) <0.05 (or otherwise novel) in the Exome Sequencing Project (ESP6500); 1000Genome databases (http://1000genomes.org); Exome Aggregation Consortium (ExAC) http://exac.broadinstitute.org; Genome Aggregation Database (gnomAD) (http://gnomad.broadinstitute.org); and an in-house control database (WEHI). Clinical accredited reports were not generated as part of this process, although patients were referred for NATA-accredited testing for confirmation of relevant findings. Confirmation of genomic variants was performed by Sanger sequencing of the relevant regions of interest, and familial segregation was carried out where appropriate. Primers of relevance to the current study are listed in Table 2.2.6.

2.4.4.2 CLINICAL GENOMICS PROJECT PIPELINE

Analysis was performed using targeted gene lists according to the patient's clinical and immunological phenotype to ensure that only variants in known disease-associated genes were identified (Categories G2 and G4, Table 2.8). Gene lists were compiled using the IUIS classification of PIDs, which is a comprehensive classification of PIDs according to the immunological defects observed in reported disorders. Gene lists were initially based on the 2015 report (Picard, Al-Herz et al. 2015), and updated after the most recent publication in 2017 (Picard, Bobby Gaspar et al. 2017). Consensus for

genes included was obtained from a multidisciplinary panel comprising a clinical geneticist (Dr Zornitza Stark), clinical immunologist (Dr Charlotte Slade), a medical scientist with expertise in immunology (Dr Vanessa Bryant), and a clinician scientist with expertise in SAIDs (Dr Fiona Moghaddas). These gene lists are now available on PanelApp Australia (https://panelapp.agha.umccr.org.)

Certain genes listed in the IUIS tables were excluded when their clinical associations were not relevant to the cohort of patients, e.g. metabolic disorders and syndromes not present in the cohort, such as CHARGE syndrome, and rare bone marrow failure disorders. Some genes were also considered to be 'incidental' findings and were only included for certain cases, where they may have been relevant to the phenotype. Of particular note, constitutional mismatch repair defects, typically presenting as early onset malignancies, caused by biallelic damaging variants in *PMS2*, *MSH6*, *MSH2* and *MLH1*, may also present with a phenotype of hyper-IgM syndrome. In heterozygous state these variants are associated with hereditary non-polyposis colon cancer (HNPCC).

In certain cases, 'bespoke' gene lists were generated for patients with specific clinical features (e.g. ectodermal dysplasia, retinal dystrophy, coagulopathy, lymphangiectasia). These additional gene lists are included in the appendix (Appendix 2). In patients with complex presenting features, in particular where comorbid intellectual disability was present, the 'mendeliome' gene list was applied.

Table 2.9 Classification of genes for analysis

Gene category	Immune Disorder		
	PID	**SAID**	**HAE**
G1	Some evidence exists to link the gene to the disease. A 'speculative' set of genes for research purposes.	Some evidence exists to link the gene to the disease. A 'speculative' set of genes for research purposes.	Not applicable
G2	Genes with a known association to flagship disease (IUIS Tables, OMIM)		
G3	**Not applicable** for Immunology Flagship *(gene lists for syndromic diagnoses generated by software programs that match phenotypic features to genes)*		
G4	Manually generated list by clinicians directed by patient phenotype *(IUIS, OMIM, Pubmed)*		

(PID, Primary Immunodeficiency; SAID, Systemic Autoinflammatory Disorder; HAE, Hereditary Angioedema; IUIS, International Union of Immunologic Societies; OMIM, Online Mendelian Inheritance in Man)

Exome sequence data were generated as variant call format (VCF) files, and run through a variant caller and filtering strategy as previously described using C-pipe, an online, publicly available resource (Sadedin, Dashnow et al. 2015). Variants were prioritized for curation using LOVD plus v3.0 by a multidisciplinary expert consensus panel comprising a clinical geneticist, clinical immunologist, a medical scientist with expertise in immunology and a medical scientist with genomic expertise. The MAF of identified variants was obtained from the population databases listed above. *In silico* prediction tools used were: SIFT (http://sift.jcvi.org), Poly-Phen (http://genetics.bwh.harvard.edu/pph2), Mutation Taster (http://www.mutationtaster.org/) and CADD scores.

The initial prioritisation was based on the gene category, the mean allele frequency in population databases, conservation scores (condel) and annovar, and prioritisation assigned from V1 to V5 (Table 2.2). In addition to the frequency and conservation, variants were prioritised according to the zygosity being consistent with the predicted mode of inheritance. In some circumstances, variants were selected for curation if the zygosity was inconsistent, but only if the clinical phenotype was a very good match for the gene in which the variant was identified.

Further curation was performed using *in silico* prediction tools, literature searches, including, but not limited to PubMed, OMIM, and Google Scholar. A standardised spreadsheet was implemented in order to generate a scoring matrix for the classification of variants (Appendix 3.)

Table 2.10 Classification of variants for prioritising for curation

Variant Priority index		**Rule**
V1	**Rare** missense variants and non-frameshift indels	Variant has frequency ≤0.01 but >0.0005 (1 in 2000) in population databases
V2	**Novel or very rare** missense variants and non-frameshift indels	Variant not present in population databases or present at a frequency of <0.0005
V3	**Novel or very rare and conserved** missense variants and non-frameshift indels which are highly conserved	V2 **and** variant has Condel score > 0.7. If missing Condel score, then V3 if Annovar conserved.
V4	**Truncating variants** Variant predicted to truncate gene product	Rare (i.e. non-recurrent <0.01) frameshift indel, nonsense, +/- 1 or 2bp splice site
V5	**Known disease causing variants**	i.e. ClinVar, OMIM, LOVD

(Condel, ; ClinVar Clinical Variome, ; OMIM, Online Mendelian Inheritance in Man ; LOVD, Leiden Online Variation Database)

2.4.4.2.1 REPORTING (ONLY RELEVANT TO CLINICAL GENOMICS PROJECT)

Reporting of genomic variants was performed according to the American College of Medical Genetics (Richards, Aziz, et al. 2015) with a 5-tier classification of variants based on the likelihood of pathogenicity using the evidence gathered by the curation process. These five criteria are:

- Class 5 – pathogenic.
- Class 4 – likely pathogenic.
- Class 3 – uncertain significance.
- Class 2 – likely benign.
- Class 1 – benign.

In order to provide further information regarding the Class 3 variants (of uncertain significance), additional classification was performed according to the following criteria:

- 3a variants were considered highly likely to be of clinical significance, but the available evidence not strong enough for the variant to be considered more than 90% likely to be disease-causing. In those cases, segregation testing, or other functional studies, were recommended.
- 3c variants were classified as such if the variant was thought to be very unlikely to be disease causing, but there was insufficient evidence to be more than 90% certain it was benign.
- 3b variants were considered between 90% likely and 90% unlikely to be disease causing.
- In most cases a 3c variant was not communicated to a patient, whereas all 3a and 3b variants were reported, unless the zygosity of the variant was inconsistent with known disease pathogenesis.

2.5 Functional studies

2.5.1 Cell preparation

Up to 100 ml of blood in EDTA tubes was collected from a patient or healthy donor at a time. PBMCs were isolated from whole blood taken from patients or healthy volunteers via Ficoll-Paque density centrifugation, with Leucosep^{TM} tubes. Isolated PBMCs were manually counted using a haemocytometer, with cell viability >90% (assessed by Trypan Blue exclusion), and suspended in fetal calf serum (FCS) with 10% dimethyl sulphoxide, at a maximum density between $2.5x10^7$ cells/mL. Cells were then frozen in cryovial tubes at a rate of -1°C per minute, to -80°C. Samples were transferred to liquid nitrogen for longer term storage. To thaw, frozen PBMCs were rapidly thawed at 37°C before suspensions were added drop-wise to a large volume of pre-warmed RPMI 1640 with 1% FCS with gentle agitation. Cells were manually counted using a haemocytometer, and cell viability was assessed by Trypan Blue exclusion.

For studies of isolated cell populations, cells were purified as follows: monocytes were isolated using the EasySep Human $CD14^+$ Monocyte isolation kit, and memory $CD4^+$ T lymphocytes were isolated using $CD4^+/CD45RO^+$ T cell isolation kit (StemCell technologies) according to the manufacturer's instructions. Purity of the isolate was confirmed by determining the percentage of the population expressing CD14, and co-expressing CD4 and CD45RO, respectively, by flow cytometry (typically >90-95%).

2.5.2 Immunophenotyping

Cells were stained with selected antibody mixes for phenotyping of immune subsets (Table 2.5). Samples were acquired on a 5-laser BD Biosciences LSR Fortessa X20 along with single colour compensation controls of antibody-conjugated beads (UltraComp eBeads, eBioscience), Live/Dead (Fluoro-Gold) controls and unstained controls. At least 400,000 events were collected per sample. The data were analysed using FlowJo software (Treestar).

2.5.3 Western Blotting

Prior to stimulation, PBMCs were counted and seeded at 1 x 10^6 cells/ml in 6-well plates and rested in RPMI 1640 supplemented with 1% FCS for up to 4 hours at 37°C, 5% CO_2 to synchronise cells into a resting state. Cells were then cultured at 37°C, 5% CO_2 in tissue culture medium alone, or stimulated by supplementing tissue culture medium with stimuli at the indicated times.

To prepare cell lysates for Western blot, cells were harvested at indicated times by washing with ice-cold PBS before being resuspended in Triton lysis buffer (Tris pH 7.4, 20 mM; NaCl, 150 mM; EDTA, 1 mM; Triton X-100, 1% (v/v); glycerol, 10% (v/v)) at 2 x 10^7 cells/mL with additional protease inhibitor and incubated for 30 minutes on ice. Lysates were then centrifuged at 13,000 rpm for 5 minutes (or stored at -80°C prior to centrifugation). The resultant supernatant was collected and 6x Laemmli buffer was added before boiling the sample at 90°C for 5 minutes. Samples were then separated by electrophoresis on 4-12% BisTris polyacrylamide gels in MOPS buffer and transferred to a nitrocellulose membrane via the Bio-Rad Trans-Blot Turbo Transfer System. Membranes were then blocked with 5% (w/v) skim milk in PBS with

0.1% (v/v) Tween-20 for at least 30 minutes at room temperature with gentle agitation. Protein detection was performed with incubation of primary antibodies overnight at 4°C. To detect primary antibodies, secondary anti-rabbit IgG antibodies conjugated to horseradish peroxidase (HRP) were used (Santa Cruz Biotechnology, #2030, or Cell Signaling Technology, #7074; both diluted to 1:3,000 in 5% (w/v) skim milk in PBS with 0.1% (v/v) Tween-20), and membranes incubated at room temperature for 1 hour. To develop membranes, approximately 1 mL of Luminata Forte Western HRP substrate (Millipore, WBLUF0500) was added to coat the membrane and incubated at room temperature for 1 minute. Excess HRP substrate was removed before acquiring membrane images using a Bio-Rad ChemiDoc system, and analysed using ImageLab software (Bio-Rad).

2.5.4 Enzyme-linked Immunosorbent Assays

Human IL-1β, IL-6 and TNF were quantified using Invitrogen™ Ready-Set-Go! ELISA kits supplied by ThermoFisher. The manufacturer's instructions were followed with the exception of increasing the top value of the standard curve to 2000 pg/ml. Absorbance was read using a Versamax plate reader (Molecular Devices, CA, U.S.A). Results were analysed using SoftMax Pro software (Molecular Devices, CA, U.S.A).

2.5.5 Bio-Plex® Cytokine Assays

The BioPlex® pro human cytokine screening panel (48-plex) was performed according to manufacturer's protocol, using undiluted human serum, and standards as provided in

the kit. Samples were quantified using a Bio-Plex® 200 plate reader, and data analysed using Bio-Plex® datapro software.

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Delayed Diagnosis and Complications of Predominantly Antibody Deficiencies in a Cohort of Australian Adults

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Background: Predominantly antibody deficiencies (PADs) are the most common type of primary immunodeficiency in adults. PADs frequently pass undetected leading to delayed diagnosis, delayed treatment, and the potential for end-organ damage including bronchiectasis. In addition, PADs are frequently accompanied by comorbid autoimmune disease, and an increased risk of malignancy.

Objectives: To characterize the diagnostic and clinical features of adult PAD patients in Victoria, Australia.

Methods: We identified adult patients receiving, or having previously received immunoglobulin replacement therapy for a PAD at four hospitals in metropolitan Melbourne, and retrospectively characterized their clinical and diagnostic features.

Results: 179 patients from The Royal Melbourne, Alfred and Austin Hospitals, and Monash Medical Centre were included in the study with a median age of 49.7 years (range: 16–87 years), of whom 98 (54.7%) were female. The majority of patients (116; 64.8%) met diagnostic criteria for common variable immunodeficiency (CVID), and 21 (11.7%) were diagnosed with X-linked agammaglobulinemia (XLA). Unclassified hypogammaglobulinemia (HGG) was described in 22 patients (12.3%), IgG subclass deficiency (IGSCD) in 12 (6.7%), and specific antibody deficiency (SpAD) in 4 individuals (2.2%). The remaining four patients had a diagnosis of Good syndrome (thymoma with immunodeficiency). There was no significant difference between the age at diagnosis of the disorders, with the exception of XLA, with a median age at diagnosis of less than 1 year. The median

age of reported symptom onset was 20 years for those with a diagnosis of CVID, with a median age at diagnosis of 35 years. CVID patients experienced significantly more non-infectious complications, such as autoimmune cytopenias and lymphoproliferative disease, than the other antibody deficiency disorders. The presence of non-infectious complications was associated with significantly reduced survival in the cohort.

Conclusion: Our data are largely consistent with the experience of other centers internationally, with clear areas for improvement, including reducing diagnostic delay for patients with PADs. It is likely that these challenges will be in part overcome by continued advances in implementation of genomic sequencing for diagnosis of PADs, and with that opportunities for targeted treatment of non-infectious complications.

Keywords: predominantly antibody deficiency, primary immunodeficiency, diagnostic delay, common variable immunodeficiency, X-linked agammaglobulinemia, immunoglobulin subclass deficiency, specific antibody deficiency

INTRODUCTION

Primary immunodeficiencies (PIDs) are a heterogeneous group of diseases, characterized by an impaired immune response to pathogens, predisposing to more frequent and severe infection, in some instances to a single pathogen, and dysregulated immune function, which may result in autoimmune disease or inflammatory conditions (1). Knowledge of the key molecular processes underpinning these varied disorders continues to evolve with advances in genomic technology. The archetypal classification of immunologic disorders has recognized predominantly antibody deficiency (PAD) as the most prevalent PID, and these patients require lifelong antibody replacement therapy (2, 3). In comparison with other countries, the prevalence of PADs in Australia has not been clearly established. There are only two reports over the last two decades with varied results that are likely to represent significant under reporting, due to ascertainment bias (4, 5).

Diagnosis is challenging because PADs have varied clinical presentations and may present from infancy to late adulthood. The hallmark clinical feature is a history of recurrent sinopulmonary bacterial infections, resulting from an ineffective antibody response, however, in many cases, non-infectious complications, such as autoimmune disease, or malignancy may complicate the clinical presentation.

The most profound PAD is agammaglobulinemia with a complete or near-complete absence of serum Ig and absence of mature B cells in blood. Most of these individuals suffer from X-linked agammaglobulinemia (XLA) which occurs due to mutations in the gene encoding Bruton's tyrosine kinase (*BTK*), an enzyme essential for B-cell development (6). Defects in other genes crucial for B-cell development and survival have been identified in a smaller proportion of patients (7–10), currently leaving only 5–10% without a genetic diagnosis.

Common variable immunodeficiency (CVID) constitutes the majority of PAD cases which require ongoing treatment, with a global incidence of approximately 1:25,000 (11) although this varies according to the population studied. A recent Finnish study found the prevalence to be as high as 6.9 per 100,000 (12). CVID has complex clinical features and pathophysiology, underpinned by identifiable monogenic defects in a minority of patients (13–15). Patients may present with a spectrum of manifestations, including recurrent infections and autoimmune cytopenias. Several diagnostic criteria, based upon a combination of clinical and laboratory features, may be used to aid in diagnosis and classification of patients with these complex presentations (16–18). A diagnosis may be made from 2 years of age (19).

The spectrum of PAD also includes milder syndromes, albeit with variable clinical features that include selective IgA deficiency; unclassified hypogammaglobulinemia (HGG), characterized by reduced levels of IgG in the presence of normal IgA and IgM; IgG subclass deficiency (IGSCD), characterized by a reduction in one or more IgG subclasses with normal total IgG; and specific antibody deficiency (SpAD), characterized by normal serum immunoglobulins with an apparent failure to produce antibody in response to vaccines. Thymoma with immunodeficiency, otherwise eponymously known as Good syndrome, is a rare PID associated with the presence of a thymoma, although the mechanism is not well understood (1, 20–22).

We sought to identify the diagnostic approaches that had been used for PADs and potential obstacles to diagnosis. In addition we aimed to characterize the infectious and non-infectious manifestations in our patients, such as organ-specific or systemic autoimmune disease as well as malignancies such as lymphomas and gastrointestinal (GI) tumors.

MATERIALS AND METHODS

Patients

We performed a comprehensive cross-sectional clinical analysis of adult patients with PADs managed under clinical immunology services in Victoria from January 2001 to February 2017. Patients who were currently receiving, or had previously received replacement immunoglobulin for PADs were identified by physicians specializing in Clinical Immunology at four major teaching hospitals in metropolitan Melbourne, Victoria: The Royal Melbourne Hospital, The Alfred Hospital, Monash Medical Centre and Austin Health. Patients with secondary hypogammaglobulinemia were

excluded, as were individuals with a defined combined immunodeficiency, or a history of hematopoietic stem-cell transplantation. To ensure that the cohort included patients with clinically significant disease, and receiving regular follow-up, we did not include patients who were not deemed to require replacement immunoglobulin therapy. The study was carried out according to the principles of the Declaration of Helsinki and was approved by local human research ethics committees (Melbourne Health projects 2009.162, 2013.245; Walter and Eliza Hall Research Institute projects 10/02, 14/01; Alfred Health 109/15, 277/17). All living patients, or their next of kin, consented to the collection of their medical information. For those individuals who were deceased at the time of the data collection, ethical approval was obtained to review the medical records without the consent of the next of kin.

Data Collection

Data were retrospectively collected from the clinical case notes using a pre-specified template to capture age of reported onset of symptoms, age at diagnosis and organ-specific manifestations that included respiratory, GI, neurological, musculoskeletal, organomegaly, and malignancy. We defined immune dysregulation on clinical notes reporting any of the following diagnoses: autoimmune disease, lymphoproliferative disease, organomegaly, granulomatous inflammation, and/or enteropathy.

Statistical Analysis

We examined differences between groups using one-way ANOVA for comparison of continuous variables; Chi square analysis for comparison of categorical variables with a high frequency, and Fisher's exact test for categorical variables with a frequency of less than 5; and Mantel–Cox log rank for comparing differences in survival between disease groups. As the data for age of onset, diagnosis and diagnostic delay were non-parametrically distributed, we have reported median values for these measures. A p value of less than 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 7 software.

RESULTS

Patient Demographics

179 patients with PAD were identified from the four centers. 98 patients were females, and 81 were males, with median age of 49 years (**Table 1**). CVID was the most frequent antibody deficiency disorder requiring clinical follow-up with 116 patients. XLA was diagnosed in 21 male patients. 22 patients had HGG, 4 had SpAD, and 12 were diagnosed with IGSCD. We also included four patients with thymoma and immunodeficiency, with a diagnosis of Good syndrome (**Table 1**).

Diagnostic Features

Age at Diagnosis and Diagnostic Delay

The median age at diagnosis for the entire cohort was 36 years, which ranged from diagnoses at birth to 80 years old. Patients with XLA were diagnosed at significantly younger ages than all other antibody deficiency disorders (**Figure 1A**; **Table 1**) and experienced significantly shorter delay from symptom onset to diagnosis (**Table 1**; Table S1 in Supplementary Material). With regards to CVID specifically, the median age of reported onset of symptoms was 20 years, and the median age of diagnosis was 35 years (**Table 1**; **Figures 1A,B**). The median diagnostic delay was available for only 83/116 CVID patients and was 9 years. The four patients with Good syndrome were diagnosed at a median age of 60.75 years (interquartile range 44.3–74), which was significantly older than XLA ($p < 0.0001$) and CVID ($p < 0.05$) diagnoses, but not the other antibody deficiency syndromes (**Table 1**; **Figures 1A,B**).

Serum Immunoglobulin Levels

The serum IgG levels at time of diagnosis of CVID were significantly lower than those recorded for patients with HGG (3.10 ± 1.55 versus 6.32 ± 4.67 g/L, respectively, $p < 0.0001$; **Figure 1D**). However, serum IgG levels for CVID and HGG were both significantly lower than in patients with a diagnosis of IgGSCD and SpAD ($p < 0.0001$; **Figure 1D**). Serum IgA levels were also significantly reduced in CVID patients, compared with other PAD, consistent with the diagnostic criteria (0.23 ± 0.28 versus 1.16 ± 1.05 g/L, $p = < 0.0001$; **Figure 1D**). Serum IgM levels in CVID (0.3 ± 0.36 g/L) were significantly lower compared with HGG (0.97 ± 0.6 g/L; $p < 0.0001$), and IGSCD (1.15 ± 0.66 g/L; $p < 0.0001$) (**Figure 1D**). Serum Ig levels at time of diagnosis were not available for most of the XLA patients, due to the historic nature of their diagnoses.

TABLE 1 | Diagnostic and demographic data of the Victorian adult predominantly antibody deficiency cohort.

Immunologic diagnosis	Number of patients (%)	Male:female ratio	Current age (years; range)	Median age at diagnosis (years)	Median age at symptom onset[a] (years; range)	Median diagnostic delay[a] (years; range)
All	179	0.82	49 (16–87)	36 (0–87)	20 (0–68)	7.5 (0–63)
Common variable immunodeficiency (CVID)	116 (64.8)	0.78	48.5 (16–80)	35 (2–80)	20 (1–65)	9 (1–63)
X-linked agammaglobulinemia (XLA)	21 (11.7)	N/A	31.5 (23–65)	0 (0–15)	0 (0–2)	1 (0–15)
Hypogammaglobulinemia (unclassified) (HGG)	22 (12.29)	0.16	54 (18–87)	43 (5–80)	19.5 (1–50)	4 (1–59)
Specific antibody deficiency (SpAD)	4 (2.23)	1.00	65.5 (43–81)	68 (33–72)	N/A	N/A
IgG subclass deficiency (IGSCD)	12 (6.7)	0.20	56 (30–81)	36 (7–74)	20 (2–68)	9 (6–16)
Good syndrome	4 (2.23)	3.0	69 (46–77)	60 (40–71)	58 (38–68)	2 (0–3)

[a]*Data for age at symptom onset and diagnostic delay available for the following numbers of patients in each group. CVID, 83/116; XLA, 16/21; HGG, 7/22; SpAD, 1/4; IGSCD, 7/12; Good syndrome 2/4.*

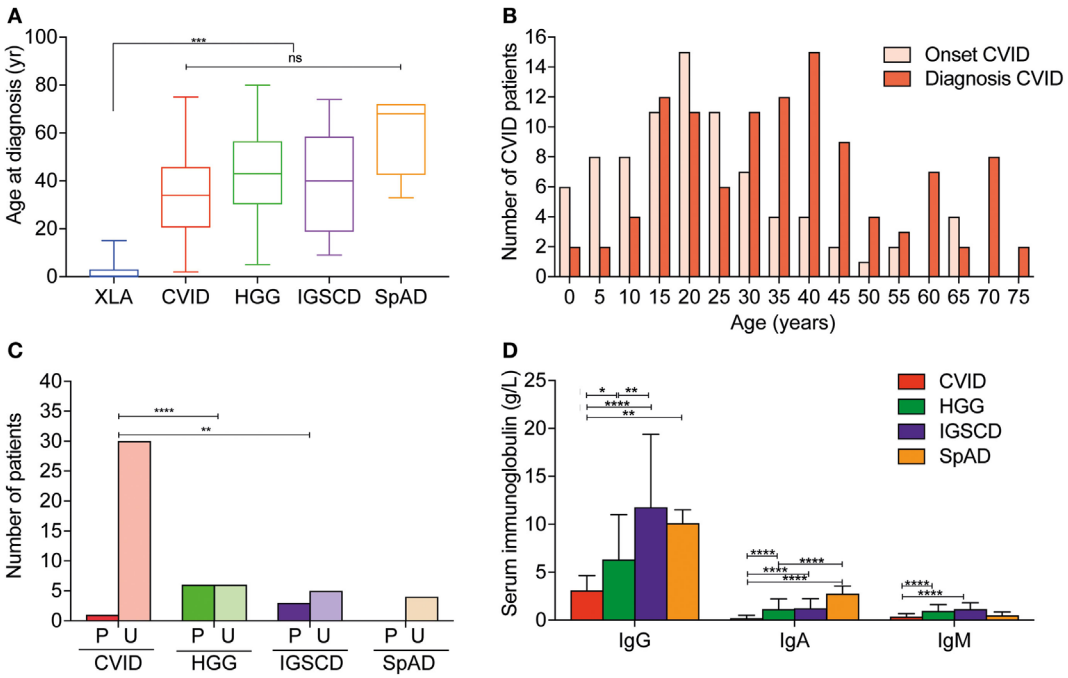


FIGURE 1 | Age at diagnosis and diagnostic features differ between disease groups in Victorian adult patients with predominantly antibody deficiencies. **(A)** Box–whisker plot of age at diagnosis according to disease classification with median and 5–95% percentiles indicated. **(B)** Histogram demonstrating age at reported symptom onset, light red, and age at diagnosis (dark red) in common variable immunodeficiency (CVID) patients. **(C)** Qualitative vaccine responses to pneumococcal polysaccharide vaccine responses measured IgG binding against PPS23 polysaccharides in CVID patients (red), unclassified hypogammaglobulinemia (HGG) patients (green), IgG subclass deficiency (IGSCD) (purple), and specific antibody deficiency (SpAD) (orange). Darker colored columns indicate protective responses, P, and lighter colored columns indicate unprotective responses, U (χ^2 analysis: $^{**}p < 0.01$ and $^{****}p < 0.0001$). **(D)** Serum IgG and IgA (g/L, mean ± SD) at diagnosis according to disease classification ($^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, using one-way ANOVA).

Specific Antigen Responses

The results of pneumococcal polysaccharide vaccine responses were available for 55 patients with PAD reported here. For CVID, specific pneumococcal polysaccharide antibody titers were available for 30 of 116 patients. From these 30 records, 29 CVID patients were reported to have unprotective vaccine responses (96.7%). Reflecting the heterogeneity of these conditions, the single patient with protective vaccine responses, in retrospect had an atypical presentation for CVID with early-onset chronic mucocutaneous candidiasis, and subsequent adult-onset hypogammaglobulinemia, was identified to have a gain-of-function (GOF) *STAT1* mutation, which is not typically associated with PAD. Furthermore, consistent with the diagnostic criteria for CVID, a higher proportion of HGG and IGSCD patients demonstrated protective vaccine responses compared with the CVID and SpAD groups, who consistently demonstrated suboptimal vaccine responses (χ^2; $p < 0.001$ and $p < 0.01$ respectively; **Figure 1C**).

Genetic Diagnoses

Although genetic testing was not uniformly used in the diagnosis of these patients, 40/178 had an identified genetic cause for PAD (Table S1 in Supplementary Material). This included 21 XLA patients with hemizygous *BTK* mutations. Of the remaining 19 patients with PAD and an identified genetic contribution, 17 had CVID, and 2 had IGSCD. Of the CVID patients, 4 had NFκB1 deficiency, and 3 were heterozygous for the (C104R) variant in *TNFSRF13B*, encoding TACI, a known risk-factor for the development of CVID (23). One of these patients also harbored a pathogenic mutation in *TCF3* (E555K), which causes an autosomal dominant form of agammaglobulinemia (24). In addition, we identified *NFKB2* mutations in three CVID and two IgGSCD patients, who had marked autoimmune clinical manifestations. Two patients initially diagnosed with CVID were found to have *CXCR4* mutations and three patients with CVID were rediagnosed with CTLA4 haploinsufficiency.

Clinical Features and Disease Complications

All patients presented with infections; however, most patients developed complications in addition; only 28% demonstrated an infection-only clinical phenotype (**Table 2**; **Figure 2A**). Respiratory tract involvement was the most common clinical manifestation followed by disease of the GI tract, skin and musculoskeletal system (**Table 2**; **Figure 2A**). Pneumonia and sinusitis were the most common infectious manifestations of the respiratory tract, present in 78% of all PAD patients, and GI infections were present in 19% of patients (data not shown). CVID patients were disproportionately affected and appeared susceptible to a broad range of infections that included *Giardia* in eight patients, *Campylobacter* and *Salmonella* in five, candidiasis in three, *Helicobacter pylori* in three and *Cryptosporidia* spp. in two patients. Bronchiectatic structural lung disease was most

TABLE 2 | Complications in Victorian adults with predominantly antibody deficiency.

	CVID, *n*(%)	XLA	HGG	IGSCD	SpAD	Good syndrome	Total, *n*(%)
Total number of patients	116	21	22	12	4	4	179
Infections only[a]	33 (28)	7 (33)	12 (55)	4 (33)	1 (25)[c]	0	57 (32)
Bronchiectasis	31 (27)	14 (67)	4 (18)	4 (33)	2 (50)	1 (25)	28 (16)
GLILD	5 (4)	0	0	0	0	0	5 (3)
Autoimmunity (total)	44 (38)	0	3 (14)	4 (33)	1 (25)	2 (50)	54 (30)
Musculoskeletal	11 (9)	0	0	2 (17)	0	1 (25)	14 (8)
Cytopenia[b]	24 (21)	0	1 (5)	0	0	1 (25)[b]	26 (15)
Endocrine	9 (8)	0	0	2 (17)	1	0	12 (7)
Gastrointestinal disease (total)	27 (23)	0	2 (9)	1 (8)	0	0	30 (17)
Enteropathy	16 (14)	0	0	1 (8)	0	0	17 (9)
Colitis	11 (9)	0	2 (9)	0	0	1 (25)[c]	14 (8)
Granulomatous disease	8 (7)	0	0	0	0	1 (25)[c]	9 (5)
Autoimmune liver disease	3 (3)	0	1 (5)	0	0	0	4 (2)
Neuropathy	4 (3)	0	0	1 (8)	0	0	5 (3)
Malignancy[c]	20 (17)	0	2 (9)	3 (25)	0	2 (50)	27 (15)
Solid organ	14 (12)	0	2 (9)	2 (17)	0	1 (25)[c]	19 (11)
Hematological	6 (5)	0	0	1 (8)	0	1 (25)[c]	8 (4)

CVID, common variable immunodeficiency; XLA, X-linked agammaglobulinemia; HGG, unclassified hypogammaglobulinemia; IGSCD, immunoglobulin subclass deficiency; GLILD, granulomatous lymphocytic interstitial lung disease; SpAD, specific antibody deficiency.

[a]*Excludes autoimmunity, gastrointestinal disease, granulomatous disease, liver disease, malignancy, and GLILD.*

[b]*Excluding lymphopenia.*

[c]*Excluding thymoma.*

common in XLA with 66.7% patients affected compared with 27% of CVID patients (**Table 2**; **Figure 2A**). Four of these patients with chronic lung disease underwent lung transplantation; one female with CVID, and three males with XLA. One of four patients with Good syndrome also developed bronchiectasis.

Neurologic manifestations were also evident in this cohort. There were five cases of meningitis, which were enterovirus-related in two patients, one of whom succumbed to a fatal progressive meningoencephalitis (**Table 3**). Neuropathy was reported in four CVID patients and a single patient with IGSCD (**Table 2**). The features of the neuropathy were not specified as to severity, pattern or progression. Seizures, optic neuritis, autoimmune hypophysitis were also recognized in individual patients from this cohort. Endocrine manifestations that were recorded included autoimmune thyroid disease in eight CVID patients, one IGSCD and one SpAD, while autoimmune adrenal insufficiency was reported in one CVID and one IGSCD patient.

Overall, manifestations of immune dysregulation were most prevalent in CVID and IGSCD patients (72 and 67%, respectively), compared with only 5/22 hypogammaglobulinemic patients, and 1/4 SpAD (**Table 2**). The proportion of patients with all non-infectious manifestations was significantly higher in the CVID group, compared with the aggregated non-CVID cohort of patients (59/116 versus 12/38, respectively; χ^2, $p < 0.05$).

We reviewed the incidence of specific clinical manifestations in the cohort according to diagnostic group (**Figure 2A**). Compared with the non-CVID antibody deficient patients, the 116 CVID patients more frequently developed cytopenia ($p < 0.001$), enteropathy ($p < 0.01$), splenomegaly ($p < 0.01$), lymphadenopathy ($p < 0.05$), and granulomatous infiltration ($p < 0.05$), but there were no significant differences between the proportions of patients with bronchiectasis, colitis, solid organ or hematological neoplasia and autoimmune liver disease.

To determine if organ complications were cumulative, we assessed the presence or absence of bronchiectasis, autoimmune liver disease, enteropathy, colitis, organomegaly, granulomas and malignancy across the key antibody deficient disease groups. Most patients suffered from <2 complications of disease; however, CVID patients were more likely to have >2 complications (**Figure 2B**).

Management of PAD

Patients were identified on the basis of current or previous immunoglobulin replacement therapy. At the time of data analysis all patients with a diagnosis of XLA, CVID and Good syndrome were receiving replacement immunoglobulin and maintaining IgG mean trough levels of 8.8 g/L (data not shown). Antibiotics were used prophylactically in 10 of 20 CVID patients with available data while systemic immunosuppression was used in 17 CVID patients. These included nine individual patients treated with prednisolone, four patients with azathioprine or methotrexate in combination with prednisolone, whereas rituximab (anti-CD20) was administered in six CVID patients for cytopenias. One patient with CVID and granulomatous lymphocytic interstitial lung disease was treated with rituximab and mycophenolate. Splenectomy was performed on two patients for refractory cytopenia. One patient had undergone liver transplantation due to cirrhosis secondary to nodular regenerative hyperplasia and has tolerated tacrolimus well. One other patient had undergone renal transplantation. Three XLA patients and one CVID patient received lung transplants and were treated with combination immunosuppression including mycophenolate, tacrolimus and prednisolone as prophylaxis against rejection.

Mortality

From this retrospective analysis, we identified 19 PAD patients who had died, including 4 from infections (cytomegalovirus,

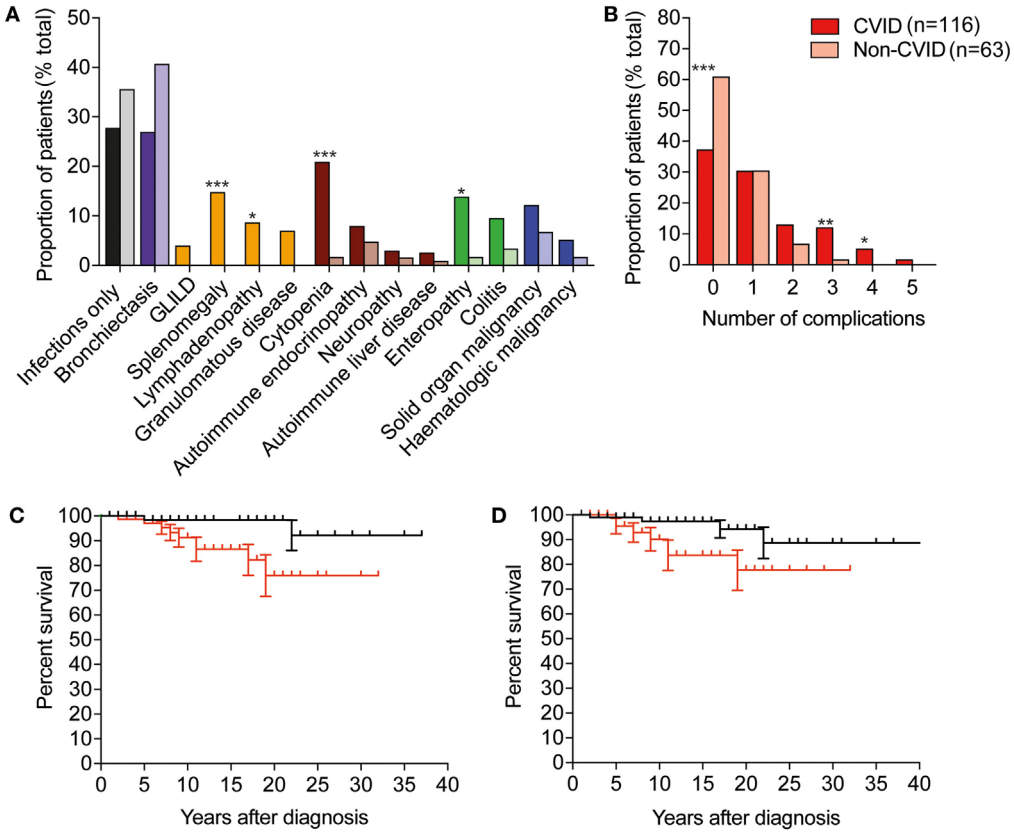


FIGURE 2 | CVID is associated with more frequent non-infectious complications than other predominantly antibody deficiencies, and these complications are associated with reduced survival. **(A)** Histogram depicting frequency of complications in CVID, darker-colored columns, compared with non-CVID cases in lighter-colored columns. Abbreviations: GLILD, granulomatous lymphocytic interstitial lung disease; CVID, common variable immunodeficiency. Purple represents bronchiectasis; yellow represents non-malignant lymphoproliferative complications; brown, autoimmune complications; green, gastrointestinal disease, and blue, malignancies. (Fisher's exact test: ***$p < 0.001$; **$p < 0.01$; and *$p < 0.05$). **(B)** Histogram depicting percentage of CVID and non-CVID patients with 0, 1, or more non-infectious complications of disease. **(C)** Survival after diagnosis in individuals with immune dysregulation is significantly reduced compared with patients with infections only ($p < 0.05$, Mantel–Cox log rank). Black line indicates survival of individuals without features of immune dysregulation, red line indicates survival of individuals with immune dysregulation. **(D)** Survival after diagnosis in individuals with bronchiectasis is significantly reduced compared with patients with infections only ($p < 0.05$, Mantel–Cox log rank). Black line indicates survival of individuals without bronchiectasis, and red indicates survival of individuals with bronchiectasis.

enteroviral encephalitis, sepsis-unspecified, and respiratory infection) and 6 patients died as a result of malignant disease (breast cancer, malignant thymoma, serous ovarian cancer, squamous esophageal cancer, lung adenocarcinoma, and diffuse large B cell lymphoma; **Table 3**). Of those, five had CVID and one had a diagnosis of Good syndrome, with a malignant thymoma. One patient died from an intracerebral hemorrhage, which was thought to be unrelated to PAD. Survival following diagnosis was significantly reduced in patients with immune dysregulation, with a 75% survival at 20 years after diagnosis compared with 98% for those with an infection-only phenotype ($p < 0.05$; **Figure 2C**). Significantly reduced survival was also noted in PAD patients with bronchiectasis as early as 10 years post-diagnosis (**Figure 2D**).

DISCUSSION

Primary immunodeficiencies are a heterogeneous mix of disorders, with >300 defined conditions. The most prevalent of these are PADs, which may present with a broad range of clinical features, age of onset, and population-dependent frequency. As a result, the underlying immunodeficiency diagnosis is typically significantly delayed or missed.

We aimed to characterize how PAD patients have been diagnosed, managed and manifested disease and sought to identify areas for improvement in clinical practice in Victoria, the second most populous state in Australia, with a population aged over 15 years of approximately 4.9 million at the end of 2016 (Australian Bureau of Statistics). Australia is a geographically expansive country, with a relatively small population; posing unique challenges for diagnosis and treatment of rare diseases. The most recent Australian study demonstrated incomplete capture of all PIDs, with the finding of 5.6 cases per 100,000 (5). We found a prevalence of PAD in Victorian adults of approximately 1 in 25,000, which is similar to other population frequencies internationally (11). The prevalence of PAD varies geographically, with a very high prevalence in Finland, a non-diverse population, in contrast to that of Australia, which is more heterogeneous (25).

As with any retrospective study, there are some limitations to this report. Data collection may be incomplete, and inconsistency may arise due to differences in practice between individual

TABLE 3 | Causes of death among antibody deficient patients in Victoria (2000–2017).

Patient no.	Diagnosis	Sex	Clinical phenotype	Age at diagnosis	Age deceased	Cause of death
46	IGSCD[a]	F	Immune dysregulation	9	28	Enteroviral encephalitis
90	CVID	F	Infections only	62	70	Breast cancer
91	Good syndrome	M	Infections only	75	77	Thymoma
92	CVID	F	Immune dysregulation	69	74	Serous ovarian cancer
93	CVID	F	Infections only	47	74	Squamous esophageal cancer
113	CVID[b]	F	Immune dysregulation	67	81	Lung adenocarcinoma
123	CVID	F	Immune dysregulation	40	65	Intracerebral hemorrhage
156	CVID	M	Immune dysregulation	42	62	Diffuse large B cell lymphoma
157	CVID	F	Immune dysregulation	?	48	Sepsis
158	CVID	M	Immune dysregulation	71	78	Respiratory failure—ILD
159	XLA	M	Infections/bronchiectasis	?	58	Infection post lung transplant
160	CVID	F	Infections only	68	73	Not available
161	XLA	M	Infections/bronchiectasis	?	35	Lung transplant complication
162	CVID	M	Immune dysregulation	27	32	Enteropathy/cachexia/sepsis
163	XLA	M	Infections/bronchiectasis	13	59	Respiratory failure
164	XLA	M	Infections/bronchiectasis	?	39	Infection post lung transplant (CMV)
165	XLA	M	Infections/bronchiectasis/cirrhosis	?	36	HCV-related cirrhosis
166	CVID	M	Immune dysregulation	36	53	Respiratory failure and refractory enteropathy
167	CVID	F	Infections/bronchiectasis	71	80	Respiratory failure

IGSCD, immunoglobulin subclass deficiency; CVID, common variable immunodeficiency; XLA, X-linked agammaglobulinemia; ILD, interstitial lung disease; CMV, cytomegalovirus; HCV, hepatitis C virus.

? indicates unknown.

[a]*NFKB2 mutation.*

[b]*NFKB1 mutation.*

physicians and centers. Under-diagnosis is likely to relate to the relative infrequency of these conditions, and therefore lack of suspicion among primary care providers, particularly as the clinical features are varied (25). There is also a significant delay in diagnosis observed in the majority of these disorders, which is associated with an increased risk of mortality, and significant morbidity (26). Thus, increased awareness among medical practitioners and improvements in diagnostic tools are of great clinical importance. Implementation of increased awareness programs is likely to raise the number of patients to be diagnosed and potentially this will lead to identification of a higher prevalence of PAD than 1 in 25,000.

While this cohort represents a small group of patients, our median diagnostic delay of 9 years in the CVID group is longer than other recently reported cohorts. European data from more than 2,000 patients with CVID identified a median diagnostic delay overall of 5 years when the diagnosis was made prior to the year 2000, and 4.2 years when the diagnosis was made during or after the year 2000 (27). However, those patients comprised a large proportion of children, whereas our cohort only included adults, which may introduce a bias to a shorter diagnostic delay in that study. Indeed, in other smaller European adult cohorts a diagnostic delay of 7 years has been reported (28, 29).

Predominantly antibody deficiency remains a diagnostic challenge unless specifically considered in light of the varied non-infectious manifestations, and involvement with physicians with expertise in diagnosis and treatment of primary immunodeficiency. Our data demonstrate that distinguishing primary hypogammaglobulinemia from CVID cannot be readily made using laboratory parameters in isolation, as there is overlap in these parameters between the two conditions. Furthermore, vaccination studies, which are used for diagnosis of PAD, are complicated by the need for paired serum analyses, and are confounded by increasing community use of pneumococcal vaccines and variability in the measurement, and interpretation, of polysaccharide responses. Taken together, there is a need to improve PAD diagnosis, which may entail the use of molecular tests and dialog between internal medicine specialists and clinical immunologists.

Molecular tests that identify pathogenic mutations will improve diagnostic precision in PAD. In this cohort, patients who were later identified to have a genetic contribution to their disease, had a shorter time to a clinical diagnosis (**Table 2**). This may be influenced by the severity of presenting illnesses and/or a positive family history pre-empting screening for antibody deficiency. In addition, molecular tests may help identify patients-at-risk for certain disease complications, as in the case of *NFKB2* mutations which are associated with a high risk of central adrenal insufficiency. Further highlighting the complexities of clinical presentation in PID, we have described a patient who was initially misdiagnosed with CVID, but later found to have a *STAT1* GOF mutation after his diagnosis was reconsidered in light of early-onset mucocutaneous candidiasis.

We also identified one individual with digenic disease due to a pathogenic E555K variant in *TCF3* and C104R variant in *TNFRSF13B*. We have previously reported another kindred affected by PAD and autoimmune disease; the proband harbored a novel non-sense *TCF3* mutation and the C104R variant in *TNFRSF13B*, with a resultant CVID-like disorder and systemic lupus erythematosus (30). We are not aware of other reports of digenic PAD due to the more common pathogenic *TCF3* E555K variant in combination with an additional genetic risk-factor.

Our findings support improved access to diagnostic genomic studies in these patients, although currently the majority of CVID cases are unlikely to be readily diagnosed due to their complex pathogenesis (31, 32). Nevertheless diagnostic rates could

increase to 30% for CVID patients selected for sequencing on the basis of certain clinical or laboratory features (14). Other studies have identified XLA in adult patients previously misdiagnosed as CVID, which has significant implications regarding missed opportunities for genetic counseling (33). The genetic diagnoses we have reported here may have been biased toward patients with more extreme phenotypes, and further work is ongoing to determine the clinical utility of genomic sequencing to aid diagnosis in the cohort more broadly.

Earlier diagnosis of PAD will facilitate earlier IgG replacement therapy and/or antibiotic therapy to mitigate the susceptibility to infections, and delay or prevent end-organ changes from infections such as bronchiectasis. However, optimal management of infections alone may not prevent complications that arise from immune dysregulation such as autoimmune disease, granulomatous inflammation and enteropathy, which affected 68% of our cohort. This is a higher proportion than reported in other cohorts and may reflect a referral bias toward patients with more severe disease, and a failure to include stable PID patients with infection-only phenotypes controlled on immunoglobulin replacement managed by non-Immunology services, or indeed those not receiving immunoglobulin at all.

Prediction of risk for the non-infectious complications of PAD is a major challenge. In a recent US study, unbiased network clustering using two large CVID patient datasets has identified novel biochemical markers of patients with lymphoproliferative, autoimmune and allergic disease (34). Whether these associations apply to the other PADs is unclear. In our cohort CVID patients suffered the most non-infectious complications, but we also identified non-infectious complications in other PADs. Non-infectious complications in IGSCD have also been recognized in other cohorts, although a recent Dutch study did not identify any non-infectious complications in a cohort of patients with HGG (35, 36). Importantly, two IGSCD patients in our cohort harbored *NFKB2* mutations, which may have predisposed these patients to the more severe clinical phenotype.

CONCLUSION

Predominantly antibody deficiencies are the most common of the primary immune deficiency diseases, yet are still likely to be underdiagnosed, or diagnosed after the development of life-threatening complications. Individuals with PADs suffer from diverse and severe complications including infection, bronchiectasis and disorders of immune dysregulation, which are associated with reduced survival. These complications may be averted by earlier and more precise diagnosis, and targeted therapeutic interventions. Early diagnosis relies upon improved knowledge and appropriate clinical suspicion among internal medical physicians and primary care providers, as well as equitable access to genetic diagnosis and the benefits of personalized medicine.

ETHICS STATEMENT

The study was carried out according to the principles of the Declaration of Helsinki and was approved by local human research ethics committees (Melbourne Health projects 2009.162, 2013.245; Walter and Eliza Hall Research Institute projects 10/02, 14/01; Alfred Health 109/15, 277/17). All living patients, or their next of kin, consented to the collection of their medical information. For those individuals who were deceased at the time of the data collection, ethical approval was obtained to review the medical records without the consent of the next of kin.

AUTHOR CONTRIBUTIONS

CS and JB designed the study, collected data, interpreted data, and wrote the manuscript. MZ and VB designed the study, interpreted the data, and wrote the manuscript; TG and EK collected and interpreted data. RS, PC, FH-L, MS, SH, SO, SB, GU, JL, MP, JM, KS, YT, PA, KN, RO, JD, and PH contributed data and assisted writing the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 4 A STUDY OF THE DIAGNOSTIC YIELD AND CLINICAL UTILITY OF EXOME SEQUENCING IN A COHORT OF AUSTRALIAN PATIENTS WITH PRIMARY IMMUNODEFICIENCY DISEASES

4.1 Introduction

The previous chapter provided a detailed analysis of the clinical features of a cohort of adult patients in Victoria, Australia, with Predominantly Antibody Deficiencies (Slade, Bosco et al. 2018). One of the striking findings from this study was the median delay of nine years from onset of symptoms to diagnosis, thus delaying appropriate treatment, and likely leading to a greater burden of complications and increased mortality. Previous studies have shown that a delay in diagnosis of one year is associated with a 4% increase in mortality in CVID (Resnick, Moshier et al. 2012). This chapter focuses on the diagnostic and clinical utility of exome sequencing for the diagnosis of PIDs, when offered early during the investigation of patients with possible PID.

Of the 158 patients with non-XLA PADs described in the previous chapter, 19 had an identifiable monogenic cause for their disease (Appendix 4). Ten of those diagnoses were achieved by way of exome sequencing through the Novel gene discovery project (Methods, Section 2.4.1), including a family with a LOF mutation in *NFKB1* (Fliegauf, Bryant et al. 2015), and the family reported in Chapter 5 with a novel dominant negative mutation in *NFKB2*. However, the majority of this cohort had not previously been offered genetic testing, so the actual rate of monogenic disease was unknown. Whilst an accurate diagnosis is important for understanding the pathogenesis of disease, it can also lead to opportunities for personalised therapies and genetic counseling.

Here we set out to investigate the diagnostic yield and clinical utility of exome sequencing in an extended group of PID patients. This 'Clinical Genomics Project' was carried out as part of the Melbourne Genomics Health Alliance (Gaff, I et al. 2017). Melbourne Genomics was established with a number of clear goals. Namely: to determine the diagnostic yield of exome sequencing for a collection of disease groups; to research the clinical utility and cost-effectiveness of genomics in a broad array of clinical settings; enable workforce training for effective and ethical implementation of such testing; and provide evidence to government for decision-making about reimbursements for testing (Gaff, I et al. 2017).

The Alliance was formed in 2013 and funded collaboratively by the following institutions: The University of Melbourne, RMH, WEHI, RCH, Murdoch Children's Research Institute (MCRI), Austin Health, Monash University, AGRF, and Commonwealth Scientific and Industrial Research Organisation (CSIRO). In 2016, the Victorian state government made a matched contribution to a total of AUD$25 million and has since contributed a further AUD$100M. The first phase of Melbourne Genomics comprised 4 pilot programs in peripheral neuropathy (n=50) (Walsh, Bell et al. 2017), focal epilepsy (n=41), hereditary colorectal cancer (n=35), AML (n=54), and childhood syndromes (n=142) (Stark, Tan et al. 2016).

The second phase of the Melbourne Genomics Health Alliance was launched in 2015, to expand the types of disease studied, and increase patient numbers. In addition to this project, which was referred to as the 'Immunology Flagship', other disease flagships were included in Phase 2: Dilated Cardiomyopathy, Congenital Deafness, Childhood

onset disease with Complex needs, and advanced cancers, but will not be discussed further here.

Significant consideration was given to the ethical risk in offering this type of testing. As such, the Hippocratic principles were applied in establishing the study:

1. First do no harm (non-maleficence) - the interests and welfare of the individual should have priority, including in respect of human rights, genetic discrimination and testing-induced harms.
2. Act in the best interest of the individual (beneficence) - all clinical genomic interventions must be scientifically sound, clinically indicated and employed after assessing the risks and benefits.

Thus, we sought to find only variants in relevant known disease-causing genes and offered the testing to patients with a spectrum of disease, reflecting a 'real world' sample of PID patients. The results presented here therefore reflect limited emphasis on identifying novel disease mechanisms and gene discovery, although further studies are underway in reanalysis of the genomic data supported by complementary functional testing in order to pursue greater understanding of the pathogenesis of disease.

4.2 Results: Patient details

4.2.1 Patient Recruitment

Two hundred fifty-five individuals were identified as potentially eligible for participating in the study. Of these, 200 individuals in total were recruited, from March 2016 through to July 2017. Three types of immune disorders were included as described in detail in Chapter 2 (section 2.4.1). The clinical features of several of the patients with

PADs who participated in the Flagship (n=90, 6 genetic diagnoses through MGHA), were described in the previous chapter, but the cohort also included individuals with a broader spectrum of PIDs, and, importantly, children, and individuals with complex phenotypes. Inclusion/exclusion criteria are outlined in section 2.4.2.

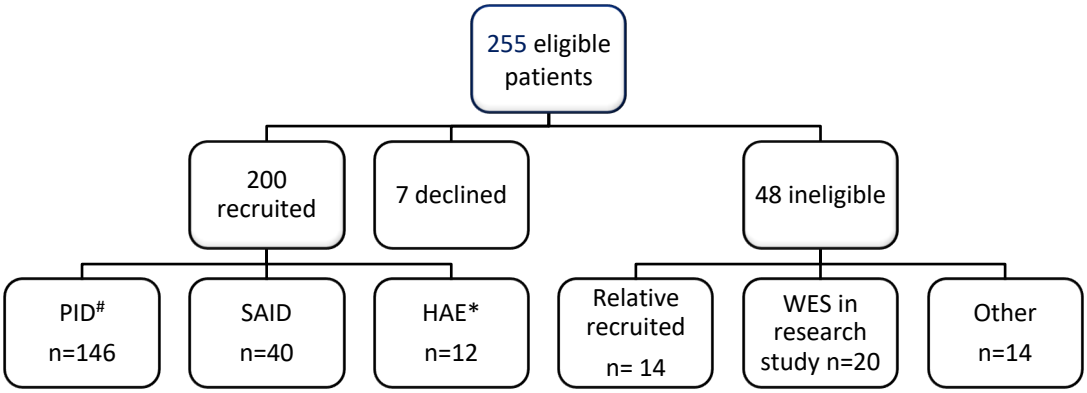


FIGURE 4.1. PATIENT RECRUITMENT

The flowchart demonstrates the process of identification of patients for participation in the study, number of patients who were ineligible, or declined participation, and the number who were recruited within each subcategory. *('Other' includes, previous genetic diagnosis made, or extensive genetic testing already performed; #1 patient diagnosed with 22q11.2 deletion after SNP microarray performed at time of recruitment; *1 patient did not have blood sample taken)*

4.2.2 Patient characteristics

In total, 123 patients with suspected or confirmed immunological conditions were recruited from RMH, seven from the Austin Hospital, 36 from Monash Medical Centre, and 34 children from the RCH (Figure 4.2). Two patients were withdrawn from the study: one failed to have a blood sample taken, and another was diagnosed by SNP

microarray with 22q11.2 deletion prior to WES. The majority of patients (n=156) were adults; 44 were children.

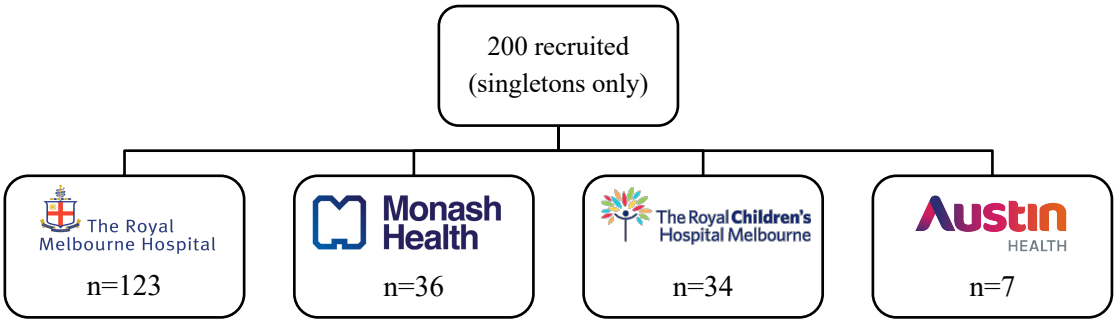


FIGURE 4.2. RECRUITMENT FROM PARTICIPATING SITE.

In total 200 patients were recruited, with the majority being adults from Royal Melbourne Hospital, and smaller numbers recruited from Monash Health, Royal Children's Hospital and Austin Health as indicated in the flowchart.

Table 4.1. Number of patients with PIDs, and expected and actual diagnostic rates.

Diagnosis	No. patients	Age range	Male:Female	Expected diagnostic rate#	Actual diagnostic rate
SCID	1	2 months	0:1	90%	100%
CID	6	8yrs-40yrs	3:3	50%	50%
PAD/CVID	100	4yrs-70yrs	45:55	10-20%	11%*
Syndromic PID	6	10yrs-50yrs	3:3	50%	33%
Immune dysregulation	3	1yr-16yrs	2:1	50%	33%
Innate immune defects	8	19-52yrs	4:4	50%	0%
Complement deficiencies	5	3yrs-38yrs	2:3	80%	40%
Neutrophil defects	7	20yrs-48yrs	2:5	60%	29%

(SCID, severe combined immunodeficiency; CID, combined immunodeficiency; PAD, Predominantly Antibody Deficiency; CVID, common variable immunodeficiency.

*#, 'expected' refers to expected currently known monogenic causes; *, 2 patients received a non-PID diagnosis)*

4.2.3 Clinical diagnoses

The majority of patients recruited to the Melbourne Genomics Flagship were classified as having a PID (n=146) (Figure 4.1), and most (n=100) had a diagnosis (or suspected diagnosis) of a PAD, with CVID being the most common (Table 4.1). Only a single patient with SCID was (prospectively) recruited. Six patients were classified as having a CID, and a further six with a PID with syndromic features. Of the patients with syndromic features, one had a clinical diagnosis of Ataxia Telangiectasia (AT), and four had ectodermal dysplasia (ED; accompanied by lymphopenia in three and specific antibody deficiency in one).

Eight patients with suspected defects of innate immunity were recruited (Table 4.1). Of these, two patients had a diagnosis of possible Hyper-IgE syndrome (HIES), and there was one patient with each of the following phenotypes: chronic mucocutaneous candidiasis (CMC); recurrent herpes simplex encephalitis; invasive salmonellosis; disseminated mycobacterial infection with myelodysplastic syndrome; recurrent pulmonary aspergillus infection and lymphocytic colitis; and recurrent episodes of staphylococcal sepsis, with the working diagnosis of a possible defect in TLR signaling.

Of the five patients with complement deficiencies (Table 4.1), two had a diagnosis of C2 deficiency, one had C7 deficiency, and two had a diagnosis of mannose-binding lectin (MBL) deficiency. The MBL deficient patients were also being investigated for other causes of their presentation (one had recurrent pregnancy losses due to placental infection, and one had recurrent bacterial meningitis). The group of patients with

neutrophil disorders comprised three individuals with chronic granulomatous disease (CGD), and four with neutropenia (one cyclic and three congenital) (Table 4.1).

Table 4.2 Diagnostic outcomes for all disease categories

Disease group	No. patients	Age (yrs)	Diagnoses by standard care	Additional diagnoses by genomic testing	Total diagnoses
PID	146	0-70	5 (3%)	15 (10%)^	20/146 (14%)
SAID	41	1-40	5 (12%)	2 (5%)	7/41 (17%)
HAE	11	3-56	8 (72%)	0*	8/11 (72%)
TOTAL	198	0-70	18 (9%)	17 (9%)	35/198 (18%)

*(PID, Primary Immunodeficiency; SAID, systemic autoinflammatory disease; HAE, hereditary angioedema; ^, one of the diagnoses made by standard care was missed by genomic testing (complement deficiency); *, only 4 of the 8 diagnoses made by standard care testing for HAE were made by genomic testing)*

4.2.4 Higher diagnostic yield from WES for patients with SCID, CID and syndromic PIDs

In the PID group, 20 (14%) diagnoses were made, and 15 (10%) would not have been detected by standard diagnostic testing (Table 4.2). In the SAID group, 7 (17%) diagnoses were made and, two would not have been detected by standard testing (Table 4.2). For HAE, more diagnoses were made through usual diagnostic testing than genomic sequencing (Table 4.2). Genomic sequencing is clearly not better than usual care for patients with HAE.

The single patient with SCID presented at the age of four months, with severe pneumonitis, and was subsequently diagnosed with T-/B-SCID. Standard diagnostic testing was performed at the same time as WES, both confirming novel homozygous *RAG1* mutations. The patient has since undergone a successful haploidentical HSCT,

and parents have received genetic counseling. They have since elected to have prenatal testing for their second pregnancy.

Four of 12 patients with CID and syndromic PID received diagnoses from WES, and one was diagnosed prior to sequencing by a SNP microarray. A 16-year-old female with a history of recurrent sinopulmonary infections, bronchiectasis and lymphopenia, was found to have a Class 5 *GATA2* mutation (p.Arg395Gln) (Table 4.3), a diagnosis which had not previously been entertained, perhaps partly due to maintenance of normal monocyte counts, despite reduced B and NK cells. Another patient with ectodermal dysplasia and lymphopaenia was found to have a rare *WNT10A* variant, not likely to explain the lymphopaenia, but may explain the autosomal dominant ED. For the patient with a clinical diagnosis of Ataxia Telangiectasia, two rare mutations in *ATM* were identified, confirmed to be in compound heterozygous state.

A patient with myelodysplastic syndrome and mycobacterial infection, was considered likely to have a *GATA2* mutation, but only a heterozygous variant in *IL12RB* was identified, that was unlikely to explain the phenotype. Two heterozygous variants in *ZAP70*, were identified in 2 individuals with combined immunodeficiency, however no second variants were identified, leaving these findings uncertain.

Table 4.3 Variants curated in patients with PIDs other than Predominantly Antibody Deficiency

Diagnosis	Gene Lists	Variants curated	*In silico* prediction	MAF	Variant Classification	Previously reported
SCID	**SCID**	***RAG1* c.1950C>A; p.Asn650Lys (hom)**	**pathogenic**	**absent**	***RAG1* Class 4**	**No**
Lymphopenia	**CVID, ALL**	***GATA2* c.1187G>A; p.Arg396Gln (het)**	**pathogenic**	**absent**	***GATA2* Class 5**	**Yes**
CGD	**NEUT**	***NCF1 c.579G>A;* p.Trp193***	**NMD**	**6.00E-05**	***NCF1* Class 5**	**Yes**
Immune dysregulation	**DYSREG, CID, ALL**	***FOXP3* c.1022C>A; p.Thr341Asn) (hemi)**	**pathogenic**	**absent**	***FOXP3* Class 4**	**No**
Lymphopenia; ED	**CID, ALL, ED**	***WNT10A* c.321C>T; p.Cys107* (het)**	**truncating**	**6.00E-04**	***WNT10A* Class 5**	**Yes**
Neutropenia	**NEUT, ALL**	***CSF3R* c.340C>T; p.Gln114* (het)**	**pathogenic**	**2.00E-04**	***CSFR* Class 4**	**No**
		***CSF3R* c.931C>T; p.Arg311Cys (het)**	**pathogenic**	**4.00E-05**	***CSFR* Class 4**	**No**
Ataxia	**ATM, CID**	***ATM* c.5228C>T; p.Thr1743Ile (het)**	**pathogenic**	**2.00E-04**	***ATM* Class 5**	**Yes**
telangiectasia		***ATM* c.6404_6405insTT; p.Arg2136* (het)**	**pathogenic**	**7.00E-05**	***ATM* Class 5**	**Yes**
Neutropenia	NEUT, CVID, ALL	*LRBA* c.4297dupA; p.Ser1433Lysfs*35 (het)	truncating	absent	*LRBA* Class 4	No
MDS, CID	GATA2, CID, ALL	*IL12RB*	conflicting	absent	*IL12RB* VUS 3b	No
CID, ED	CID, ALL, ED	ZAP70 c.899C>T; p.Thr300Met (het)	benign	4.00E-06	*ZAP70* VUS 3c	No
CID	CID, ALL	*ZAP70* c.512A>G; p.Glu171Gly (het)	damaging	5. 00E-06	*ZAP70* VUS 3b	No

(CGD, chronic granulomatous disease; MDS, myelodysplastic syndrome; CID, combined immunodeficiency; SCID, severe combined immunodeficiency; ED, ectodermal dysplasia; NMD, non-sense mediated decay; reclassified from VUS3a after segregation; MAF, minor allele frequency; Pathogenic (Class 5) and likely pathogenic (Class 4) variants are indicated in bold)

4.2.4.1 SYNDROMIC DIAGNOSES – PEDIATRIC DISEASES DIAGNOSED IN ADULTHOOD

In two adults a diagnosis was made of a syndrome typically identified in childhood. One patient with a complex phenotype, characterised by moderate intellectual disability, short stature, ectodermal dysplasia, staphylococcal septic arthritis and an elevated IgE level, was found to harbour a likely pathogenic variant in *KMT2A* (c.5572C>T; p.Arg1858*), a gene associated with Wiedemann-Steiner syndrome (OMIM #605130), resulting in an unexpected diagnosis in this 50-year-old male. A 38-year-old female with facial dysmorphism, recurrent severe HPV infection, and associated cervical and vulval cancer, was recruited to the study, but a SNP microarray was performed prior to exome sequencing, and identified a deletion of 22q11.2, leading to the diagnosis of 22q11.2 deletion syndrome. Together, these results suggest value in testing for copy number variation in adults, particularly in individuals with complex phenotypes.

4.2.4.2 MOST PAD-ASSOCIATED VARIANTS WERE CLASSIFIED 'OF UNCERTAIN SIGNIFICANCE', AND REFLECT THE HETEROGENEITY AND COMPLEX PATHOGENESIS OF THESE CONDITIONS

Thirty-nine variants from 34 patients (34% of patients) were selected for curation in the PAD group (Table 4.4). Only nine variants in PID-associated genes were directly classified as pathogenic or likely pathogenic (i.e. Class 5 or Class 4), the remaining 30 variants require further functional studies/or familial segregation to confirm or exclude pathogenicity (Table 4.4, Figure 4.2). This uncertainty may reflect the rarity of these conditions, the relative infancy of genomic sequencing for the diagnosis of PADs, and subsequent paucity of evidence in medical literature; according to the ACMG guidelines a prior report of a single case or family is insufficient to allow reporting of

a variant in that gene as even likely pathogenic (Richards, Aziz et al. 2015). The variants identified reflect the pathogenic mechanisms known to contribute to the development of PADs, in particular in genes involved in B- and T-cell signaling and NF-κB signaling (Figure 4.2).

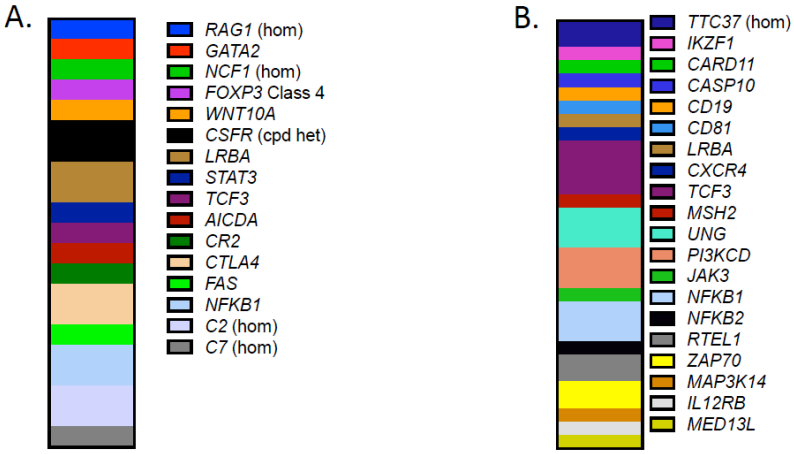


FIGURE 4.3 VARIANTS OF UNCERTAIN SIGNIFICANCE ARE MORE ABUNDANT THAN PATHOGENIC VARIANTS IN PIDS, AND ARE FOUND IN MANY GENES.

Bar graphs depicting, (A), pathogenic variants, and, (B), variants of uncertain significance identified in patients with PIDs. (Each colour represents a different gene, with the height of the bar reflecting the number of variants identified in each gene.)

Of the pathogenic variants, only three variants had been previously reported as disease-causing in PID patients, in *STAT3*, *TCF3* and *AICDA* (Table 4.4). Of note, the *AICDA* variant was initially dismissed, due to being in heterozygous state, and AID deficiency typically being a recessive condition. However upon investigation of the literature that variant was confirmed to cause disease in a dominant negative manner (Imai, Zhu et al. 2005). All the other likely pathogenic variants were novel. The *FAS* variant was determined to be *de novo*. The proband has 2 young children who will be offered predictive testing (immunologic investigations to this point have been unremarkable). The *CTLA4* variants identified have led to changes in care for those individuals, as

described in the next section. Both *NFKB1* variants had not been reported as disease-causing at the time.

In addition to known PID-associated genes, two other pathogenic variants were identified in two patients from the PAD group. In one adult male with an autosomal dominant coagulopathy and CVID, a likely pathogenic *THBD* variant was identified in the gene encoding thrombomodulin (OMIM #614486; Table 4.4); accounting for the coagulopathy, although it is unclear how this variant is relevant to the antibody deficiency, although it is recognised that thrombomodulin modulates the behaviour of classical dendritic cells, which are well known to play a role in B cell activation (Dubois, Vanbervliet et al. 1997, Toda, D'Alessandro-Gabazza et al. 2014). A young female patient with intellectual disability, Arnold Chiari malformation and scoliosis, in addition to CVID, was found to harbour a novel *MED13L* variant (p.Glu1657Lys), in addition to the TACI C104R risk factor (Table 4.4). The *MED13L* variant was located at a residue highly conserved in vertebrates, but not within a functional domain. The gene encodes a subunit of the so-called Mediator, a multi-protein complex that coordinates transcription of many genes in a number of tissues, by linking DNA-binding transcription factors and RNA polymerase II (Sato, Tomomori-Sato et al. 2004). Haploinsufficiency of *MED13L* (OMIM #616789) is an established cause of a syndrome encompassing intellectual disability, frequently associated with congenital cardiac abnormalities and diverse neurological features (Asadollahi, Zweier et al. 2017).

Thus, these patients received a diagnosis for a condition apparently unrelated to PID, although the phenotypic spectrum of most genetic conditions is expanding with more

patients receiving exome and genome sequencing. *THBD* is, in fact, listed as a disease-causing gene in the IUIS classification since it encodes a component of the complement and coagulation pathways, and as stated above, it is an attractive hypothesis that defects in thrombomodulin could impact B cell behaviour indirectly via dendritic cells (Bousfiha, Jeddane et al. 2018). Intriguingly, *MED13L* was identified as a potential PID gene in a study of the human gene connectome (Itan and Casanova 2015), due to its 'connectedness' to *AIRE*, the gene encoding the autoimmune regulator and certainly warrants further investigation.

Finally, in addition to the diagnostic results, in two patients, heterozygous variants of uncertain significance in *ZAP70* were identified (Table 4.3). These variants, p.Glu171Gly, and p.Thr300Met, were classified as 3b and 3c respectively, on the basis of *in silico* predictions and population frequencies. Thus, whilst not currently classified as pathogenic, if a second damaging variant were to be identified upon reanalysis, or with implementation of alternative testing techniques (e.g. Sanger or whole genome sequencing), this classification may change.

Table 4.4. Variants curated in patients with Predominantly Antibody Deficiencies

Diagnosis	Variants curated	*In silico* predictions*	MAF#	Classification
CVID	***STAT3* c.454C>T; p.Arg152Trp**	**pathogenic**	**absent**	***STAT3* Class 5**
Agamma	***TCF3* c.1633G>A; p.Glu333Lys**	**damaging**	**absent**	***TCF3* class 5**
CVID	***AICDA* c.568C>T; p.Arg190***	**truncating**	**absent**	***AICDA* Class 5**
CVID	***CR2* c.2297G>A; p.Trp766***	**truncating**	**7.66E-04**	***CR2* Class 4**
CVID	***CTLA4* c.410C>T; p.Pro137Leu**	**pathogenic**	**absent**	***CTLA4* Class 4**
CVID	***CTLA4* c.410C>T; p.Pro137Leu**	**pathogenic**	**absent**	***CTLA4* Class 4**
CVID	***FAS* c.809C>T; p.Thr270Ile**	**conflicting**	**absent**	***FAS* Class 4**
CVID	***NFKB1* c.1676_1679delinsCCTAC, p.Leu559Profs*15**	**truncating**	**absent**	***NFKB1* Class 4**

CVID	***NFKB1 c.*904dupT; p.Ser307Phefs*7** *TTC37* c.1507G>T*;* p.Tyr503Cys (hom)	**truncating** damaging	**4.00E-06** 2.00E-05	***NFKB1* Class 4** *TTC37* VUS3a
CVID	*TCF3* c.1820A>G; p.Asn607Ser	conflicting	absent	*TCF3* VUS3a
CVID	*PLCG2* c.3397C>T; p.Arg1133Cys	pathogenic	4.0E-06	*PLCG2* VUS3a
CVID	*IKZF1* c.1280C>T; p.Ser427Leu	benign	absent	*IKZF1* VUS3b
CVID	*TTC7A* 1.c.2014C>T; p.Ser672Pro *TTC7A* 2.c.1817C>G; p.Lys606Arg	pathogenic pathogenic	2.10E-03 2.10E-03	*TTC7A* VUS3b *TTC7A* VUS3b
CVID	*CARD11* c.2597C>A; p.Arg866Gln	conflicting	absent	*CARD11* VUS 3b
CVID	*CASP10* c.684+1G>A	donor splice site loss	5.0E-05	*CASP10* VUS3b
CVID	*CD19* c.487C>G; p.Arg163Gly *CD81* c.409G>A; p.Asp137Asn	benign benign	absent 5.425E-5	*CD19* VUS3b *CD81* VUS3c
CVID	*CXCR4* c.586A>G; p.Asn196Asp)	benign	absent	*CXCR4* VUS3c
CVID	*LRBA* c.8584C>T; p.Arg2862Cys	pathogenic	1.332E-4	*LRBA* VUS3b
CVID	*MAP3K14* c.2146C>G; p.Leu716Val	conflicting	1.60E-4	*MAP3K14* VUS3c
CVID	*MSH2* c.1148G>A; p.Arg383Gln *UNG* c.262C>T; p.Arg88Cys	benign damaging	1.218E-5 1.11E-4	*MSH2* VUS3c *UNG* VUS3a
CVID	*NFKB1* c.2608A>G; p.Val870Ile	benign	2.00E-06	*NFKB1* VUS3b
CVID	*NFKB1* c.692G>A; p.Arg231His	conflicting	3.60E-05	*NFKB1* VUS3b
CVID	*NFKB1:* c.2831C>A; p.Thr944Asn	benign	3.00E-04	*NFKB1* VUS3c
CVID	*NFKB2* c.1714G>A; p.Ala572Thr	benign	5.45E-05	*NFKB2* VUS3c
CVID	*PI3KCD:* c.2873G>A; p.Gly958Asp	conflicting	5.00E-04	*PI3KCD* VUS3b
CVID	*PIK3CD* c.719C>T; p.Thr240Met	conflicting	5.00E-06	*PIK3CD* VUS3b
CVID	*PIK3CD* c.780+3G>A p.?	splice site	absent	*PIK3CD* VUS3c
CVID	*JAK3* c.649G>A; p.Val217Met	damaging	2.60E-04	*JAK3* VUS3b
CVID	*RTEL1* c.2789A>G; p.Glu930Gly	benign	8.35E-06	*RTEL1* VUS3b
CVID	*TCF3* c.1523T>C; p. (Thr508Met)	benign	1.00E-04	*TCF3* VUS3c
CVID	*TCF3* c.23C>T; p.Ala8Val	pathogenic	8.00E-5	*TCF3* VUS3b
CVID	*TCF3* c.604delT; p.Ser202Profs*82	NMD	absent	*TCF3* VUS3b
CVID	*TCF3* c.1154G>A; p.Gly385Asp *JAK3* c.1793 T>A; p.Met598Lys	conflicting damaging	2.5E-03 absent	*TCF3* VUS3c *JAK3* VUS3a
CVID	*UNG* c.231C>G; p.Asn77Lys	conflicting	1.00E-05	*UNG* VUS3b
CVID	*MED13L* c.4969G>A; p.Glu1657Lys	damaging	absent	*MED13L* VUS3a
CVID	*THBD c. ;p.*	damaging	absent	*THBD* VUS3a

*(*In silico prediction tools used: PolyPhen, CADD, SIFT and Mutation Taster; MAF, minor allele frequency from gnomAD; CVID, Common Variable Immunodeficiency; Agamma, agammglobulinemia; Pathogenic and likely pathogenic variants are indicated in bold.)*

4.3 Management changes consequent to genetic diagnoses

Seventeen of the 27 PID and SAID patients diagnosed have been offered precision care and for 13 patients this differs from what would have been recommended following standard diagnostic testing and are summarised in Table 4.3. An additional three patients with PID received diagnoses that may enable the use of precision therapies when required. Two relatives diagnosed as a result of this project have also received targeted therapies, as described in the following section.

4.3.1 Genomic diagnoses enabling curative therapies: Haematopoietic stem cell transplantation in IPEX and GATA2 deficiency

A 13-year-old male with a complex phenotype, including myopathy, hypogammaglobulinemia, growth restriction (unresponsive to growth hormone therapy) and colitis, was identified to harbor a likely pathogenic variant in *FOXP3*, leading to a probable diagnosis of X-linked immunodysregulation, polyendocrinopathy, and enteropathy (IPEX; OMIM# 304790; Table 4.3). The variant was initially reported as of uncertain significance, however segregation studies led to reclassification as it was also detected in a brother, who was similarly affected. The diagnosis enabled an immediate change to therapy; one brother was initially treated with sirolimus, which is recognised to be a safe and effective therapy in IPEX (Yong, Russo et al. 2008). Both boys have now undergone successful HSCT as a result of recognition of the genetic cause of their condition, and its dire prognosis without definitive therapy. Interestingly their growth has also increased since transplantation. This family demonstrates the expanded clinical spectrum of IPEX, which had been reported as a condition typically

fatal in the first decade of life (Bennett, Christie et al. 2001, Levy-Lahad and Wildin 2001). Indeed, the syndrome was not initially suspected due to the more dominant neurological symptoms, which led to multiple other investigations, and consideration of other variants as a cause of the condition.

Another patient, a 16-year old female was diagnosed with GATA2 deficiency (OMIM #614172) and has also now undergone a successful HSCT. The genomic result prompted a bone marrow biopsy being performed, which demonstrated early changes of myelodysplasia, leading to the decision for definitive therapy.

Table 4.5 Diagnostic genomic findings and therapeutic impact in PADs

Diagnosis	Genomic result (classification)	Therapeutic impact above usual care	Treatment change
CVID	*STAT3* (Class 5)	*Y*	*tofacitinib or tocilizumab*
Agamma	*TCF3* Class 5	*N*	*N/A*
CVID	*AICDA* Class 5	*possible*	*?lenialosib*
CVID	*CR2* Class 4	*N*	*N/A*
CVID	*CTLA4* Class 4	*Y*	*abatacept/discontinued harmful therapy*
CVID	*CTLA4* Class 4	*Y*	*abatacept*
CVID	*FAS* Class 4	*Y*	*sirolimus*
CVID	*NFKB1* Class 4	*possible*	*?tocilizumab/ anakinra*
CVID	*NFKB1* Class 4	*possible*	*?tocilizumab/ anakinra*

(CVID, common variable immunodeficiency; Agamma, agammaglobulinemia. Y, yes; N, no; N/A, not applicable; ?, possible therapy.)

4.3.2 Examples of precision medicine

The example of CTLA4-haploinsufficiency (OMIM #616100), also known as 'CHAI' for *C*TLA4 *H*aploinsufficiency with *A*utoimmune *I*nfiltration (Lee, Moon et al. 2016), is an ideal example of the potential for precision medicine. As a result of the diagnoses reached by way of Melbourne Genomics, two patients were successfully treated with abatacept, a CTLA4-Ig fusion molecule that has demonstrated benefits in 'CHAI' patients (Lee, Moon et al. 2016). A 19-year-old male with severe GI manifestations and a high requirement of IVIG (0.7 mg/kg/4weeks) had significant improvement in diarrhea, with a 50% reduction in frequency of bowel actions, and, importantly, a large decrease in IVIG requirements (reduced from 0.7 mg/kg/4weeks to 0.5 mg/kg/4weeks post therapy). His sister was recruited as an exception to the inclusion criterion of only recruiting one member per family, because the siblings were estranged and at the time

she was acutely unwell with agranulocytosis, requiring admission to an isolation bed in the Intensive Care Unit. She had previously received a diagnosis of relapsing remitting multiple sclerosis (RRMS). Understanding the mechanism of her disease was thought to have potential implications for treatment and also led to awareness of therapies with potentially dire adverse effects. The anti-IL-2Rα monoclonal antibody daclizumab, had been trialled in this patient, after other standard therapies for MS had failed (prior to the finding of CTLA4-haploinsufficiency). It is likely that this therapy exacerbated the autoimmune disease and led to a potentially fatal complication of agranulocytosis (Watson, Slade et al. 2018). Unfortunately, several months later, she was found to have metastatic melanoma, which led to her death within a year of her genetic diagnosis. Due to her immunodeficiency, she was not treated with chemotherapy, nor was immunotherapy with the anti-CTLA4 monoclonal antibody considered appropriate given her underlying genetic disease.

4.3.3 Genetic diagnosis leads to personalised management, and monitoring for specific susceptibilities

Awareness of potential complications known to be more strongly associated with some monogenic conditions is a particularly valuable aspect of personalised medicine. For example, HPV susceptibility in WHIM syndrome has led to a different approach in offering additional HPV vaccines to affected individuals (Handisurya, Schellenbacher et al. 2010). The heightened awareness of central adrenal insufficiency in patients with mutations in *NFKB2* is a particularly important, given the potential for coma and death in patients with untreated hypo-adrenalism (Brue, Quentien et al. 2014). Patients with *NFKB1* haploinsufficiency are susceptible to liver disease: one patient from our original

report developed hepatopulmonary syndrome in the context of nodular regenerative hyperplasia and has subsequently undergone a successful liver transplant (Fliegauf, Bryant et al. 2015). It is attractive to hypothesise that the specific defect in NF-κB signaling leads to derangement of inflammatory pathways that may be targets to treat, or even prevent the development of liver disease in the first instance. Indeed, this has been studied in a mouse model of NF-κB1 deficiency and is responsive to high dose COX inhibition (Jurk, Wilson et al, 2015).

4.4 Discussion:

This study demonstrates that exome sequencing provides significant diagnostic and clinical utility to individuals with PID and SAID. Overall a specific diagnosis was reached in 14% of patients with PID, although this was higher in those with CID, immune dysregulation or syndromic features. As compared to standard care, this was a five-fold increase in diagnoses, since many patients would not have otherwise been offered any genetic testing at all. Only 11% of patients with PADs received a diagnostic result. Interestingly, however, in 34% of participants with PAD, a genomic variant of interest was identified but did not meet criteria for pathogenicity in accordance with American College of Genetics criteria, and these variants require further investigation to determine their functional impact (Figure 4.2).

Other studies of the utility of exome or genome sequencing performed in PID cohorts have demonstrated higher overall diagnostic rates than we have reported here. In a study from Mt Sinai Hospital, New York City, 30% of CVID patients who had early onset disease, complicated by autoimmunity, or with a family history, received a genetic diagnosis (Maffucci, Filion et al. 2016). Another larger cohort had a diagnostic rate of

40%, although that study included a higher proportion of combined immunodeficiencies, higher rates of consanguinity and included trio analysis (Stray-Pedersen, Sorte et al. 2017).

In this study, the lower diagnostic yield in PAD/CVID is likely due to the population selected for inclusion: the majority of the cohort were adult patients with a diagnosis of PAD (mostly CVID), which in most cases was sporadic. This was intentional; we wanted to be able to offer testing to a group of patients for whom genomic sequencing was inaccessible. Pediatric patients with CID or syndromic PID were likely to have previously been offered genetic testing (and therefore many have been excluded from the study), so the cohort is enriched for patients in whom a genetic diagnosis was not previously considered likely, or, who had a clinical diagnosis of a condition where genetic testing was not previously readily available, or, where genetic testing was of dubious clinical utility. Furthermore, if genetic testing had been offered at all, this was on an *ad hoc* basis, depending on the site, clinician perspective and expertise, and availability of specialist genetics services. Singleton analysis may also have led to lower diagnostic rates, although segregation after identification of a strong candidate variant was performed in some cases. Additionally, there is the potential benefit of reanalyzing the genomic data in a way that enables interrogation of causes of polygenic disease.

Another approach that has been trialled in CVID, was WGS in conjunction with RNA sequencing, and led to more insights into pathogenesis in a smaller group of pts, n=34, but may be some time before widespread clinical application due to the cost and intensive sample processing involved (van Schouwenburg, Davenport et al. 2015). Nevertheless, the use of RNA studies, to complement genomic analysis is an attractive

approach, and may offer rapid validation of novel genetic findings. Indeed, we are undertaking further studies to determine whether RNA sequencing (of both bulk and single cells) could be a tool for validation of the VUS findings from genomic sequencing.

Identifying the genetic aetiology of PID may have a multitude of benefits for patients. Many PIDs are diagnosed using descriptive terms, often after a significant 'diagnostic odyssey'. A molecular diagnosis can be powerful in validating a clear cause for disease in a patient and may be of both psychological and clinical benefit. These diagnoses may replace a rather vague, descriptive diagnosis, such as CVID, which is essentially an 'umbrella' term for a heterogeneous collection of disorders. This cohort demonstrates that identification of a specific genomic diagnosis can transform treatment options by enabling the use of personalised therapies otherwise not considered or in the case of HSCT, potentially curative.

In the classic SAIDs, precision therapies targeted to the overproduction of a single, or narrow range of cytokines are well-established in clinical practice, but the delineation between autoinflammatory disease and immunodeficiency is not always distinct. Blocking the effects of inflammatory cytokines may be of relevance in conditions where these features overlap, such as in patients with *PLCG2* mutations, *NFKB1* haploinsufficiency, and ADA2 deficiency; all may result in a combination of infectious susceptibility and autoinflammation.

Antagonising intracellular signaling pathways is also a useful therapeutic approach, as has been demonstrated in patients with GOF *STAT1* and *STAT3* mutations (Forbes,

Vogel et al. 2018) (Table 4.5). Another PID with great potential for personalised therapy, is *A*ctivated *P*I3-kinase *D*elta *S*yndrome (APDS, OMIM #615513), arising as an autosomal dominant immunodeficiency due to *PIK3CD* mutations that lead to a GOF of the p110δ subunit of PI3kinase (Angulo, Vadas et al. 2013), or LOF in *PIK3R1*, the regulatory subunit of p110δ (Lucas, Zhang et al. 2014). These patients benefit from targeted inhibitors, already used in clinical practice for lymphoproliferative disease, such as idealisalib (Dornan, Siempelkamp et al. 2017).

The complexity of the immune response is further highlighted by the striking variability in penetrance, severity, and age of onset of the autosomal dominant monogenic diseases, such as *CTLA4* and *NFKB1* haploinsufficiency, as well as in the case of IPEX syndrome. Understanding the factors that mediate reduced penetrance and severity will lead to opportunities to identify potential therapeutic targets, and eventually enable clinical trials, in order to gather further evidence for stratification of therapies according to genotype. In these conditions, the utility of true genomics is likely to be evident in the future. Whilst epigenetic factors may influence disease phenotype, the role of additional germline genetic factors should not be ignored, and interpreting polygenic risk is a future challenge in this area, whereby the nature of genetic variation, may be more thoroughly used to predict disease outcomes. It is likely that relatively common genetic variants, such as those already identified in *TNFRSF13B*, will be incorporated in genetic test reports to patients, and considered as disease modifiers.

Genetic counselling and the potential for prenatal diagnosis and pre-implantation genetic diagnosis is not only empowering for patients, but also has greater potential benefits from a cost-benefit perspective. A pre-emptive diagnosis of SCID is life-

saving, yet Australia has no national newborn screening program at the time of writing; however a pilot program is underway in NSW, and will hopefully lead to national implementation. The benefit of facilitating decision-making about reproductive options is difficult to measure and is likely to be highly dependent on the experience of prospective parents and their families.

Further studies are ongoing to determine the overall health economic benefit of offering genomic sequencing for PID diagnosis. Continued reanalysis of the sequence data with expanded and more inclusive gene lists, may lead to additional diagnoses, as well as novel genetic discoveries. It is likely that this will increase the diagnostic yield, as well as enhance clinical utility. Data from other Melbourne Genomics disease flagships have demonstrated cost savings of application of exome sequencing early in the patient journey. 52% of children suspected of having a monogenic disorder were diagnosed by exome sequencing, and if sequencing was performed at the initial referral to a tertiary centre, prior to additional diagnostic work-up, saved close to AU$10,000 per diagnosis (Tan, Dillon et al. 2017).

The immediate benefit of identifying a genetic diagnosis for patients is clear for a number of cases, where therapy has changed as a result of understanding the mechanism of disease and led to an improvement in physical well-being and long-term prognosis. More work is now being undertaken to study the psychological benefits, in-depth health economic analysis and modeling, as well as reanalysis of the sequence data in order to identify variants in more recently reported genetic causes of PIDs, as well as for gene discovery.

CHAPTER 5. NF-KAPPAB2 DEFICIENCY: CLINICAL MANIFESTATIONS IN HUMAN DISEASE AND QUANTITATIVE ANALYSIS OF LYMPHOCYTE BEHAVIOR IN AN ANIMAL MODEL OF THE DISEASE

5.1 Introduction

In Chapter 3, the challenges associated with diagnosing PADs, as well as the frequent non-infectious complications of CVID, were highlighted. As further expanded upon in Chapter 4, early implementation of genetic testing in the diagnostic process is likely to lead to more rapid, precise diagnoses for patients, bypassing the 'diagnostic odyssey', and ideally leading to safe, targeted therapies. Only a minority of the patients, studied in Chapter 3, had been offered genetic testing at the time of the study; in 19 patients with (non-XLA) PAD, a genetic diagnosis had been made (Chapter 3, Table S1). Of these, five individuals, from three families, were identified to harbor damaging heterozygous variants in *NFKB2*, encoding the non-canonical NF-κB transcription factor, p100/p52. An additional patient was identified to harbor a VUS in *NFKB2* through the Melbourne Genomics study reported in Chapter 4.

As discussed in Chapter 1 (section 1.5.1), mutations in *NFKB2* were first reported to cause CVID in 2013, by a group in Salt Lake City, USA (Chen, Coonrod et al. (2013). Subsequently a number of other families have been reported from North America, Europe, Asia and Australia. With these reports, the spectrum of clinical manifestations associated with NF-κB2 deficiency expanded; and in addition to early-onset hypogammaglobulinema, ACTH deficiency and alopecia; also includes ectodermal abnormalities in some patients (Lee, Fulcher et al. 2014, Lindsley, Qian et al. 2014, Liu, Hanson et al. 2014, Shi, Wang et al. 2016). Understanding the underlying genetic

cause of disease is clearly of benefit with regard to awareness of gene-specific disease complications, such as the potential severe morbidity from ACTH deficiency. Furthermore, it may also lead to insights into specific infectious susceptibilities, and potential novel therapeutic strategies.

5.1.1 Human NF-κB2 deficiency

In this chapter NF-κB2 defects are studied in more detail: the first part comprises the manuscript entitled, *Fatal enteroviral encephalitis in a patient with Common Variable Immunodeficiency harboring a novel mutation in NFKB2 (Slade, McLean et al. 2019)* This report highlights the cellular and clinical phenotypic variability in NF-κB2 deficient individuals, and the potential risk of severe viral infections even with apparently adequate immunoglobulin replacement. The reason for this specific viral susceptibility could therefore be attributed to a cellular defect rather than the antibody defect *per se*, warranting further investigations of lymphocyte function.

5.1.2 Quantitative assays of NF-κB2 deficient lymphocytes

The second part of this chapter focuses on the establishment of a quantitative assay of lymphocyte proliferation, to test for small differences in the sensitivity of genetically diverse lymphocytes to stimulatory signals. One limitation of studying human samples was the variably low numbers of primary cells. The proband of the family presented here, had a complete absence of B cells, and her sister demonstrated an absence of memory B cells. Using an animal model deficient in NF-κB2 provided an opportunity to study lymphocyte behavior in the absence of the potential confounders of using

patient samples, such as comorbid infection, immune-modulatory therapies, and the complete absence of some cell populations.

We hypothesised that measurement of *Nfkb2*$^{-/-}$ and *Nfkb2*$^{+/-}$ lymphocyte responses to activating signals would lead to insights into the pathogenesis of specific infectious susceptibility in human NF-κB2 deficiency. Due to the important role of CD8^{+} T lymphocytes in anti-viral immunity, and the fatal enteroviral infection in our patient, we questioned whether NF-κB2 deficient CD8^{+} T lymphocyte behavior could account, at least in addition to the obvious B cell defect, for this infectious susceptibility. We chose to investigate this first in the *Nfkb2*$^{-/-}$ mouse model (kindly provided by Dr Lorraine O'Reilly, WEHI) (Caamano 1998, Franzoso, Carlson et al. 1998).

5.2 Results Part 1: "Fatal enteroviral encephalitis in a patient with Common Variable Immunodeficiency harboring a novel mutation in NFKB2"

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ORIGINAL ARTICLE

Fatal Enteroviral Encephalitis in a Patient with Common Variable Immunodeficiency Harbouring a Novel Mutation in *NFKB2*

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Abstract

Common variable immunodeficiency is the most prevalent of the primary immunodeficiency diseases, yet its pathogenesis is largely poorly understood. Of the cases that are monogenic, many arise due to pathogenic variants in *NFKB1* and *NFKB2*. Here, we report enteroviral encephalomyelitis as the cause of a fatal neurodegenerative condition in a patient with a novel heterozygous mutation in *NFKB2* (c.2543insG, p.P850Sfs36*) that disrupts non-canonical NF-κB signaling. Investigations of primary and secondary lymphoid tissue demonstrated a complete absence of B cells and germinal centers. Despite multiple negative viral PCR testing of cerebrospinal fluid during her disease progression, post-mortem analysis of cerebral tissue revealed a chronic lymphocytic meningoencephalitis, in the presence of Cocksackie A16 virus, as the cause of death. The clinical features, and progression of disease reported here, demonstrate divergent clinical and immunological phenotypes of individuals within a single family. This is the first reported case of fatal enteroviral encephalomyelitis in a patient with NF-κB2 deficiency and mandates a low threshold for early brain biopsy and the administration of increased immunoglobulin replacement in any patient with a defect in this pathway and deterioration of neurological status.

Keywords Immunodeficiency · CVID · NFKB2 · enterovirus · encephalitis · NF-kappaB2 deficiency

Introduction

Nuclear factor kappa B 2 (NF-κB2) is essential for the activation and differentiation of B cells, and development of secondary lymphoid organs [1]. Mutations affecting the C-terminus of the NF-κB2 protein result in common variable immunodeficiency (CVID) due to impaired activation of the alternative NF-κB pathway (CVID10, OMIM ID: 615577) [2–9]. The predominant feature reported in these patients with a deficiency of NF-κB2 function, is early-onset hypogammaglobulinemia. In the majority of cases, there is a reduction of isotype-switched memory B cells, and in a few, a complete absence of B cells. The function of other cells is also impaired; reduced $CD4^+$ T cell proliferation has been demonstrated in some [3, 4], NK-cell function has been shown to be defective [8], and reduction in the proportion of regulatory T cells and T follicular helper cells has been reported in several patients [4, 5]. In contrast, mutations leading to an apparent gain-of-function in NF-κB2 signaling, appear to cause a more severe immunodeficiency, characterized by susceptibility to Epstein-Barr Virus (EBV), and cytomegalovirus (CMV) infections [9].

Accompanying the abnormalities in immune cell types in patients reported with NF-κB2 deficiency is a susceptibility to endocrine disease, which is likely to be due to an autoimmune process in many cases, and alopecia areata and universalis.

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Defective central tolerance most likely plays a role in the pathogenesis of these autoimmune complications; reduction in expression of the autoimmune regulator (*AIRE*) has been observed in animal models of NF-κB2 deficiency [10, 11]. Furthermore, there is considerable overlap of clinical manifestations reported in individuals with NF-κB2 deficiency and those with autoimmune polyglandular syndrome-1 (APS-1 or APECED, OMIM ID: 240300) due to homozygous and compound heterozygous mutations of *AIRE* [12], including a recent report of anti-cytokine antibodies in a patient with NF-κB2 deficiency [13]. Loss of peripheral tolerance is also likely to be a significant contributing factor to autoimmune manifestations, with the observation of reduced numbers of circulating regulatory T cells in NF-κB2 deficient individuals [4].

Methods

Ethics and Informed Consent

Patients and their healthy relatives were recruited from the Department of Clinical Immunology and Allergy, Royal Melbourne Hospital, Victoria, Australia. Unrelated healthy donors were recruited from the Volunteer Blood Donor Registry, (VBDR). Informed consent was obtained from all individual participants included in the study for genomic analysis and functional immunologic studies prior to inclusion in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of Human Research Ethics Committees at Melbourne Health and The Walter and Eliza Hall Institute for Medical Research (Approved projects 2009.162, 10/02) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Genomic Analysis

Genomic DNA (gDNA) was extracted from whole blood from family members using QIAamp minikit (QIAGEN). Whole-exome sequencing was performed on gDNA from the proband and her sister (Illumina HiSeq 2000) at the Australian Genome Research Facility (AGRF, Melbourne). Analysis was performed as previously described [14]. Briefly, a filtering pipeline was applied to identify non-synonymous variants in protein-coding or splice-site regions with minor allele frequency of less than 1 % in publicly available databases (ESP6500; 1000 Genomes). Priority was given to frameshift and truncating variants. Additional in silico prediction tools were used for missense variants. Sanger sequencing was performed (AGRF) in mother and father to confirm familial segregation.

Cell Culture and Western Blotting

Prior to stimulation, peripheral blood mononuclear cells (PBMCs) were counted and seeded at 1×10^6 cells/mL, rested in RPMI 1640 supplemented with 1% FCS for up to 4 h at 37 °C, 5% CO_2 to synchronise cells into a resting state. Cells were then cultured at 37 °C, 5% CO_2 in tissue culture medium alone or stimulated by supplementing tissue culture medium with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (1 ng/mL), or CD40 ligand (100 ng/ml, MegaCD40L, Enzo Life Sciences), and cells were harvested at indicated times. Cell lysates, suspended in 6x Laemmli buffer, were loaded onto 4–12% BisTris polyacrylamide gels in MOPS buffer and transferred to a nitrocellulose membrane via the Bio-Rad Trans-Blot Turbo Transfer System, as previously described [14, 15]. Protein detection was performed using the following primary antibodies from Cell Signaling Technologies: phospho-p100 #4810; p100/p52, #4766; phospho-p105, #4806; p105/p50, #3035; phospho-p65; and p65, kit#9936, and detected with secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP, Santa Cruz Biotechnology). Images were obtained with the Bio-Rad ChemiDoc system and analysed using Image Lab software (Bio-Rad). Beta actin was used as a loading control.

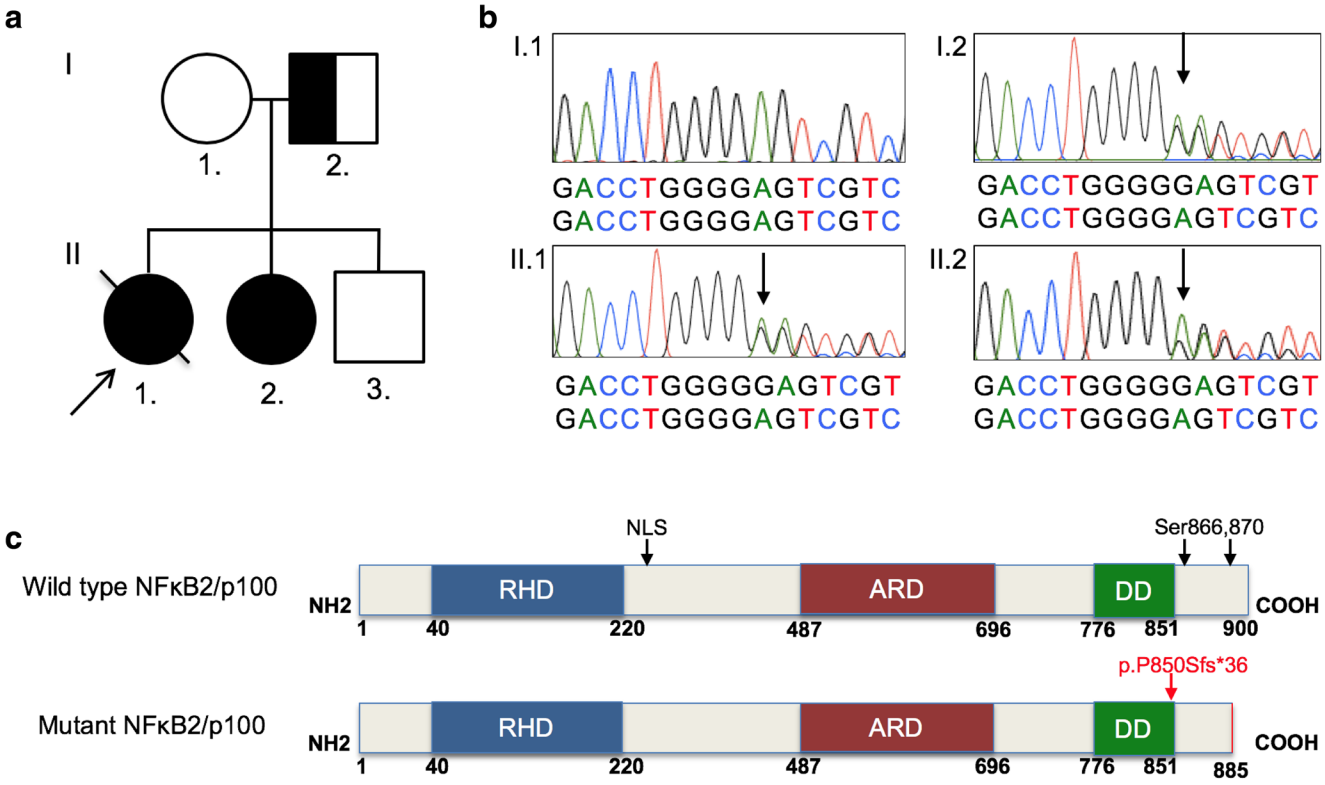
Immunophenotyping

PBMCs were separated from whole blood and stained with the following antibodies according to standard protocols: BV650 anti-CD19 (BioLegend; clone HIB19); APC-H7 anti-CD27 (Miltenyi Biotec; MT271); BUV395 anti-CD3 (BD Pharmingen, SK7); PerCP Cy5.5 anti-CD4 (BD Pharmingen, RPAT4); PE anti-CD8 (BD Pharmingen, SK1); FITC anti-CXCR5 (BD Pharmingen, RF8B2); FITC anti-CD45RA (BD Pharmingen, HI100); PE anti-CD45RO (BD Pharmingen, UCHL1); PE-Cy7 anti-CD127(BD Pharmingen, AO19D5); BV421 anti-CD25 (BD Pharmingen, BC96); and BV711 anti-PD1 (BD Pharmingen, EH12.2H7). Samples were acquired on a five-laser BD Biosciences LSR Fortessa X-20 along with single-color compensation controls of antibody-conjugated beads (UltraComp eBeads, eBioscience), live/dead (FluoroGold) controls, and unstained controls.

Results

Clinical Presentation of Proband

The proband (II.1, Fig. 1a), the first-born child of non-consanguineous parents of northern European origin, was



d

human	830	GLLEALSDMGLEEGVRLLRG**P**ETRDKLPSTAEVKEDSAYGSQSVE–QE–AEKLGPPPEPPGGLCHGHPQPQVH	900
c.2543insG	830	GLLEALSDMGLEEGVRLLRGSRNPRQAAQHSRGEGRQCVREPVSGAGGREAGPTP*	885
monkey	830	GLLEALSDMGLEEGVRLLRG**P**ETRDKLPST----------GKGASLEGGSGLGGWEA---	876
wolf	830	GLLCALSDMGLEEGVRLLRG**P**EARDKLPSTAEVKEDSAYGSQSVE–QE–AEKLGSPPEPPGGLCHGRPQPQVH	900
cow	831	GLLNALSDMGLEEGVRLLKG**P**ETQEKLPST–EVKEDSAYGSQSVE–QE–AEKLGPPPEPPGGLCHGHPQPQVH	900
mouse	830	GLLEALSDMGLHEGVRLLKG**P**ETRDKLPSTAEVKEDSAYGSQSVE–QE–AEKLCPPPEPPGGLCHGHPQPQVH	900
rat	829	GLLEALSDMGLHEGVRLLKA**P**ETRDKLPST–EVKEDSAYGSQSVE–QE–AEKLCPPPEPPGGLCHGHPQPQVH	898
chicken	837	GLLEALDSMGLRGAVRMLRKPEPLEKLQST–EVKEDSAYGSESVE-EEQAAALKPRPVPEGELPHSQQQQQVH	907
zebrafish	848	GLVEALQALGVREGVRLLRDSQPREDKQSS–DSTEDSGFGSQSIG–EE-------MANPAVGN----------H	902
frog	884	ELINTLQSMGLNKGVELLCKSETYAKHQSPAEGKNDSAYESQSMEVDQSSAELSPTTEPAIGQ----------	946

Fig. 1 Autosomal dominant inheritance of a novel *NFKB2* variant in a multiplex CVID family. **a** Pedigree of family with a heterozygous variant in *NFKB2* segregating with CVID in 2 affected sisters. The proband (II.2) is indicated by the arrow, and the line through shape indicates deceased. Shaded circles represent affected females, and unfilled circle represents unaffected female. Unfilled square represents an unaffected male, and the half-filled square represents unaffected male harbouring the *NFKB2* c.2543insG variant (I.2). **b** Sanger sequencing showing heterozygous insertion of G at position c.2543 (indicated by arrow) in exon 22 of *NFKB2* in the proband (II.1), her sister (II.2) and father (I.2), and wild type sequence in the mother (I.1). **c** Schematic illustration of NF-κB2 p100 wild type protein (top) and p.P850Sfs*36 mutant protein (bottom), depicting the following domains: NLS, nuclear localisation signal; RHD, rel homology domain; ARD, ankyrin repeat domain; DD, death domain. Ser866 and 870 indicate phosphorylation sites at serine residues, positions 866 and 870. The mutated residue, c.2543insG, p.P850Sfs*36, is indicated and is predicted to result in a truncated protein with loss of serine residues at positions 866 and 870. **d** Evolutionary conservation of NF-κB2, p100. The predicted protein sequence resulting from *NFKB2* c.2543insG (P850S) is depicted in red (mutalyzer). Arrow indicates the wild type P residue at position 850; conserved residues in bold

diagnosed with "possible CVID" in 1997, at the age of 9 years, after investigation for failure to thrive, recurrent lower respiratory tract infections and widespread molluscum contagiosum. Immunologic investigations at diagnosis revealed reduced IgG2 and IgA levels, reduced total B cell numbers, normal T and NK cell numbers, and normal T cell proliferative responses to PHA (phytohemagglutinin) (Table 1). Production of specific antibody in response to pneumococcal polysaccharide, conjugated tetanus and diphtheria vaccines, was absent. After an initial unsuccessful trial of continuous antibiotic therapy, intravenous immunoglobulin (IVIG) replacement therapy was introduced in 1998. She responded well to the treatment initially, with significantly fewer respiratory tract infections. Over time, there was also resolution of the molluscum contagiosum.

Table 1 Laboratory investigations of patients and family members

Family Member	II.1			II.2		I.1	I.2	II.3
Diagnosis/status	"Possible" CVID			CVID		Healthy	Healthy	Healthy
Date of test (month/year)	02/1997	10/2015		02/1997	10/2015	10/2015	10/2015	10/2015
IgG (5.5–16.3 g/L)	11.4	11*		*5.4*	10.7*	11.6	8.8	12.5
IgG2 (1.1–5.5 g/L)	*0.08*			*0.06*				
IgA (0.7–4.2 g/L)	*< 0.1*	*0.1*		*0.34*	*0.1*	1.9	2.5	1.8
IgM (0.3–2.9 g/L)	*0.27*	*0.1*		*0.3*	*0.1*	1.9	0.9	1.7
Lymphocyte count (1.2–4.0 × 10^9/L)	2.9	2.4		3.5	2.1	2.9	3.4	2.1
Total B cells, $CD19^+$ (% total lymphocytes, 7–23%)	*1%*	*absent*		7%	7.4%	15.5%	13.9%	–
Memory B cells, CD20+CD27+ (% total B cells, 16–55%)	–	*absent*		–	*absent*	27%	17.5%	–
Total T cells, $CD3^+$ (% total lymphocytes, 60–85%)	*57%*	63.4%		89%	82%	73.7%	68%	–
T helper cells, $CD3^+CD4^+$ (% total lymphocytes, 28–58%)	37%	39.3%		*64%*	*60.6%*	*59.3%*	50.9%	–
T regulatory cells, $CD3^+CD4^+$ $CD25^+$ $CD127^{lo}$ (%	–	–		–	*1.42%*	10.6%	7.22%	
TFH cells, $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ (%$CD4^+$ cells)	–	–		–	*0.17%*	4.02%	7.55%	–
Cytotoxic T cells, $CD3^+CD8^+$ (% total lymphocytes, 19–48%)	20%	24.9%		26%	21%	*12.3%*	19.6%	–
Natural Killer cells, $CD16^+CD56^+$ (% total lymphocytes, 6–29%)	*42%*	*34.3%*		*3%*	8%	7.5%	12.8%	–
Date of test (month/year)	2/2014	7/2014	3/2015	2000	2015	–	–	–
Cerebral MRI	NAD	Small foci of diffusion restriction	Diffuse cortical atrophy	NAD	NAD	–	–	–
CSF findings:				–	–	–	–	–
Lymphocytes	*24 × 10^6/mL*	*6 × 10^6/mL*	*4 × 10^6/mL*					
Polymorphonuclear cells	0 × 10^6/mL	*3 × 10^6/mL*	*9 × 10^6/mL*					
Erythrocytes	0 × 10^6/mL	*1 × 10^6/mL*	0 × 10^6/mL					
Protein (< 0.45 g/L)	*0.48 g/L*	*0.54 g/L*	*0.58 g/L*					
Glucose (2.2–3.9 mmol/L)	3.5 mmol/L	2.7 mmol/L	3.0 mmol/L					
Gram stain, ZN stain, cryptococcal antigen, and PCR[¶]	All negative	All negative	All negative					
Autoantibodies: anti-GAD antibodies (<5U/ml)	*(242 U/mL)*	–	*177 U/mL*	–	*60 U/mL*	–	–	–

Units of measurement and reference ranges in parentheses; CSF, cerebrospinal fluid; *MRI*, magnetic resonance imaging; *CVID*, common variable immunodeficiency; *NAD*, nothing abnormal detected; *PCR*, polymerase chain reaction; *GAD*, glutamic acid decarboxylase; *lymph*, lymphocytes; *PMNs*, polymorphonuclear cells; *ZN*, Ziehl-Nielsen; *TFH*, T follicular helper [¶] PCR was performed for herpes simplex viruses-1 and -2, varicella zoster virus, herpes zoster virus, enterovirus, parechovirus ,and polyoma (JC and BK) viruses; italic indicates abnormal values; *on intravenous immunoglobulin replacement; −, not performed

Clinical Features of Other Family Members

The proband's younger sister (II.2, Fig. 1a), aged 7, was also diagnosed with CVID after a similar history of respiratory tract infections and reduced total IgG, IgG2, and IgA levels (Table 1). At the age of 11, she was diagnosed with hypothyroidism and central adrenal insufficiency. At 13, she developed autoimmune thrombocytopenia, which was treated successfully with rituximab after an initial lack of response to corticosteroids and IVIG.

At diagnosis, and at long-term follow-up after treatment with rituximab, the younger sister had normal total B cell numbers, although there was an absence of memory B cells (Table 1). Apart from a reduced proportion of NK cells at diagnosis, all other lymphocyte populations tested were normal in number (Table 1). However, more extensive immunophenotyping revealed a reduction in the proportions of T follicular helper cells and T regulatory cells (Table 1).

The parents and younger brother were healthy. In particular, their immunoglobulin levels and proportions of B and T lymphocytes were normal, whereas the proband developed a complete absence of peripheral B cells (Table 1).

Clinical Progression of Proband

In February 2014, at the age of 27 years, the proband presented with a 2-month history of weight loss, anorexia, headache, and visual disturbance. She had been receiving 0.5 mg/kg monthly Intragam P™ (Commonwealth Serum Laboratories, CSL Behring, Australia) with adequate trough levels between 8 and 9 g/L. Neurologic examination revealed no focal deficits; however, a vitreal hemorrhage was observed by fundoscopy. Cerebrospinal fluid (CSF) was notable for the presence of lymphocytes (24×10^6/mL) in the absence of erythrocytes and polymorphonuclear cells (Table 1). CSF bacterial culture and PCR for herpes simplex viruses (HSV-1, HSV-2), herpes zoster virus (HZV), varicella zoster virus (VZV), polyoma (JC and BK) virus, and entero- and parechoviruses were negative (Table 1). Cryptococcal antigen was not detected, and there was no evidence of mycobacteria by Ziehl-Nielsen staining and PCR. Cerebral imaging by CT and MRI was normal (Fig. 2a). She was treated with IV benzylpenicillin for 72 h, given the possibility of bacterial meningitis; however, after review of the results of CSF analysis, a presumptive diagnosis of aseptic meningitis was made. Despite an initial resolution of the symptoms, 6 months later, the patient represented with a recurrence of headaches. No additional new neurologic features were present. Further imaging and repeat analyses of CSF revealed no significant abnormalities (Table 1).

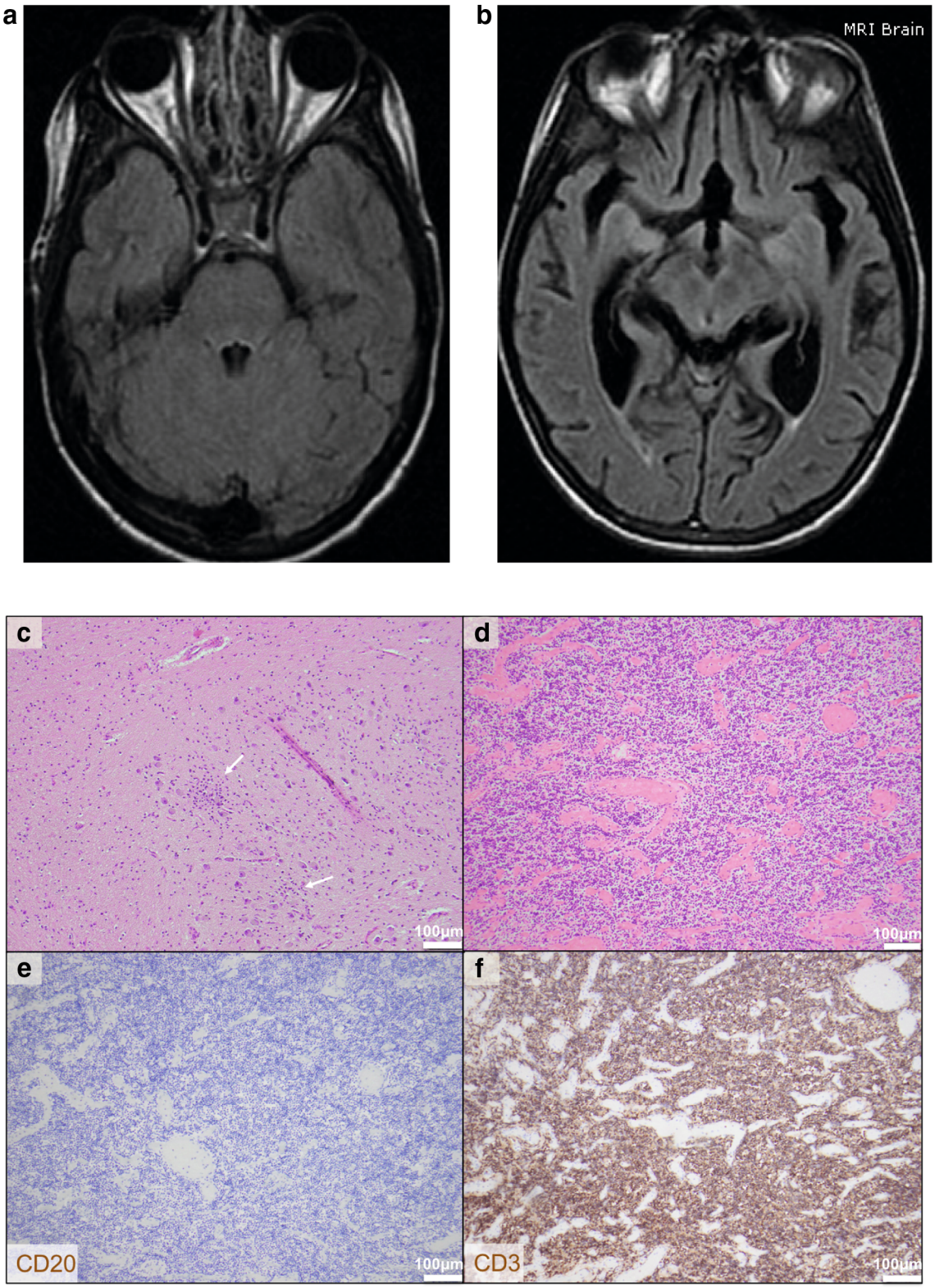
Twelve months after the initial presentation, the proband returned to the clinic with significant neurologic deterioration. Her family reported personality changes characterized by social withdrawal, and impaired memory, which had developed over a 1-month period. Neurological examination revealed an unsteady broad-based shuffling gait, dysmetria, and dysarthria. Tone and reflexes were generally increased. MRI revealed inflammation of bilateral mesial temporal lobes and cerebellum (Fig. 2a), likely accounting for the personality and gait changes respectively. Serum and CSF testing for antineuronal antibodies was negative, although anti-GAD antibodies were detected in serum (Table 1). Brain biopsy was recommended, but not performed due to the patient and family's wishes.

On the basis of these clinical features, the abnormalities on cerebral imaging, and lack of evidence of an infectious cause, a preliminary diagnosis of autoimmune encephalitis was made. High-dose IVIG (2 g/kg) and methylprednisolone 2 mg/kg was administered; however, the patient's condition continued to worsen. She was unable to ambulate, had significantly impaired vocalization and swallowing had become uncoordinated. A single dose of cyclophosphamide (500 mg) was administered, but the patient's condition deteriorated further over the following 2–3 weeks, and she died 4 weeks after withdrawal of active treatment.

At post-mortem, a chronic lymphocytic meningoencephalitis was diagnosed, with a T lymphocyte-rich, perivascular and parenchymal lymphocytic infiltration, neuronophagia and microglial upregulation, most prominent in the brainstem and cerebellum (Fig. 2c). No specific viral inclusions or viral immunoreactivity was found for HSV, HZV, CMV, measles virus (MeV), or polyoma virus. Real-time PCR detected enteroviral RNA from cerebral tissue, which was subsequently identified by Sanger sequencing as Coxsackie A virus, subtype A16. Reticulo-endothelial system changes included an absence of $CD20^+$ B lymphocytes in the splenic lymphoid tissue and an absence of germinal centers and $CD20^+$ B lymphocytes in sampled lymph nodes, as detected by histological analyses (Fig. 2d–f). The bone marrow was normocellular, the thymus normal for age, and the pituitary was normal. In addition to the encephalitis, bacterial bronchopneumonia, pulmonary edema, multiple renal cortical adenomata, and multiple plantar verrucae were also noted.

Identification of Novel *NFKB2* Mutation by Whole-Exome Sequencing

In order to identify the genetic etiology of the clinical phenotypes in this family, we performed whole-exome sequencing of gDNA from the proband and her younger sister, as described above. We identified a heterozygous insertion of a guanine at c.2543 in exon 22 of *NFKB2*. Sanger sequencing confirmed that the variant segregated with disease in both sisters, and was also identified in the



healthy father (Fig. 1b). The variant is predicted to lead to disruption of the phosphorylation sites required for initiating degradation of p100 to its active subunit, p52 (Fig. 1c). Proline at amino acid position 850 is highly conserved in mammals and insertion of G at c.2543 and results in proline to serine substitution and the creation of a premature stop codon, 36 residues along the resulting protein (Fig. 1d).

◀ **Fig. 2** NF-κB2 deficiency is associated with reduced B cell numbers, absence of germinal centers, and the development of enteroviral encephalitis in the proband. Cerebral MRI of proband from (**a**) February 2014 is normal in appearance, and, in (**b**) March 2015 shows marked cerebral atrophy. **c** Histology of cerebellar dentate nucleus shows features of encephalitis including perivascular and parenchymal lymphocytic infiltration (arrows) with neuronophagia and upregulated microglia (Hematoxylin and Eosin, H&E, × 100 actual magnification). (**d–f**) Post-mortem of carinal lymph node reveals (**d**) small lymphoid cells (H&E × 100 actual magnification), demonstrating complete absence of lymphoid follicles and germinal center architecture, (**e**) CD20 immunoperoxidase staining (positive staining, brown; negative, blue) depicting a complete absence of B lymphocytes (× 100 actual magnification), and (**f**) a dominant population of small T lymphocytes (CD3 immunoperoxidase, positive staining, brown; × 100 actual magnification)

Immunophenotyping Identifies a Variable B Cell Defect Associated with NF-κB2 Deficiency, with a Reduction in T Follicular Helper Cells and Regulatory T Cells in the Surviving Sister

Given the divergent clinical history of these patients, we sought to compare the proportions of B and T cell subsets in this kindred to that of a cohort of CVID patients ($n = 16$) and unrelated healthy donors ($n = 35$). Consistent with clinical phenotyping previously performed, the deceased sister had a complete absence of peripheral B cells (0.11% of total lymphocytes), whereas her sister had a reduced proportion of B cells (3.83%; Fig. 3a). Although no significant differences were observed in the proportion of B cells between

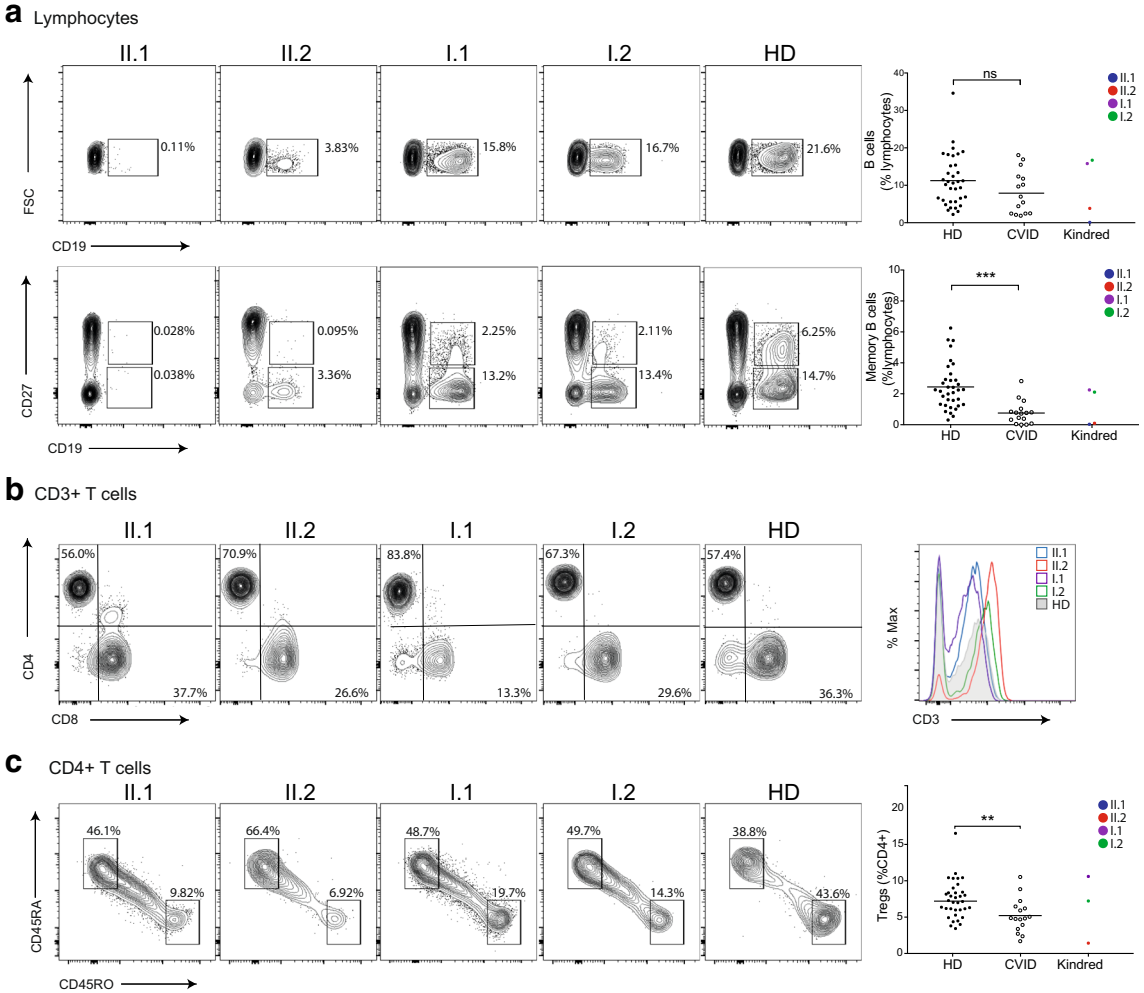


Fig. 3 Immunophenotyping reveals a variable B cell defect and reduced T follicular helper cells and regulatory T cells in NF-κB2 deficiency. Immunophenotyping of PBMCs depicting the proportion of (**a**) total B cells ($CD19^+$), naive B cells ($CD19^+CD27^-$) and memory B cells ($CD19^+CD27^+$). **b** Helper ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$) and (**c**) naïve ($CD4^+CD45RA^+$) and memory helper T cells ($CD4^+CD45RO^+$). Family members are labeled according to pedigree in Fig. 1. HD indicates healthy donor. Representative flow plots are shown for each family member tested, and an unrelated healthy donor (HD) and summary graphs are shown for HD ($n = 35$), unrelated CVID patients ($n = 16$) and family members from kindred as indicated. (ns, non-significant; *, p<0.05; **, p<0.01; ***, p<0.005)

healthy donors and a cohort of CVID patients, the affected sister and their parents were within the lower and upper limits of the group, respectively. Not surprisingly, a significant reduction in the percentage of memory B cells was observed in the CVID group, compared to healthy donors, again with the affected individuals showing an almost complete absence of memory B cells and normal proportions of memory B cells observed in their unaffected parents. While total helper ($CD3^+CD4^+$) and cytotoxic ($CD3^+CD8^+$) T cells were normal in all family members (Fig. 3b), a reduction in memory T helper cells and regulatory T cells was observed in affected sisters, but not in their parents, or unrelated healthy donor (Fig. 3c).

Functional Validation of the *NFKB2* Variant Demonstrates Impaired Phosphorylation of p100 after Short-Term Stimulation

To determine the functional effect of the *NFKB2* variant on NF-κB signaling, we next performed western blot analysis of lysates from PMA/ionomycin-stimulated PBMCs isolated from II.2 and an unrelated healthy donor at both early (10–20 min), and later (72 h) timepoints, given the biphasic expression of phospohorylated p100 following stimulation (Fig. 4a). Phosphorylation of p100 was severely reduced in stimulated PBMCs from II.2, compared to an unrelated healthy donor, with almost no protein detected after 20 min of stimulation (Fig. 4b). After 3 days, however, the amount of

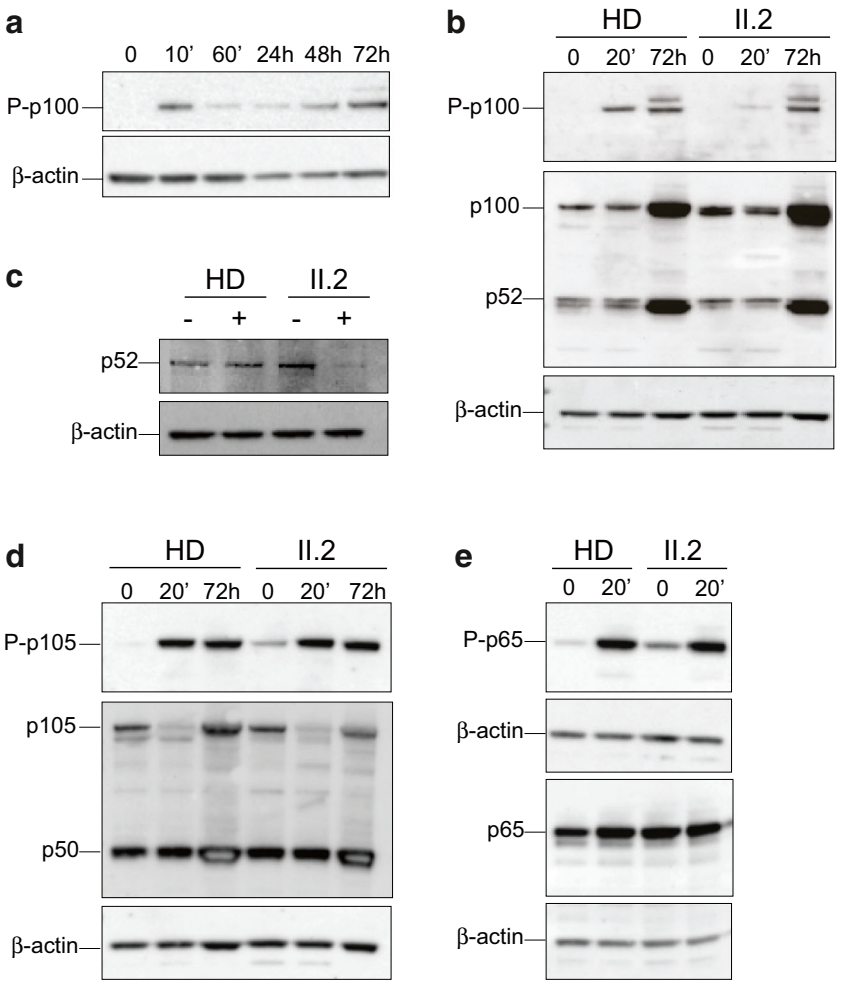


Fig. 4 A defect in early activation of non-canonical NF-κB signaling is observed in the presence of *NFKB2* c.2543insG. **a** Kinetics of phosphorylated p100 (P-p100) expression following stimulation with PMA/ionomycin demonstrates biphasic expression both at early and later timepoints. **(b)** Western blot of PBMCs from the patient, II.2, and a healthy donor, either unstimulated, or stimulated with PMA/ionomycin, for indicated times to measure early and late activation of NF-κB signaling. Proteins detected to measure non-canonical signaling are phosphorylated full-length p100 (P-p100) and expression of full-length p100 and p52 subunit. **c** Expression of p52 active subunit was evaluated in PBMCs from II.2 and another unrelated healthy donor either unstimulated or following incubation with CD40L for 48 h as indicated (**d**, **e**). To determine the effect on canonical signaling, phosphorylation of p105 (P-p105) and p65 (P-p65) as well as expression of p105, p50, and p65 was measured as indicated. Beta-actin was used as a loading control. Western blot analysis was performed in four independent experiments

phosphorylated p100 was comparable to the healthy donor and only a marginal reduction in processed p52 was observed. Both the full-length p100 and p52 subunit were detected in the affected individual and healthy donor. However, in II.2, expression of p100 was uniformly higher than in healthy donor individuals, suggesting an accumulation of non-processed p100 protein over time. A defect in p52 processing was also observed following CD40L activation (Fig. 4c), further supporting a functional defect in NF-κB2 signaling and functional p52 deficiency. The canonical pathway appeared unaffected, as no differences were observed for p105/p50 expression and signaling via the NF-κB1 pathway was comparable to healthy donor (Fig. 4d, e). Together, these results suggest that *NFKB2*^{P850Sfs36*} impairs NF-κB2 signaling at early timepoints, but not under prolonged stimulation conditions, and this may result in a reciprocal accumulation of p100, but does not affect canonical signaling.

Discussion

CVID is a disease of complex pathogenesis, which in the majority of cases is poorly understood. Monogenic causes account for a minority; however, the implementation of whole-exome and whole-genome sequencing continues to expand our understanding of the mechanisms by which this condition may develop [16]. Fourteen monogenic causes have been identified according to OMIM, of which NF-κB2 deficiency is one. We have previously discovered mutations in *NFKB1,* which cause CVID due to haploinsufficiency of the active subunit of the canonical NF-κB1 signalling pathway, p50 [15]. Together, *NFKB1* and *NFKB2* mutations are likely to be the predominant monogenic causes of CVID, particularly as they lead to autosomal dominant inheritance of the condition [17]. One of the features of dominantly inherited conditions is a variable penetrance of clinical features, as is reflected in this family. While certain features of monogenic CVID are common to all forms of the disease, polygenic and monogenic alike, some complications are restricted to single genes or pathways, such as the adrenocorticotropic hormone (ACTH) deficiency and alopecia seen in patients with *NFKB2* mutations affecting the C-terminal component of the protein, leading to disease by a dominant negative mechanism. In fact, more than half of the patients so far reported with NF-κB2 deficiency due to dominant negative mutations were reported to have ACTH deficiency and several have alopecia areata or totalis (Table 2) [2–9, 13]. Interestingly, alopecia is also a feature of NF-κB1 deficiency, but ACTH deficiency has not been reported in these individuals [15, 17, 18]. Here, we demonstrate the normal histologic appearance of the pituitary and thymus in a patient with NF-κB2 deficiency, in addition to complete absence of B cells and germinal centers in secondary lymphoid tissue (Fig. 2d–f). We have also demonstrated an impairment of activation of non-canonical NF-κB signaling after both PMA/ionomycin and CD40L stimulation, as evidenced by reduced phosphorylation of p100 early after activation, with a corresponding accumulation of total p100 and reduced active p52 (Fig. 4b,c) with canonical signalling largely intact (Fig. 4d,e). Together, these findings suggest a functional insufficiency of p52. The frameshift mutation identified in this family, while novel, is only 3 amino acids upstream from the most commonly reported *NFKB2* mutation, namely the nonsense mutant, p.Arg853*, and 5 amino acids from the first frameshift mutation reported, p.Lys855Serfs7* [2, 6, 8, 13, 19]. Furthermore, reported pathogenic *NFKB2* mutations in the NIK domain, downstream of the P850Sfs*36 mutation described here, are restricted to damaging only a single phosphorylation site (p.A867Cfs19*) [3], or located immediately adjacent to a phosphorylation site (D865G) [4, 6], and thus may be predicted to have a less pronounced effect on p100 phosphorylation than the mutation in the current report, which disrupts both Ser866 and Ser879 sites. Our observation of accumulated p100 following long-term stimulation in patient cells also supports disruption of impaired p100 processing to p52 in these individuals. Thus, the findings reported here are likely to be broadly applicable to patients with other dominant negative *NFKB2* mutations.

Autoimmune complications affect at least 25% of individuals with CVID [20] and pose therapeutic challenges due to the risk of immunosuppression in individuals with pre-existing immunologic incompetence. This family, with three phenotypically diverse individuals with the same *NFKB2* mutation, demonstrates the complexity of CVID, even when a monogenic etiology is found. The more widespread application of genomic technologies for the diagnosis of CVID, and other PIDs, will continue to reveal the spectrum of phenotypes associated with heterozygous disease-causing variants. The term monogenic is more appropriate here than Mendelian, as penetrance and expressivity are clearly variable. A pertinent example is CTLA4 haploinsufficiency, with a recent large cohort study revealing a clinical penetrance of 67% [21]. This is intriguing and raises many questions for future studies regarding the impact of genetic modifiers, in particular with respect to the contribution of environmental exposures and the potential for epigenetic effects.

Cocksackie A16 virus is a ubiquitous enterovirus, typically causing self-limited hand, foot, and mouth disease in children, although in rare cases it may lead to neurologic disease even in ostensibly immunocompetent individuals [22]. It is likely that most individuals are exposed to the virus and recover without serious adverse outcomes. The absence of B cells and immunoglobulin leads to an increased susceptibility to infection; enteroviral encephalitis is recognized as a potential complication of X-linked agammaglobulinemia (XLA); however, most reported cases have occurred in the setting of limited or no immunoglobulin replacement [23–25]. Adequate

Table 2 Clinical features of patients with dominant negative *NFKB2* mutations

NFKB2 variant protein change [Ref]	c.2564delA K855Sfs* [2, 7]	c.2557C > T R853* [2, 8, 13]	c.2593_2600del, D865Vfs*17 [5]	c.2598_2599insT, pA867Cfs*19 [3]	c.2594A > G, p.D865G [4, 6]	c.2600C > T p.A867V [6]	c2556_2563 p.R853Afs*29 [6]	c.2563A > T, p.R853* [6]	c.2596A > C p.S866R [9]	c.2543insG, p.P850Sfs*36 (current report)
Onset (years)	< 10, 1.5, 2	< 2, 2, 2, 23	1, 0.5	1	11, 20, 2, ?	8, 7	4, ?	2	8	2, 3
Diagnosis (years)	30, 6, 3	10, 4, 2, 23	9, 7	1.5	40, 20, 5, ?	9, 7	4, ?	18	10	7, 9
ACTH deficiency	3/3	3/4	1/2	0/1	1/4	1/2	1/2	1/1	1/1	1/2
Alopecia	1/3	4/4	0/2	1/1	3/4	0/2	0/2	1/1	0/1	0/2
Trachyonychia	1/3	4/4	0/2	1/1	0/4	0/2	0/2	1/1	0/1	2/2
Autoantibodies	2/3	4/4	0/2	0/1	0/4	0/2	0/2	1/1 (anti-IA2)	0/1	2/2 (anti-GAD)
Neurologic disease	Bell's palsy 1/3	Aseptic meningitis 1/4	CNS inflammation[¶] 1/2	0/1	Aseptic meningitis 1/4	0/2	0/2	0/1	0/1	Enteroviral encephalitis 1/2
Skin disorder	Vitiligo 1/3	MC 1/4 ED 3/4 psoriatic rash 1/4	0/2	0/1	Aquagenic urticaria 1/4	0/2	0/2	ED 1/1	MC 1/1	MC 1/2 psoriatic rash 1/2 BCC 1/2 plantar warts1/2

(ACTH, adrenocorticotropic hormone; *CNS*, central nervous system; *GAD*, glutamic acid decarboxylase; *IA2*, islet antigen 2; [¶] unspecified cause; *ED*, ectodermal dysplasia; *MC*, molluscum contagiosum; *BCC*, basal cell carcinoma;*?*, uncertain)

immunoglobulin replacement is usually sufficient to prevent the development of chronic infection. In this reported case, trough immunoglobulin levels were maintained between 8 and 9 g/L throughout the initial course of disease, and were above 10 g/L in the last 3 months of illness (Table 1). Other reports of disseminated enteroviral infections related to B cell depletion for the treatment of hematological malignancies and autoimmune disease provide further evidence that B cells provide a role other than antibody secretion in their defence against this infection [26, 27]. A single case of chronic enteroviral infection has been reported in a patient with BLNK deficiency, an extremely rare cause of autosomal recessive agammaglobulinemia (OMIM ID: 613502), and was associated with arthritis and dermatitis, in the absence of neurologic involvement [28]. Another report of a young female patient, who only months after receiving a diagnosis of CVID succumbed to enteroviral meningoencephalitis (Cocksackie A16 virus), highlights the necessity to be vigilant for this potential diagnosis [29]. It is unclear whether any genetic investigations were performed in this case, and B cell numbers and immunoglobulin levels were not reported.

Due to the ubiquitous nature of enteroviruses, immunoglobulin derived from multiple healthy donors should provide protection against most enteroviral subtypes. The use of purified, specific immunoglobulin has been implemented in other chronic viral infectious disease, such as cytomegalovirus [30], although no enteroviral specific product is currently available. An inactivated EV71 vaccine has been demonstrated to be effective in protecting against hand, foot, and mouth disease in a large cohort of infants and children in China, but was not effective in reducing severe cases of disease, and offered no protection against Cocksackie A16 infection [31]. Other therapeutic measures have been attempted in isolated cases of enteroviral encephalitis, including high-dose IVIG and intraventricular immunoglobulin, with variable, but predominantly poor outcomes [23, 32]. The use of ribavirin and pleconaril, which have in vitro activity against enterovirus [33], has not been well studied in human enteroviral infections. Other agents used as adjunctive therapy to IVIG include fluoxetine, which has been successfully used in a single case of an XLA patient with enteroviral encephalitis [34], and itraconazole, which has also been demonstrated to have in vitro anti-enteroviral activity [35].

Differentiating autoimmune disease from infections in immunodeficiency is crucial to avoid outcomes such as that reported here. A recently reported case of a CVID patient, who was also being treated with mycophenolic acid for lymphocytic interstitial pneumonitis and adalimumab for colitis, is suggestive of autoimmune demyelination with no evidence of infection on brain biopsy or deep metagenomic sequencing of CSF for potential pathogens [36]. From the current report, it is clear that the sensitivity of PCR from CSF samples may be insufficient for detection of infectious etiologies of encephalitis, including enterovirus. In our patient, viral PCR was performed on CSF samples on three separate occasions (February 2014, July 2014, March 2015; Table 1), and enterovirus was only amplified from a direct sample of brain tissue at autopsy, likely due to the greater sensitivity of testing a tissue sample. Unfortunately, a brain biopsy was declined during the course of the patient's illness. Several other cases of presumed autoimmune encephalitis in CVID patients are reported, including anti-GAD antibody associated limbic encephalitis developing as the presenting feature of CVID [37]. Detection of low-titer anti-GAD antibodies in the case of our patient (Table 1), when interpreted with the cerebral MRI (Fig. 2b), was an additional factor in the diagnostic challenge which arose, but the clinical significance of these autoantibodies, if any, remains unclear.

Conclusions

The challenge of differentiating autoimmune disease from infection is not new to physicians caring for patients with CVID, nor other immunosuppressed individuals with pre-existing autoimmune disease. Awareness of the particular risk of enteroviral infection, a low threshold for early brain biopsy, and administration of therapy with high-dose IVIG and antiviral therapy should be strongly considered in any patient with CVID and deterioration of neurological status.

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Compliance with Ethical Standards

Patients and their healthy relatives were recruited from the Department of Clinical Immunology and Allergy, Royal Melbourne Hospital, Victoria, Australia. Informed consent was obtained from all individual participants included in the study for genomic analysis and functional immunologic studies prior to inclusion in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of Human Research Ethics Committees at Melbourne Health and The Walter and Eliza Hall Institute for Medical Research (Approved projects 2009.162, 10/02) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of Interest The authors declare that they have no conflict of interest.

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5.3 Results Part 2: Quantitative studies in a murine model of NF-κB2 deficiency

5.3.1 Comparison of C57BL/6 and Nfkb2$^{-/-}$ naïve B cell responses confirm a defect in T-cell dependent responses due to loss of NF-κB2.

To test the effect of NF-κB2 deficiency on the behavior of B cells, naïve B cells were isolated from splenocytes of C57BL/6 (wild-type) and *Nfkb2*$^{-/-}$ mice and cultured as described in Chapter 2 (section 2.2.). To specifically measure the effect of loss of NF-κB2 on B cell survival and proliferation in response to both T-cell independent and T-cell dependent stimulus, isolated naïve B cells were cultured in the presence of either CpG alone, or the combination of anti-CD40 and recombinant IL-4, respectively. Briefly, purified naïve B cells were labelled with the division tracking dye, CTV, and cultured at 10 x10^3 cells per well in complete B cell media with either CpG alone (3 μM) or anti-CD40 mAb (clone 1C10, 5 μg/ml) and IL-4 (500 U/ml), harvested daily for up to 140 hours and proliferation analysed by flow cytometry.

After 40 hours, CpG-stimulated, naïve B cells had begun to proliferate, as demonstrated by dilution of CTV (Figure 5.1A), consistent with previous work (Turner, Hawkins et al. 2008). Both wild-type and *Nfkb2*$^{-/-}$ B cells stimulated with CpG continued to divide until approximately 60 hours, reaching a maximum of 3 divisions, well within the resolution limits of the CTV dye. This pattern of response, where CpG stimulated cells divide 3-4 times and then stop, returning to quiescence and go on to die, is typical of this stimulation protocol (Hawkins, Hommel et al. 2007, Hawkins, Markham et al. 2009, Hawkins, Turner et al. 2013)

Analysis of the total number of live cells over time reveals the net effect of cell division and death of stimulated cells. Under CpG stimulation, a small increase in cell number was observed until 90 hours.

Overall, the magnitude of the response was greater in wild-type compared with NF-κB2-deficient B cells (Figure 5.1A). This raised the question of whether the difference in cell counts was due to reduced sensitivity to CpG in *Nfkb2*$^{-/-}$ cells.

The mean division number (MDN) measures the average division at the population level over time. Furthermore, monitoring the MDN of dividing cells over time allows simultaneous measurement of multiple division parameters – division rate (*i.e. the slope of the line*), DD time (*i.e. the time at which the MDN plateaus*), and DD (*i.e. the average division number after which point the MDN plateaus*). These parameters are described in more detail in Chapter 2 (Section 2.2.2) and in publications (Hawkins, Hommel et al. 2007, Hawkins, Turner et al. 2007).

Analysis of cohort number is an indirect measurement of cell survival and death of in vitro cultured cells across the time course, removing the effect of division from total cell counts. Assessment of MDN and cohort number thus allows the distinction of division and survival as independent parameters, so that the effect of different stimuli on each parameter can be directly measured and visualised independently. No difference in MDN between the strains was observed when B cells were stimulated with CpG alone (Figure 5.1B), suggesting that signaling via TLR9 is not dependent on NF-κB2.

However, a consistently lower precursor cohort number was observed across each timepoint tested, suggesting that the difference in total cell counts observed was principally due to a small survival advantage of wild-type cells (Figure 5.1C).

In contrast to the weak effects of *Nfkb2* deficiency on CpG stimulated B cells, a large difference in the overall magnitude of the response was observed between wild type and *Nfkb2*$^{-/-}$ B cells when

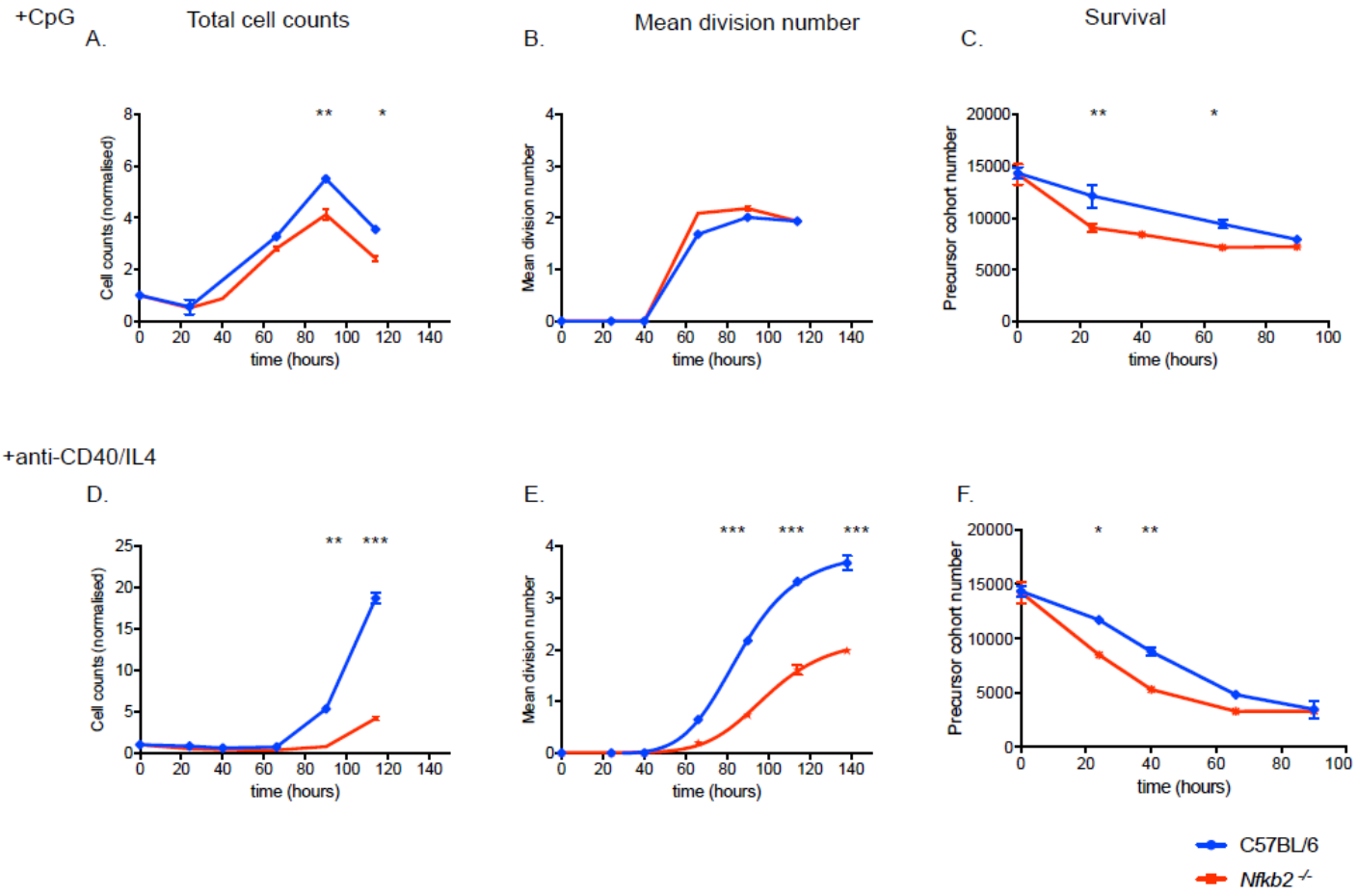
stimulated with anti-CD40 and IL-4 (Figure 5.1D). Naïve B cells stimulated with CD40L and IL4 started proliferating after 40 hours, and continued to proliferate over the time course, such that the wild-type cells had overcrowded wells by 120 hours. The *Nfkb2*$^{-/-}$ B cells also began dividing after 40 hours, but the response was significantly diminished compared to wild-type (Figure 5.1D).

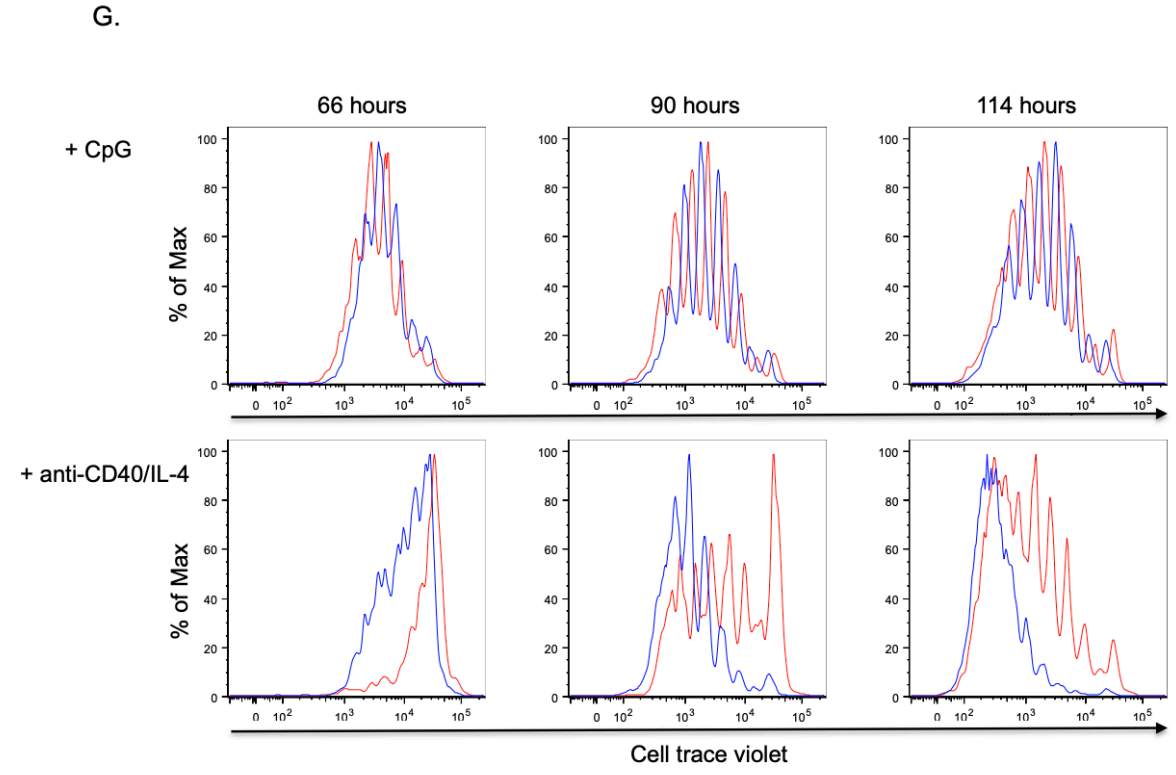
Analysis of MDN revealed wild-type naïve B cells stimulated with anti-CD40+IL-4 divided, on average 3.8 times after 100 hours, whereas naïve B cells from *Nfkb2*$^{-/-}$ mice cultured under the same conditions reached, on average, 2 less divisions (Figure 5.1E) at the same time point. B cells from both strains were continuing to divide after 100 hours (i.e. the cells had not reached division destiny), however the wild-type cells were likely constrained due to overcrowding of cultures. In this case, the major contributor to the difference in the overall response, was a defect in proliferative responses (Figure 5.1E), although a small survival advantage of wild-type cells was again observed (Figure 5.1F).

The observed differences in B cell behaviour confirmed that the loss of NF-κB2 has a significant impact on T cell dependent B cell responses, transmitted via CD40 engagement. Next, we asked, what the impact of loss of NF-κB2 on CD8 T cell responses.

FIGURE 5.1 DIFFERENTIAL PROLIFERATIVE RESPONSES OF NFKB2$^{-/-}$ *NAÏVE B CELLS TO A T-CELL INDEPENDENT STIMULUS AND A T-CELL DEPENDENT STIMULUS*

CTV-labelled naïve B cells were cultured with CpG alone, **(A-C),** or in the presence of anti-CD40 and IL-4 as indicated **(D-F).** Cells were harvested daily and live cell count over time determined for each stimulation condition as indicated. (G). Representative CTV plots demonstrate division history. (Mean +/- SEM, triplicate measures; data representative of 2 independent experiments; only significant differences are indicated: *, 0.01<p<0.05; **0.001<p<0.01; ***p<0.001)


+CpG
A.
Total cell counts
B.
Mean division number
C.
Survival
Cell counts (normalised)
Mean division number
Precursor cohort number
time (hours)
+anti-CD40/IL4
D.
E.
F.
C57BL/6
Nfkb2 -/-


G.
66 hours
90 hours
114 hours
+ CpG
+ anti-CD40/IL-4
% of Max
Cell trace violet

5.3.2 Establishment of an assay to measure proliferation, survival and division destiny in C57BL/6 CD8$^+$ T cells

CD8$^+$ T cell proliferation and integration of stimulatory signals had previously been demonstrated in a murine system using CD8$^+$ T cells with a transgenic TCR responsive to the ovalbumin peptide, SIINFEKL (Marchingo, Kan et al. 2014). In order to apply analogous assays using T cells without a transgenic TCR, an anti-CD3 agonistic antibody (clone 145-2C11) was used to mimic a TCR signal, and cytokine and co-stimulatory signals titrated to enable measurement of DD (Section 2.3.3, Figure 2.1). Our goal was to determine optimised conditions for a robust assay of T cell signal sensitivity that measured programming of ttfd, division rate, times to die and the progression to DD after a defined and standardised level of stimulation.

To ensure that differences in cell number and DD were a result of an autonomous programmed cessation in division and death (as seen for CpG stimulated B cells and N4 stimulated OT1 cells), and not a consequence of overcrowding or other constraint of the culture system that may mimic this behavior, we first examined the effect of cell density on DD and survival. Naive CD8+ T cells isolated from C57Bl/6 mice were seeded at doubling concentrations of starting cell counts from $1.25x10^3$ to $2.4x10^4$ cells/well, and cultured either with continuous anti-CD3 stimulation alone (Figure 5.2A-C); or with the addition of anti-CD28 (6 μg/ml; Figure 5.2D-F); or anti-CD28 + IL-2 (10 U/ml; Figure 5.2G-H). All cultures were supplemented with anti-murine IL-2 mAb (clone S4B6; 25mcg/ml) to block autocrine Il-2 signaling.

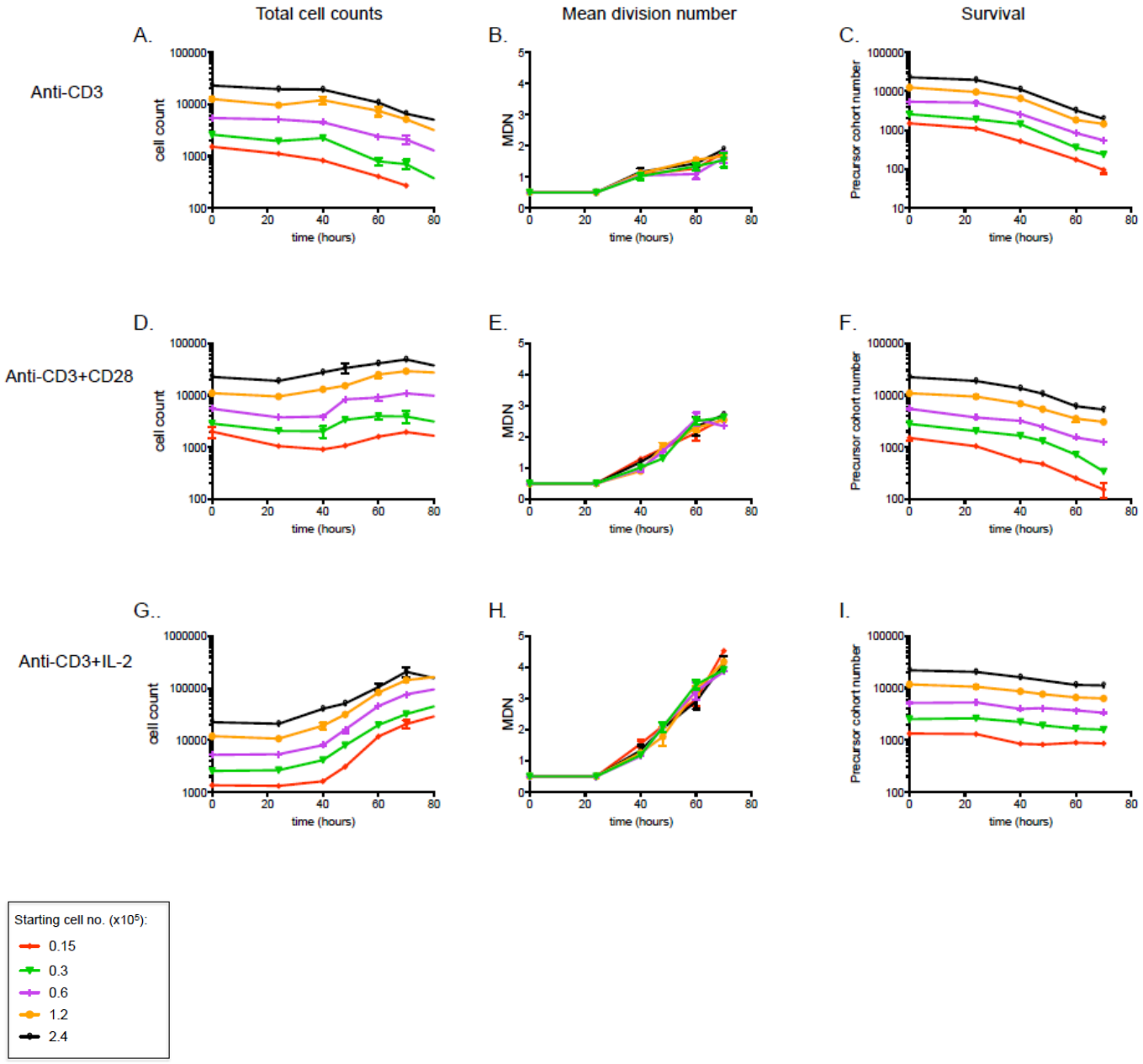


FIGURE 5.2 PROLIFERATION AND SURVIVAL ARE UNAFFECTED BY CELL DENSITY

CTV labeled murine naïve CD8+ T cells were cultured on continuous anti-CD3 alone **(A-C)**, or in the presence of addition anti-CD28 (6mcg/ml) **(D-F),** or IL-2 (10U/ml) **(G-I)**, as indicated. Cells were harvested daily; cell count, mean division number and survival over time determined for each stimulation condition as labeled. (Mean +/- SEM, triplicate measures, data from one experiment; representative of two independent experiments.)

Under continuous CD3 stimulation, cell counts decreased at the same rate, regardless of cell density (Figure 5.3A). Similarly, under these conditions there was no difference observed between the population MDN (Figure 5.3B), and the rate of cell death, as measured using the precursor cohort method, was not affected by cell density (Figure 5.3C).

When cells were cultured with anti-CD3 and anti-CD28, there was no observable difference in the rate of increase and decrease in the cell count over time (Figure 5.3D). The population MDN was approximately 2.5 for all cell densities, again demonstrating that division was not affected by cell density with the starting cell numbers used (Figure 5.3E), and the rate of cell death was similarly unaffected by the starting cell number, as measured using the precursor cohort method (Figure 5.3F).

However, some differences were observed in cultures in the presence of IL-2. The rate of increase in cell count was similar for all cell densities until 50 hours of culture. After 50 hours cultures containing the highest cell densities (those starting with >1.2-2.4 x 10^3 cells/well) demonstrated a sharper decline in total cell count (coinciding with the cell count per well surpassing $1.0x10^5$; Figure 5.3G). However, even at these high cell densities, the population MDN remained within the range of all other densities tested, suggesting that division, may be less affected by the constraints of the culture, or at least within the sensitivity of this assay as no difference could be observed. Despite the loss of cell numbers observed at higher concentrations, population survival, as measured by the precursor cohort method was not affected.

Overall, under all stimulation conditions, there was no observable difference in the kinetic characteristics of the immune response when the total cell count per well was below $1.0x10^5$ (Figure 5.2). This was true for total cell counts (Figure 5.2A, D, G), as well as MDN (Figure 5.2B, E, H); and survival (Figure 5.2C, F, I). Together, these data suggest that the parameters of division and survival were not influenced by cell density under these conditions. This is particularly pertinent for analysis in immune-deficient models, where lymphocyte numbers may be decreased, or predicted to be less responsive to stimulation.

5.3.2 Comparison of C57BL/6 and Nfkb2$^{-/-}$ CD8$^+$ T cell immune responses

Next, experiments to confirm the optimal conditions for measurement of DD were performed. As depicted in Figure 2.1C, the plateau of MDN is observed in C57BL/6 $CD8^+$ T cells cultured with continuous plate-bound anti-CD3 with the addition of IL-2 (3.16 U/ml), or anti-CD28 (6 μg/ml). Using this assay, we could then address the question of whether loss of NF-κB2 in $CD8^+$ T cells impacted cell survival or proliferative responses under a variety of stimulatory conditions. First, when total cell number was compared, no difference was observed between unstimulated wild type and *Nfkb2*$^{-/-}$ $CD8^+$ T cells at any time (Figure 5.3A), indicating no intrinsic survival defect in these mice. Interestingly, however, with anti-CD3 stimulation alone, a greater number of *Nfkb2*$^{-/-}$ $CD8^+$ T cells were observed, compared to wild type (Figure 5.3B). Similarly, there was no difference observed with the addition of low or higher concentrations of IL-2 (3.16 U/ml or 10 U/ml respectively) to continuous anti-CD3 stimulation (Figure 5.3C and D). Most notable, however, was the difference in cell

count between wild type and mutant cells when stimulated with continuous anti-CD3 and either low or high anti CD28 (2 or 6 μg/ml) (Figure 5.3 E, F).

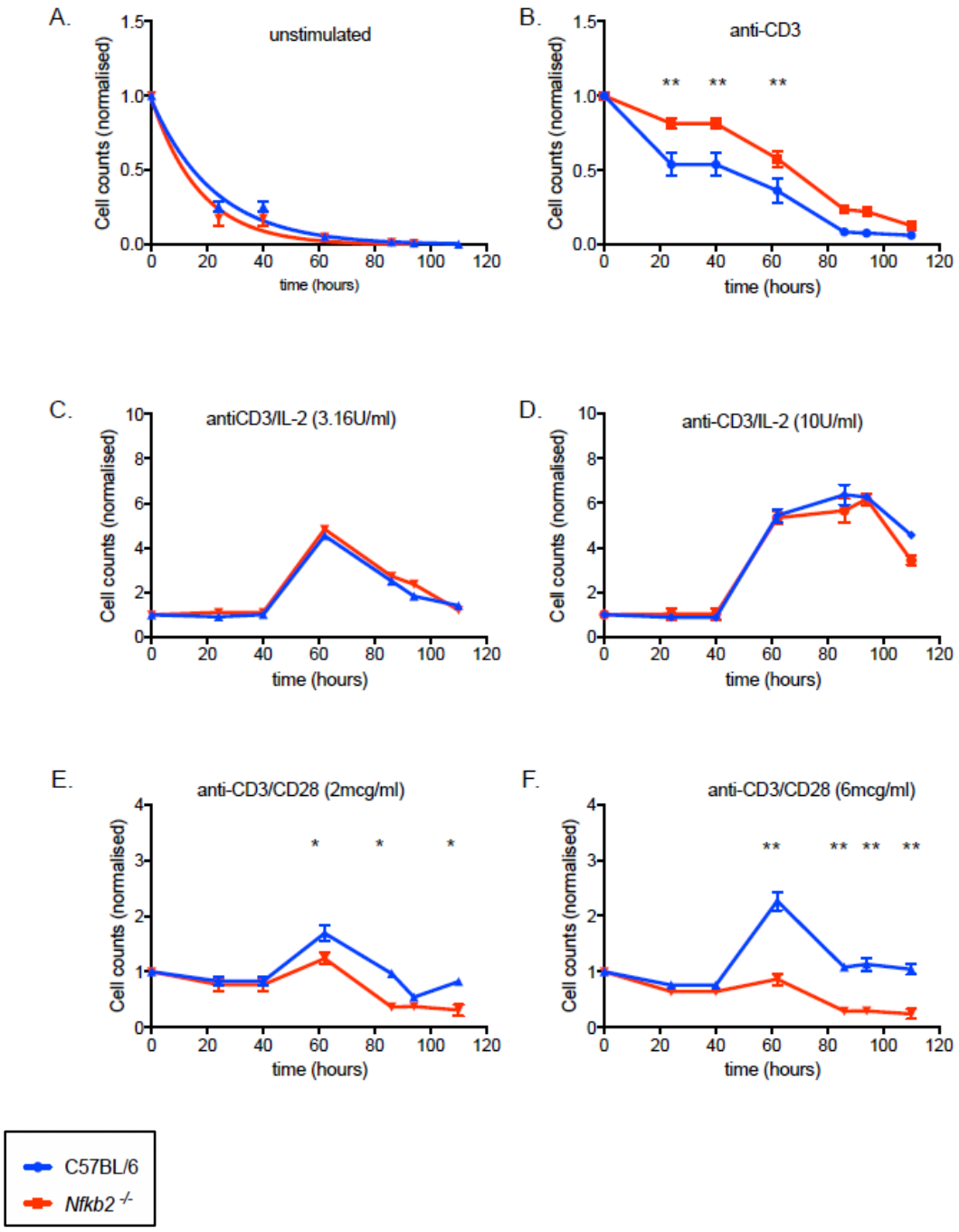


FIGURE 5.3. CELL COUNTS OVER TIME DEMONSTRATE DIFFERENCES IN IMMUNE RESPONSE OF NFKB2$^{-/-}$ CD8^{+} T CELLS TO STIMULATION WITH ANTI-CD3 ALONE, OR WITH THE ADDITION OF ANTI-CD28.

CTV labeled naïve CD8^{+} T cells were either unstimulated, (**A)**; cultured on continuous plate-bound anti-CD3 antibody alone, **(B)**; or with the addition of IL-2 at low (3.16 U/ml), (**C)**; or intermediate concentration (10 U/ml), **(D)**; and anti-CD3 with anti-CD28 at low (2 μg/ml), (**E);** or intermediate concentrations (6 μg/ml), (**F).** (Cells were harvested at indicated times, and measured in triplicate. Mean +/- SEM, from one experiment, representative of three independent experiments. Only significant differences are indicated: *, $0.01<p<0.05$; **$0.001<p<0.01$; ***$p<0.001$)

What led to these differences in cell number? Did they arise due to impaired cell survival in response to anti-CD28, or impaired proliferation, or a combination of both? First, when assessing responses to anti-CD3 stimulation alone, the small observed difference in cell number (Figure 5.3B) appeared to be solely accounted for by enhanced survival in *Nfkb2*$^{-/-}$ CD8^{+} T cells over wild-type cells, as demonstrated by a small increase in total precursor cohort number (Figure 5.4A). There was no difference in MDN noted under anti-CD3 stimulation (Figure 5.5A). (Note cell numbers were too low to accurately measure division after 60 hours). Further, there was no significant difference observed in cell survival at either concentration of IL-2 (Figure 5.4 B,C), or upon stimulation with anti-CD3 and anti-CD28 co-stimulation (Figure 5.4 D,E).

When we measured MDN and DD of naïve CD8^{+} T cells stimulated with either anti-CD3 alone or IL-2 at either concentration there were no detectable differences between *Nfkb2*$^{-/-}$ and wild-type cells (Figure 5.5A-C). However, in the presence of anti-CD28 co-stimulation, it was evident that *Nfkb2*$^{-/-}$ CD8^{+} T cells had a significantly blunted response, with cells undergoing 1.5 to 2 fewer divisions, and that this accounted for the differences in cell number observed (Figure 5.5D, E).

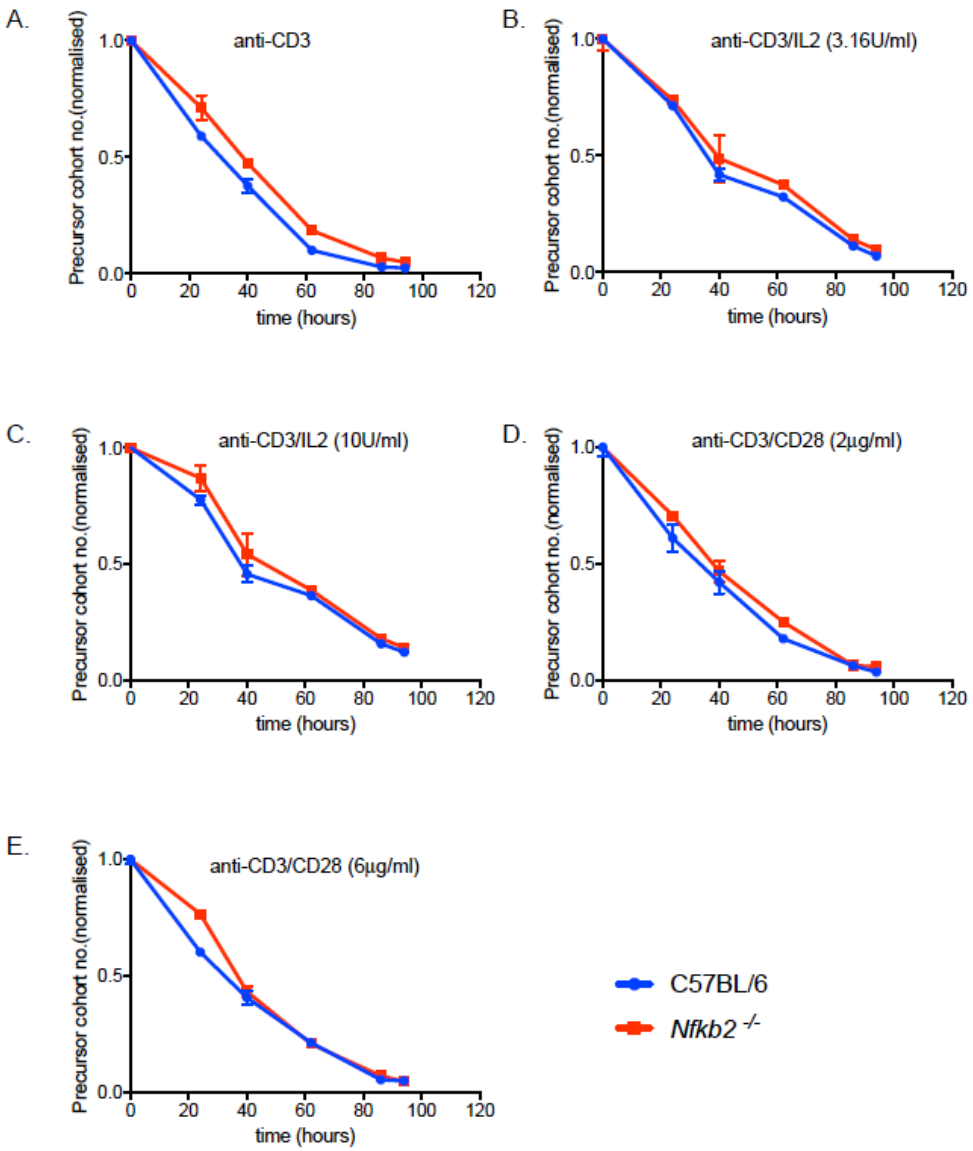


FIGURE 5.4. NFKB2$^{-/-}$ CD8^{+} T CELL SURVIVAL IS ENHANCED UPON ANTI-CD3 STIMULATION ALONE BUT IS SIMILAR TO WILD TYPE WITH THE ADDITION OF IL-2 OR ANTI-CD28.

Precursor cohort number, representing survival, of CD8^{+} T cells cultured on **(A).** continuous plate-bound anti-CD3 antibody alone; or with the addition of IL-2 at **(B).** low (3.16 U/ml), or **(C)**. intermediate concentration (10 U/ml); or in the presence of anti-CD28 at **(D).** low (2 µg/ml), or **(E).** intermediate (6 µg /ml) concentrations. (Cells were harvested at indicated times, and measured in triplicate. Mean +/- SEM, from one experiment, representative of three independent experiments.)

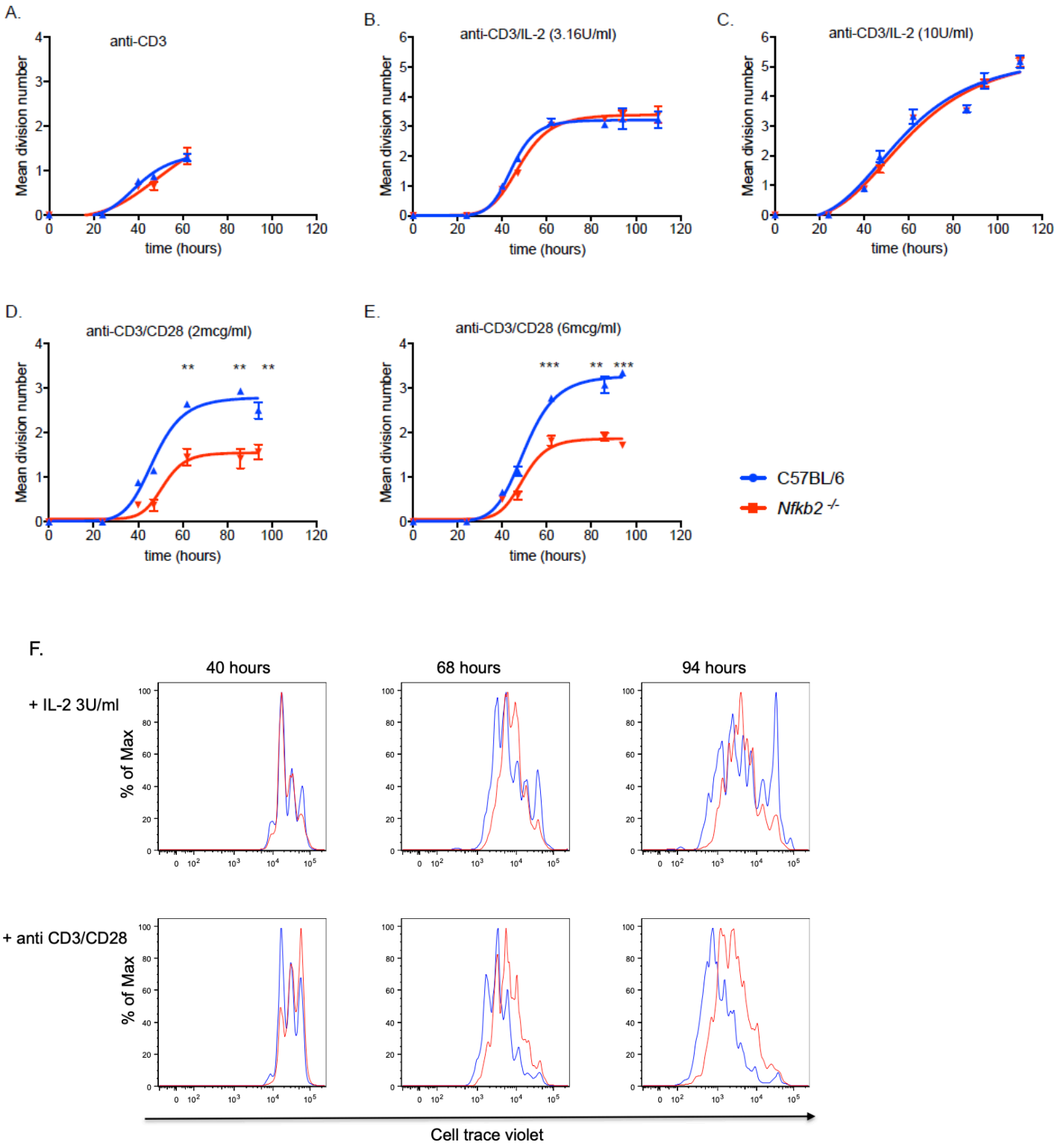

A.
anti-CD3
Mean division number
time (hours)
B.
anti-CD3/IL-2 (3.16U/ml)
C.
anti-CD3/IL-2 (10U/ml)
D.
anti-CD3/CD28 (2mcg/ml)
**
E.
anti-CD3/CD28 (6mcg/ml)
***
C57BL/6
Nfkb2 -/-
F.
40 hours
68 hours
94 hours
+ IL-2 3U/ml
+ anti CD3/CD28
% of Max
Cell trace violet

FIGURE 5.5. NFKB2$^{-/-}$ CD8^{+} T CELLS ARE INSENSITIVE TO CO-STIMULATION BY ANTI-CD28 SIGNALING

Mean division number over time of naïve CD8^{+} T cells (blue line indicates wildtype responses; red indicates *Nfkb2*$^{-/-}$ responses), **(A)** continuous plate-bound anti-CD3 antibody alone; or with the addition of IL-2 at **(B)** low (3.16 U/ml), or **(C)** intermediate concentration (10 U/ml); or in the presence of anti-CD28 at **(D)** low (2 μg/ml), or **(E)** intermediate (6 μg /ml) concentrations. **(F)** Representative Cell Trace Violet (CTV) division histograms of CD8^{+} T cells. (Cells were harvested at indicated times and measured in triplicate. Mean +/- SEM, from one experiment, representative of three independent experiments. Only significant differences are indicated: *, 0.01<P<0.05; **0.001<p<0.01; ***p<0.001.)

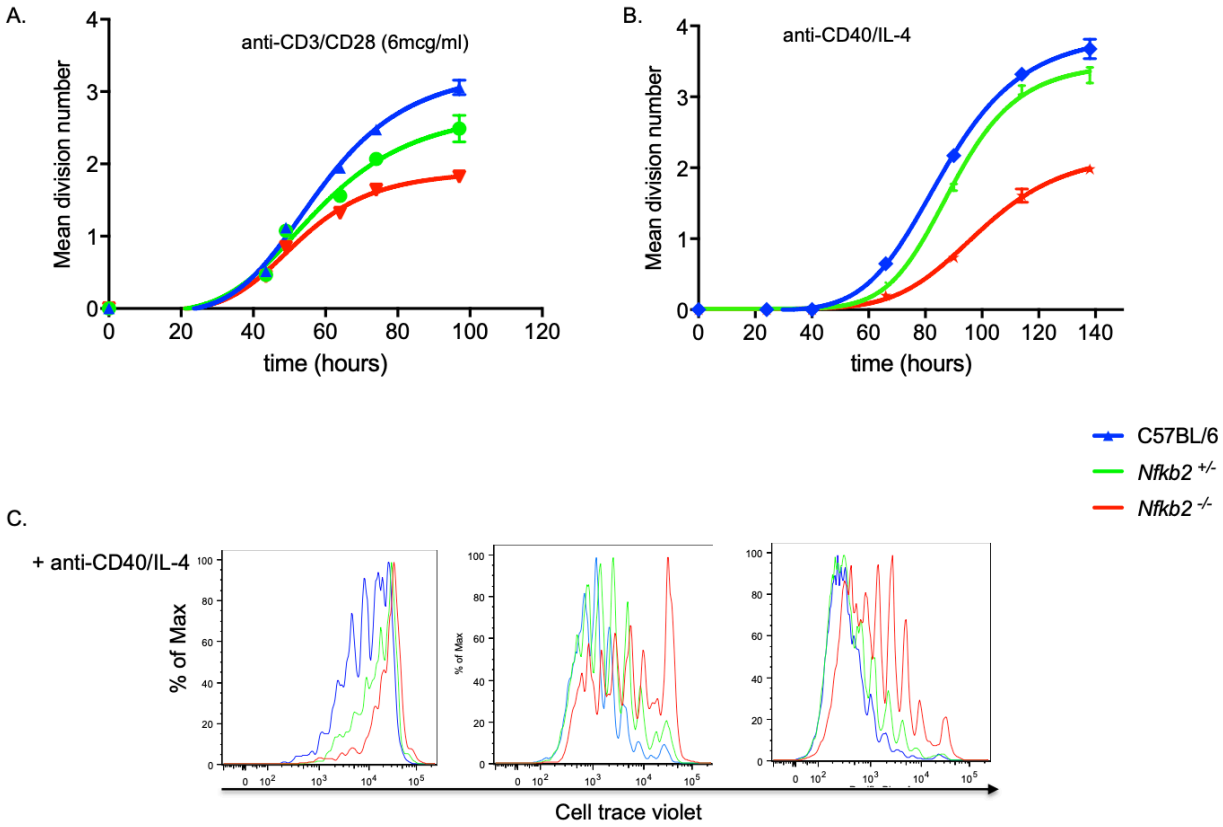


FIGURE 5.6. NFKB2 'GENE – DOSAGE' EFFECT ON PROLIFERATIVE RESPONSES OF NAÏVE CD8⁺ T CELLS, AND B CELLS.

Mean division number over time of **(A)** naïve $CD8^+$ T cells, cultured on continuous plate-bound anti-CD3 antibody and intermediate dose anti-CD28 (6 μg/ml), and **(B)** naïve B cells, stimulated with anti-CD40 and IL-4.**(C)** Representative Cell Trace Violet (CTV) division histograms at indicated times after stimulation. (Mean +/- SEM, triplicate measures. (In A, data are from one experiment, representative of three independent experiments. In B, data are from one independent experiment.)

5.3.3 Gene-dose dependency of lymphocyte responses

The differences in sensitivity to stimulatory signals between gene-deficient and wild-type cells were striking, however one of the aims of utilising quantitative assays was to identify small differences in sensitivity of B and T lymphocytes to stimulatory signals.

To determine if this assay is of sufficient sensitivity to detect the effect of loss of a single *Nfkb2* allele, we next compared proliferation and survival of naïve B and $CD8^+$ T cells from *Nfkb2*$^{-/-}$, *Nfkb2*$^{+/-}$ to C57BL/6 cells. Here, we observe, as predicted, that the $CD8^+$ T cell proliferative response of heterozygous cells to co-stimulation was intermediate between the *Nfkb2* deficient and wild type, suggesting that the response is indeed gene-dose dependent (Figure 5.6A). Furthermore, there was also a small, but measurable effect of loss of one allele of *Nfkb2* in B cells, when stimulated with CD40 and IL-4 (Figure 5.6B), although more subtle than the observed difference in $CD8^+$ T cell sensitivity to CD28 co-stimulation.

5.3.4 Mathematical modeling the Nfkb2$^{-/-}$ immune response using Cyton 1.5: Division destiny time accounts for the difference in sensitivity to anti-CD28 costimulation

From the experimental data, it was apparent that the difference in the outcome of *Nfkb2*$^{-/-}$ $CD8^+$ T cell responses was largely due to the population undergoing, on average, fewer divisions following CD28 co-stimulation. Given the parameters affecting division under these conditions are all time dependent (Heinzel, Binh Giang et al. 2017), we

next wanted to determine if this difference could be accounted for by the addition of small differences in each of the division parameters. This was important, since it remained possible differences in MDN could be explained by a delayed ttfd; a slower division rate or an earlier DD time, or a combination of each. To address this question, the response in NF-κB2 deficiency was investigated using a modified mathematical Cyton model (Hawkins, Turner et al. 2007) that assumes three timed responses govern the T cell response to stimuli (Heinzel, Binh Giang et al. 2017). In this model time to first division, time to division destiny and time to death are considered independent random variables that conform to lognormal distributions. Once dividing, cells undergo further divisions with a determined division time, which is also a parameter of the model (Heinzel, Binh Giang et al. 2017). This model was applied to data from Figure 5.5 using least squares optimisation. A summary of the estimated lognormal times to first division, time to DD and time to die, for each strain, is shown in Figure 5.7. The fitted ttfd distribution was comparable for both genotypes tested, and unchanged by the addition of anti-CD28 to anti-CD3 stimulation. The DD time upon stimulation with anti-CD3 alone was similar between both genotypes. The best fit to our data revealed that the major factor affected by loss of NF-κB2 on sensitivity to co-stimulation, was a decrease in DD time compared with responses from wild type animals (Figure 5.7A, B). The addition of anti-CD28 extended DD time by approximately 20 hrs in wild-type (Figure 5.7C), while NF-κB2 deficient $CD8^+$ T cells showed a minimal increase in DD time with addition of co-stimulation (Figure 5.7D). Together, these results further confirm the importance of division destiny on the overall outcome of the immune response and provide a direct link between NF-κB2 action and timing of DD.

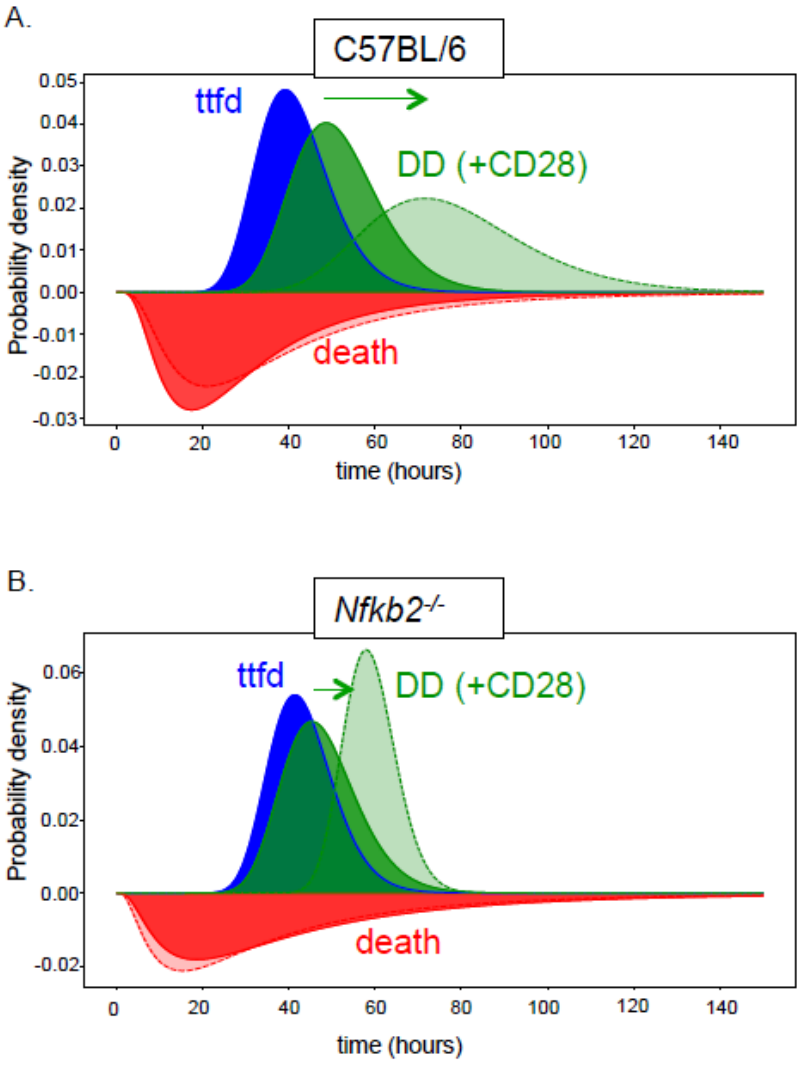


FIGURE 5.7. MODELING THE CD8⁺ T CELL IMMUNE RESPONSE REVEALS LOSS OF NF-κB2 REDUCES RESPONSIVENESS TO ANTI-CD28 STIMULATION BY SHORTENING OF DD TIME.

(A). Fitted population distribution frequencies of C57BL/6 $CD8^+$ T cell responses to anti CD-3 stimulation alone and with the addition of anti CD28 (6 μg/ml). **(B).** *Nfkb2*$^{-/-}$ $CD8^+$ T cell responses to anti CD-3 stimulation alone and with the addition of anti-CD28 (6 μg/ml). Time to first division (ttfd) in blue (for both stimulation conditions); division destiny (DD) time, with anti-CD3 stimulation alone, dark green; DD time, with anti-CD3 and anti-CD28, light green; death time with anti-CD3 stimulation alone in dark red; and light red, death time with anti-CD3 and anti-CD28.

5.4 Discussion:

NFKB2 mutations cause a human immunodeficiency, which often presents as CVID, although there is variation in expression of the phenotype, even within individuals from the same family. Here, we studied a family with two affected daughters, with clinical features of an antibody deficiency disorder, although one also demonstrated susceptibility to viral infections, having suffered severe widespread molluscum contagiosum as a child, and finally succumbed to enteroviral encephalitis as a young adult. The diagnosis of the infective cause was not made until post-mortem examination confirmed the presence of enteroviral DNA in cerebral tissue. Prior to her death she had been treated for presumed autoimmune encephalitis. This case is a pertinent example of the challenges of PIDs, at the crossroads between immune dysregulation and immune deficiency.

The mechanism of autoimmune disease has not been extensively studied in humans with *NFKB2* mutations but is likely due to a number of factors. A decrease in the number and frequency of regulatory T cells has been observed in the family reported here, and another Australian family previously reported (Lee, Fulcher et al. 2014). Another group has identified anti-cytokine antibodies in human NF-κB2 deficiency (Ramakrishnan, Rae et al. 2018). They postulated that these autoantibodies led to a heightened susceptibility to infection and reported that the patient had a successful outcome after receiving therapeutic doses of a monoclonal anti-CD20 antibody. The development of anti-cytokine antibodies is reminiscent of the autosomal recessive condition, APECED, due to loss of function mutations in AIRE (Bjorses, Aaltonen et al. 1998). Indeed, there is evidence of reduced AIRE expression in murine *Nfkb2*$^{-/-}$

thymocytes (Zhu, Chin et al. 2006). Anti-cytokine antibodies were not measured in the family reported here.

The co-occurrence of autoimmune disease and infection highlights the complexities of PADs/CVID: presumably an absence of B cells exacerbates susceptibility to viral infections, as enteroviral infection has been a recognised complication of XLA, yet the development and differentiation of B cells in the presence of 'poorly-educated' thymocytes may also drive the autoantibody responses that exacerbate susceptibility to other infections. Further to the complexity of overlapping immunodeficiency and immune dysregulation, the family demonstrates a number of features relevant to autosomal dominant PIDs, namely, variability of disease penetrance and severity.

The case also points to the potential benefit of understanding the genetic cause of disease in order to predict potential disease-specific complications, and target treatment accordingly. Other autosomal dominant monogenic PIDs, like NF-κB1 deficiency and CTLA4 haploinsufficiency, demonstrate a variable onset, penetrance, and severity of disease (Fliegauf, Bryant et al. 2015, Schwab, Gabrysch et al. 2018, Tuijnenburg, Lango Allen et al. 2018). This raises the interesting question of how this heterogeneity arises and suggests the presence of not only 'gene-dosage' effects, but also other genetic modifiers, and the influence of epigenetic factors that influence disease development and severity.

Despite the influence of additional phenotypic modifiers, such as epigenetic alterations, and environmental exposure to infections, it is clear that genetic differences underlie variation in lymphocyte behavior and account for the phenotypes observed in animal

models, and in many cases of human monogenic PIDs. In addition, there are important similarities shared between *Nfkb2*$^{-/-}$ animal models and the clinical and immunological phenotype of the family under investigation. For example, similar to what was described in the family presented here, *Nfkb2*$^{-/-}$ mice show impaired production of some immunoglobulin isotypes, impaired vaccine responses (Franzoso, Carlson et al. 1998) and impaired generation of germinal centres, specifically with disrupted splenic and peripheral lymph node architecture (Caamano 1998). Infectious susceptibility has been investigated also; poor control of leishmania has been shown to be due to a deficiency in production of IL-12 in response to infection, and is also associated with susceptibility to chronic infection with the opportunistic parasite *Toxoplasma gondii* (Franzoso, Carlson et al. 1998, Caamano, Tato et al. 2000); it appears that the development of chronic, severe infection was associated with enhanced apoptosis of lymphocytes at later stages of infection (Caamano, Tato et al. 2000). Intriguingly however, *Nfkb2*$^{-/-}$ DCs demonstrate an activated phenotype, and induce CD4^{+} T cells to proliferate and secrete more IFNγ than wild type (Speirs, Lieberman et al. 2004). These disparate cellular phenotypes demonstrate the importance of understanding the impact of these mutations on different lymphocyte populations in order to reveal the pathogenesis of the clinical manifestations of NF-κB2 deficiency.

Here we have shown, in a murine model, that complete loss, or haploinsufficiency, of NF-κB2, underlie variation in CD8^{+} T cell responses to anti-CD28 co-stimulation in a gene-dose dependent manner, without significant loss of sensitivity to TCR or IL-2 signals, under controlled *in vitro* conditions. These differences in lymphocyte behavior are likely to contribute to the infectious susceptibility observed. Furthermore, the loss of responsiveness to stimulation by anti-CD28 may also lead to susceptibility to

autoimmune disease. In murine models, signaling via CD28 promotes FoxP3 expression, and the development of thymic T regulatory cells (Tai, Cowan et al. 2005), as well as promoting the survival of T-regs in the periphery. It is possible, therefore, that impaired responsiveness to CD28 co-stimulation in NF-κB2 deficient humans, could account, at least in part, for the reduced numbers of T-regs observed in some patients, including in one sister tested in our current study (Lee, Fulcher et al. 2014). In addition, the importance of CD28 signaling in the development of GCs and T-dependent antigen responses is well established and has been discussed in detail in Chapter 1.3.1.

The ability to precisely determine characteristics of the immune response, holds great potential for understanding the complexity of disease pathogenesis and the clinical heterogeneity of human immune diseases. Furthermore, it is likely to enable predictions of immune responses due to inborn immune defects; differences in cellular behavior due to variation in sensitivity to signals may be predicted to occur in cases of human disease, where a genetic variant leads to disease due to haploinsufficiency, or even when disease arises in the setting of a missense variant or polymorphism, that does not lead to significant loss of protein expression, but may lead to a small effect on protein function. Currently, further studies are underway to analyse the behavior of naïve human B and T cells in PID and autoimmune diseases, in order to understand the intrinsic behaviors of lymphocytes in individuals with these conditions, and potentially to identify specific characteristics that may be therapeutically targetable.

CHAPTER 6. A NOVEL PRIMARY IMMUNODEFICIENCY DUE TO A HOMOZYGOUS MUTATION IN *NFKBID*

6.1 Introduction

The significant role of mutations that affect NF-κB signaling, leading to PID, was introduced in Chapter 1. Results presented in Chapters 3, 4 and 5 have further demonstrated the large contribution of genetic defects in *NFKB1,* encoding p105/p50, a key component of the canonical signaling pathway, and *NFKB2*, encoding p100/p52, a key component of the non-canonical pathway, in the pathogenesis of PADs (Figure 1.6). Briefly, the canonical pathway is critical for innate and adaptive immune responses, and is triggered after signaling via TLRs, BCR, TCR, and coordinates the transcription of several 100s of genes involved in inflammatory responses (Zhang, Lenardo et al. 2017). The non-canonical pathway is also triggered by antigen recognition by the BCR and TCR, as well as other co-stimulatory receptors and coordinates the appropriate activation of adaptive immune responses and lymphoid organogenesis (Figure 1.6). Most of the monogenic PIDs presented up to this point in this thesis, have arisen due to deficient activation of the pathways. However, loss of appropriate regulation (and hence hyperactivation) of this crucial family of transcription factors can also have detrimental effects on the immune response, and lead to PID, as discussed in Chapter 1.6. Furthermore, due to the exquisite balance required in the NF-κB pathway, deficient activation of one arm may lead to dysregulation of the entire system. To add even more complexity, some components of the pathway may behave differently in different cell types.

Regulation of NF-κB signaling occurs by the coordinated function of a number of

inhibitory proteins, discussed in detail in Chapter 1 (Figure 1.4). These proteins are characterised by a variable number of ankyrin repeat domains (ARDs), which bind to NF-κB dimers. The classical inhibitory proteins, IκBα, IκBβ, IκBε, IκBγ, and the full-length NF-κB1 and NF-κB2 proteins, p105 and p100 respectively, are expressed ubiquitously. Under steady state conditions, inhibitory proteins are bound to the RHD domains of NF-κB1 and NF-κB2 full length protein homo- and heterodimers in the cytoplasm (Hayden and Ghosh 2008). Upon receipt of a stimulatory signal, these inhibitory proteins are phosphorylated by upstream kinases, and then subsequently are degraded and undergo ubiquitination. This process exposes the nuclear localisation signal (NLS) of the active NF-κB dimers, thus enabling trafficking into the nucleus and transcription at NF-κB target sites (Hayden and Ghosh 2008).

In contrast to their classical counterparts, the non-classical inhibitors, IκBNS (or IκBδ), IκBζ and Bcl3, are thought to be located primarily in the nucleus. The function of nuclear IκBs has been shown to involve regulation of NF-κB via physical interaction with homo- and heterodimers of the active subunits, p50, p52, p65, and c-rel, so as to restrict their access to κb sites in the DNA (Hatada, Nieters et al. 1992, Yamazaki, Muta et al. 2001). In addition to the specificity of the atypical inhibitors for regulating p50 and p52 homodimers, it has been demonstrated more recently that these inhibitors can also regulate expression of some genes specifically, such as IL-2 and IL-6 (Yamamoto and Takeda 2008, Kohda, Yamazaki et al. 2016).

Here we describe a novel immunodeficiency caused by mutations in a gene encoding an atypical inhibitor of NF-κB, with overlapping clinical features of autoimmune

disease, inflammation, and immune deficiency that arise due to an imbalance in this complex pathway.

6.2 Results

6.2.1 Clinical features of proband (Kindred 1)

The proband, the second child of first cousins of Turkish Cypriot ethnicity, was diagnosed at age 16 with a possible CVID after suffering several episodes of bacterial pneumonia, autoimmune thrombocytopenia, and severe atopic dermatitis in infancy and early childhood (Figure 6.1). He also had trachyonychia, although no other significant features of ectodermal dystrophy, and a number of food and medication allergies.

Immunologic investigations revealed reduced levels of IgA (0.5 g/L), IgM (0.1 g/L), and IgG2 (0.4 g/L). Total IgG was within the normal range (6.8 g/L) and the total IgE was elevated (2403kU/L; Table 6.1). Specific antibody responses to 23-valent pneumococcal polysaccharide vaccine were impaired, suggesting a defect in T-independent B cell responses, however the T-dependent response to tetanus toxoid was normal (Table 6.1). At diagnosis, T-lymphocyte proliferation, as measured by tritiated thymidine assay, demonstrated a normal response to phytohaemagglutinin (PHA) stimulation (Table 6.1). This has been repeated subsequently by measurement of BrDU incorporation, and was comparable to a healthy control upon anti-CD3 and anti-CD28 stimulation (Table 6.1).

Table 6.1. Immunologic investigations in family members (Kindred 1)

Family Member / **Investigation**	**II.2 (PROBAND) At 'CVID' diagnosis**	**II.2 2014**	**II.2 2015**	**I.1**	**I.2**	**II.1**	**II.3**
Immunoglobulins							
Total IgG (RR: 5.4-18.2g/L)	6.8	8*	7*	10.2	8.3	7.3	11.5
IgG1 (RR: 3.8-9.3g/L)	4.9	4.35*	ND	ND	6.7	6.8	8.2
IgG2 (RR: 2.4–7g/L)	**0.4**	2.44*	ND	ND	2.6	**<0.09**	**2.26**
IgG3 (RR: 0.22-1.76g/L)	0.31	0.28*	ND	ND	0.73	1.2	0.92
IgG4 (RR: 0.04-0.86g/L)	0.4	0.09*	ND	ND	0.4	**<0.01**	0.13
IgA (RR: 0.7-4.2g/L)	**0.5**	**0.34**	**0.3**	1.8	2.2	3.9	1.8
IgM (RR: 0.3-2.9g/L)	**0.1**	**<0.05**	**<0.05**	**0.3**	0.5	0.7	**0.27**
IgE (RR: 0-120 kU/l)	**2403**	**120**	**120**	**259**	**142**	79	ND
Absolute lymphocyte count x10^9/L (% total lymphocytes) RR: 1.2-4.0 x10^9/L	1.6 (100)	**0.32**	**0.3**	2.6	2.8	2.5	3.7
CD19+ B cells RR: 0.1-0.9 x10^9/L	0.24 (15.3)	**0.02 (5)**	**0.0 (1)**	0.23 (8.7)	0.36 (12.9)	0.46 (18.2)	0.51 (13.9)
CD3+ T cells RR: 0.7-3.58 x10^9/L	1.16 (72.3)	**0.24 (74)**	**0.26 (85)**	2.04 (78.5)	2.3 (82.3)	1.8 (72.9)	2.7 (73.7)
CD4+ T helper cells RR: 0.35-2.63 x10^9/L	0.89 (55.5)	**0.19 (58)**	**0.22 (72)**	1.52 (58.5)	1.57 (55.9)	1.33 (53)	1.94 (52.5)
CD8+ cytotoxic T cells RR: 0.12-1.47	0.2 (12.2)	**0.05 (14)**	**0.04 (11)**	0.52 (14.4)	0.62 (22.1)	0.41 (16.3)	0.62 (16.7)
CD4+/CD8+ ratio RR: 1.0-2.4	**4.55**	**4.19**	**6.1**	**2.95**	**2.53**	**3.25**	**3.14**
CD16+/56+ Natural Killer cells RR:0-0.69	0.15 (9.6)	0.06 (19)	0.02 (7.2)	ND	0.09 (3.2)	0.13 (5.1)	0.43 (11.6)
Pneumococcal vaccine responses	**Absent**	ND	ND	ND	ND	Normal	Normal
T cell proliferation (PHA stimulation)	Normal	Normal	ND	ND	ND	ND	ND

*(RR, reference range; ND, not done; PHA, phytohaemaglutinin; * on IVIG; **bold,** indicates values outside reference ranges)*

Despite the initial diagnosis of a PAD, the proband's clinical phenotype progressed over time to be more complex (Figure 6.1). He developed acute hepatitis after primary Epstein Barr virus (EBV) infection at 17 years of age. The possibility of X-linked lymphoproliferative syndrome (XLP) was considered; SLAM-associated peptide (SAP) and X-linked inhibitor of apoptosis (XIAP) expression by immunoblot and flow cytometric methods, were normal, essentially excluding XLP-1, and XLP-2, due to mutations in *SH2D1A* and *BIRC4* respectively. Liver biopsy revealed tracts of inflammatory cell infiltrates around portal tracts, principally composed of lymphocytes, together with small numbers of histiocytes and a few eosinophils (not shown). One portal tract contained a medium sized lymphoid follicle, composed predominantly of T cells. Epstein Barr encoding region (EBER) *in situ* hybridization was negative, suggesting that the hepatitis was not directly attributed to viral infection, but may have been a secondary phenomenon as a result of immune dysregulation.

At age 20, he presented with acute renal failure and haemoptysis. Renal biopsy demonstrated a pauci-immune glomerulonephritis (Figure 6.1). A preliminary diagnosis of 'anti-neutrophil cytoplasmic antibody (ANCA)-negative vasculitis' was made. Although he had an initial clinical response to immunosuppression with plasma exchange and cyclophosphamide, he suffered a recurrence of disease eighteen months later, and became hemodialysis-dependent. At age 28, he had an acute presentation with autoimmune haemolytic anemia and thrombocytopenia, which responded to systemic corticosteroid therapy. The complex clinical presentation was further complicated by chronic cytomegalovirus infection, manifesting as two episodes of pneumonitis in the context of PCR positive bronchial washings, and one episode of colitis, with abdominal pain, diarrhea and an increase in viral load. He had a clinical and virological response

to short courses of therapy with intravenous ganciclovir and oral valganciclovir, however this was complicated by the development of neutropenia.

Over time, the proband developed portal hypertension in the setting of mild hepatic fibrosis, with normal synthetic function, as demonstrated by normal coagulation studies (data not shown). Recurrent ascites and increasing splenomegaly caused severe abdominal distension and pain (Figure 6.2A,B). Pancytopenia developed, likely exacerbated by splenomegaly, and possible destruction in the bone marrow: prominent lymphocytic infiltrates were observed on bone marrow examination (Figure 6.3C,D), with a predominance of $CD4^+$ T cells (Figure 6.2E-G), and near absence of $CD20^+$ B cells (Figure 6.2H). There was no evidence of haemophagocytosis. Unfortunately, he was not a candidate for splenectomy, due to the presence of large abdominal wall varices, nor for definitive therapy of HSCT, due to the comorbid renal and hepatic damage.

Attempts to modulate the patient's immune dysregulation, with systemic corticosteroids, and monoclonal anti-IL6R antibody (tocilizumab) therapy, were met with complications of infection: gram negative septicaemia due to *Proteus mirabilis* infection, and staphylococcal bacteremia occurred within days to weeks of commencing these treatments. Eventually he became dependent on high doses of opioid analgesia, and was unable to tolerate haemodialysis. He passed away in March 2019 at the age of 32.

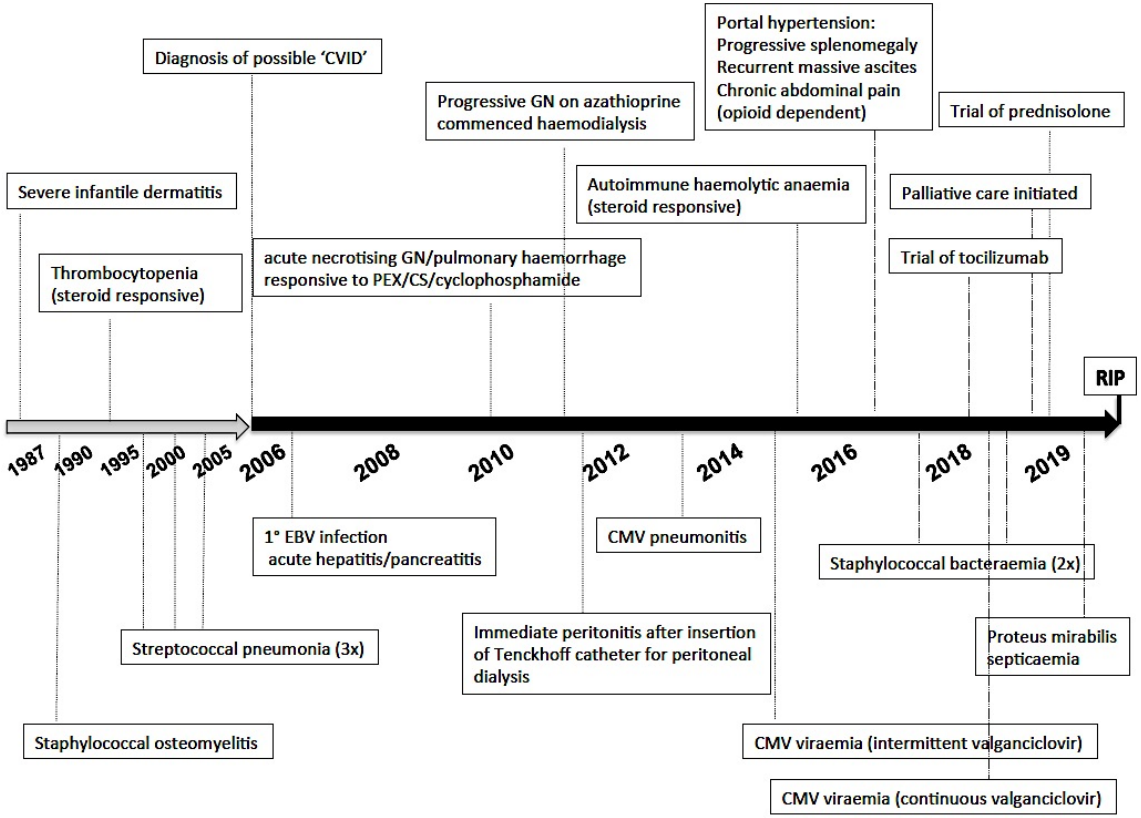


FIGURE 6.1 TIMELINE OF MAJOR CLINICAL MANIFESTATIONS IN THE PROBAND.

Major clinical manifestations are summarised, from year of birth in 1987, to year of death, 2019. Inflammatory and autoimmune conditions, and associated therapies are listed above the timeline, and infectious complications are listed below. *(CVID, Common Variable immunodeficiency; EBV, Epstein Barr virus; GN, glomerulonephritis; PEX, plasma exchange; CS, corticosteroids; CMV, cytomegalovirus)*

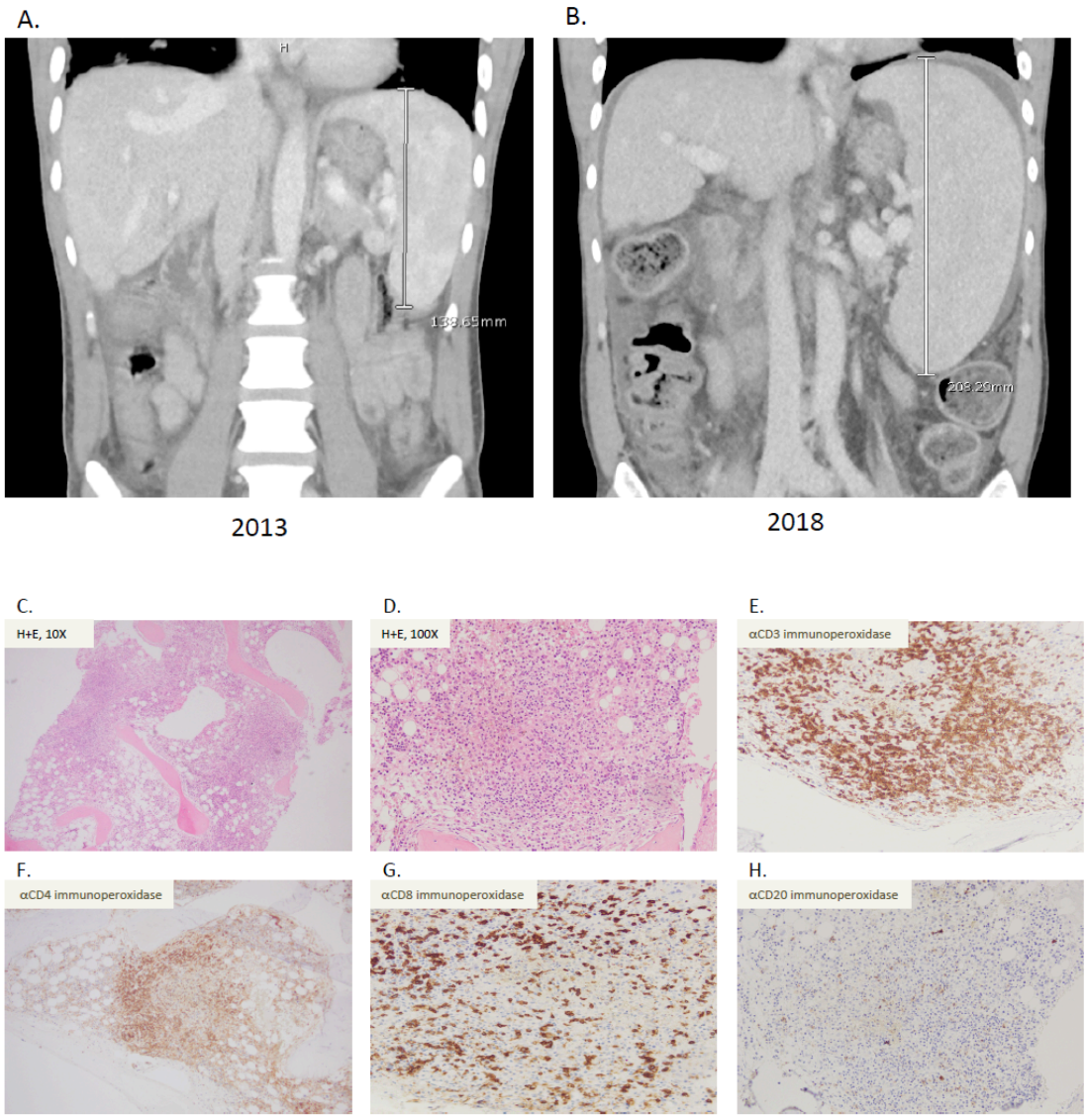


FIGURE 6.2 SPLENOMEGALY AND LYMPHOID FOLLICLES IN BONE MARROW IN IBNS DEFICIENCY

(A). CT performed in 2013 reveals enlarged spleen, with craniocaudal length of 13.8cm. (**B**). Over 5 years the spleen had increased in size, to a craniocaudal length of 20.8 cm. (**C).** Haematoxylin and eosin staining; 10X magnification; and (**D),** 100X magnification. (**E).** Anti-CD3 immunoperoxidase staining (brown; 100X magnification) (**F).** Anti-CD4 immunoperoxidase staining (brown; 20X magnification) (**G).** Anti-CD8 immunoperoxidase staining (brown; 100X magnification). (**H).** Anti-CD20 immunoperoxidase staining (brown, 100X magnification).

6.2.2 Whole exome sequencing identified a novel homozygous mutation in NFKBID

The severe clinical phenotype of the proband raised the suspicion of a monogenic etiology for his condition. The diagnoses of XLP1 and XLP2 had been previously ruled out by protein and genetic studies. Furthermore, we hypothesised that the consanguineous parentage was likely to be associated with an autosomal recessive condition, and whole exome sequencing was an opportunity to identify both known causes of PID, and potentially novel gene defects. Genomic DNA from the proband was sequenced at the Australian Genome Research Facility (AGRF) using Illumina technology (HiSeq sequencing with a mean depth of 50X, as described in Chapter 2.4).

Filtering of variants was undertaken using a model of autosomal recessive inheritance, given the consanguineous parentage (Figure 6.4A). After selecting only variants within linkage regions (+/- 50 kilobases), 164,341 variants remained. When only rare variants were selected, 42,032 variants remained (Nb. At the time of analysis the ESP6500 TGP and an in-house database were used). After removing synonymous, heterozygous or low quality variants, 98 variants remained. A single, novel candidate gene variant stood out (Table 6.2).

Table 6.2 Filtering of variants identified in proband

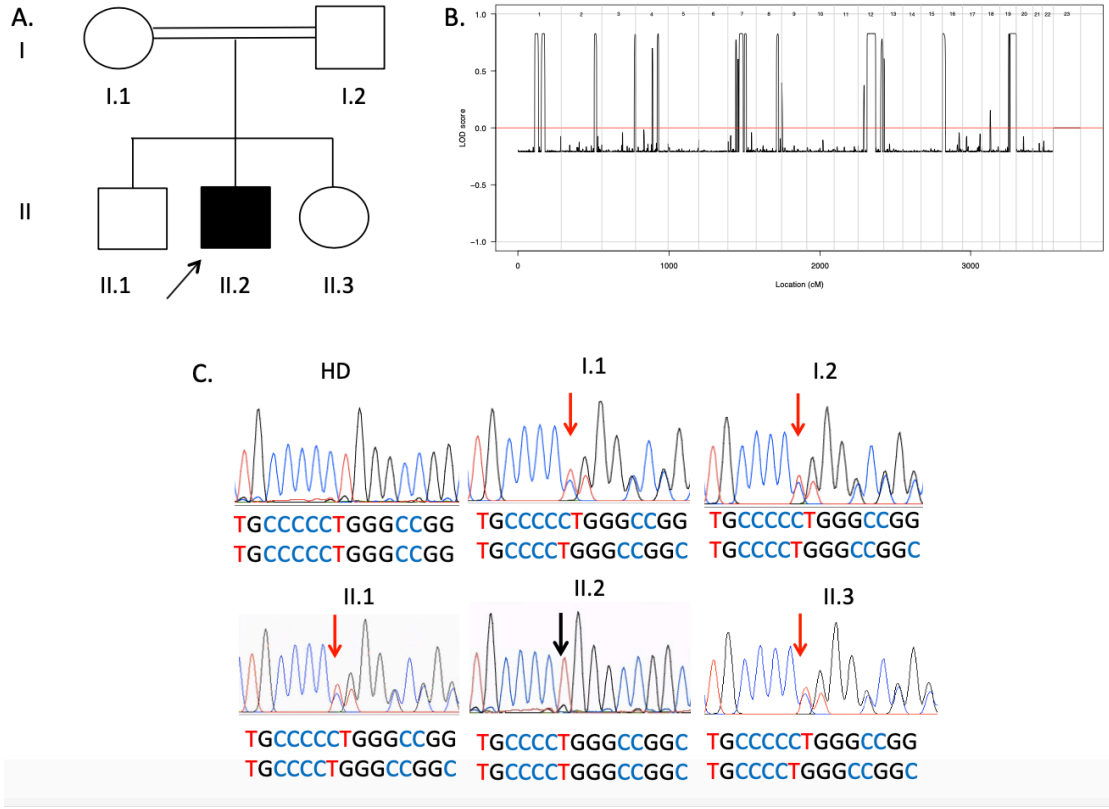
Filter	# variants
Nil	1 672 728
Linkage Region only	164 341
MAF <0.05 ESP6500 TGP	45 167
MAF <0.10 in house DB	42 032
Exon/splice/UTR only	1427
Non synonymous only	253
Homozygous	108
>30 coverage	98
Candidate genes	**1**

The candidate variant was a homozygous deletion, c.767delC, leading to a frameshift, and premature stop in *NFKBID* (Figure 6.3C). It was not present in population databases, nor an in-house control database (Table 6.2).

NFKBID encodes a non-classical inhibitor of NF-κB

NFKBID encodes the non-classical inhibitor of NF-κB signaling, IκBNS, also known as IκBδ. IκBNS in humans is 313 amino acids in length, constituted by seven ARDs, and a nuclear localisation signal (NLS). The identified variant introduces a frameshift at a highly conserved residue (Pro256Leufs*46), disrupting the distal ankyrin repeat domain, and generating a truncated protein of 301 amino acids in length (Figure 6.3E,F). This variant was predicted to be highly significant, as the gene is highly intolerant to loss of function, as the observed/expected proportion of LOF variants in the gnomAD database is 0.23, indicating that loss of function variants are not observed

as frequently as predicted by the background variation observed in the gene. Furthermore, no homozygous LOF variants have been identified in gnomAD or EXac.



E.

Human(wt)	246	TALHMAAALPPGPAQEAIVRHLLAAGADPTLRNLENEQPVHLLRPGPGPEGLRQLLKRSRVAPPGLSS 313
p.P256Lfs*46	246	TALHMAAALPLGRPRRPSCGTCWQLGRTPHCATWRMSSPFTCCGPGRALRGSGSC 303
chimpanzee	261	TALHMAAALPPGPAQEAIVRHLLAAGADPTLRNLENEQPVHLLRPGPGPEGLRQLLKRSRVAPPGLSS 328
macaque	261	TALHMAAALPPGPAQEAIVRHLLAAGADPTLRNLENEQPVHLLRPGPGPEGLRQLLKRSRVAPPGLSS 328
cow	261	TALHMAAALPPGPAQEAIVRCLLAAGADPTLRNLENEQPVHLLRPGPGPEGLRQLLKRSRVAPPGLSS 328
mouse	260	TALHMAAALPPGPPQEAIVRHLLAAGADPTLRNLENEQPVHLLRPGPGPEGLRQLLKRSRTAPPGLSS 327
rat	260	TALHMAAALPPGPPQEAIVRHLLAAGADPTLRNLENEQPVHLLRPGPGPEGLRQLLKRSRAAPPGLSS 327
frog	225	TALHMAAALPPHRSTEYLIQLLLSSGGDPSMRNLENEQPAHLVPPGEFTDQIKLLLKRRRVASSTRH 290
zebrafish	242	TALHMAAGLHGNPYQEELIQLLLKHGADPSIRNLENDQPAHLLQSGDKGE 290

F.

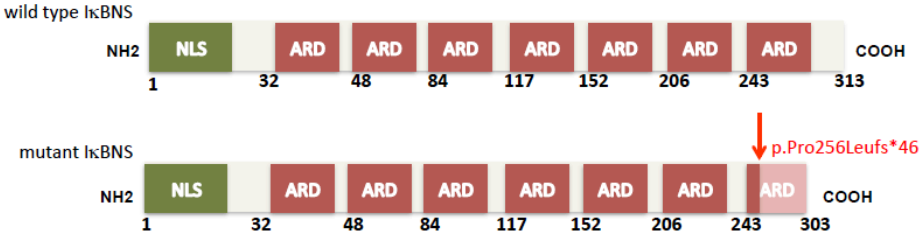


FIGURE 6.3 A NOVEL VARIANT IN NFKBID, C.767DELC, SEGREGATES WITH DISEASE IN A CONSANGUINEOUS FAMILY, AND INTRODUCES A FRAMESHIFT AT A HIGHLY CONSERVED REGION OF THE PROTEIN, IκBNS.

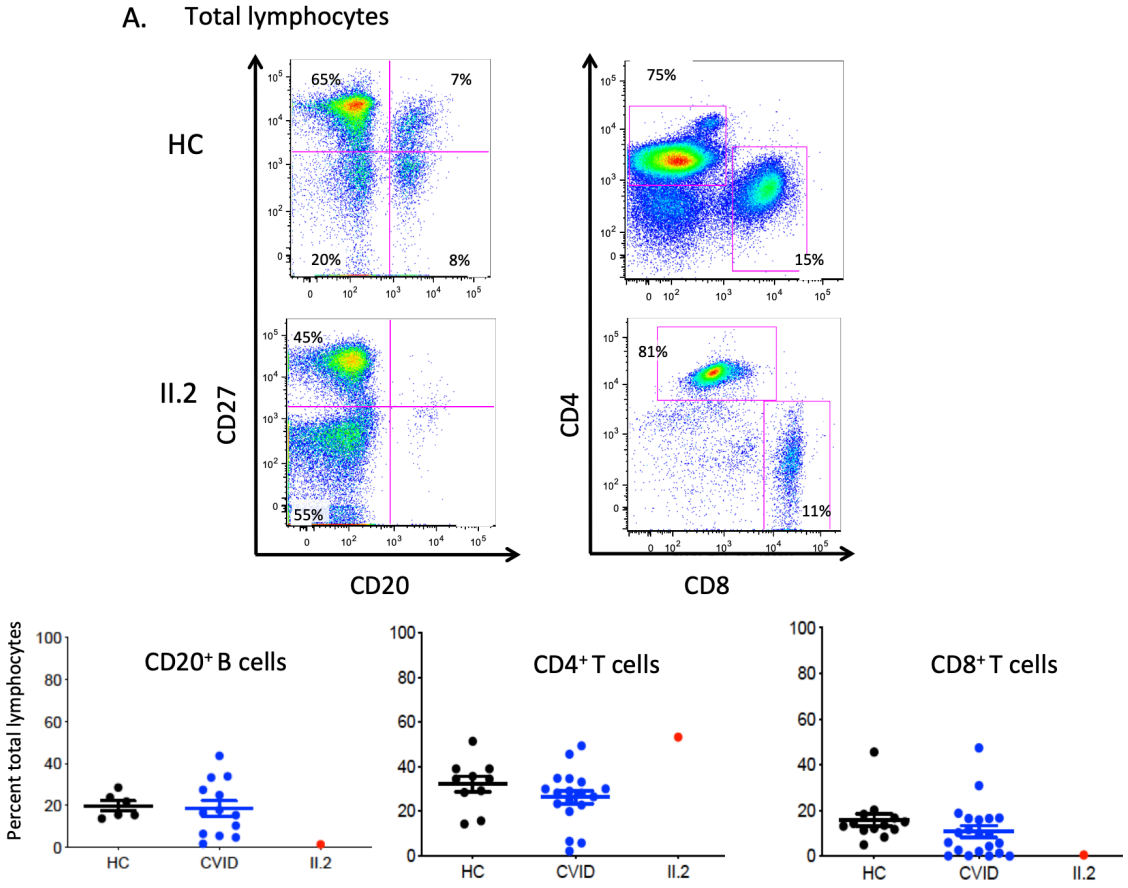
(A). Pedigree of kindred. Circles represent females and squares, males. Arrow indicates proband. Double-lines indicate consanguinity. (**B).** Linkage analysis demonstrates multiple regions of homozygosity in the proband, II.2 as indicated by peaks above 0. (**C).** Sanger sequencing confirmed the presence of the homozygous deletion in the proband (black arrow), and heterozygosity (red arrow) of all the first-degree relatives. A healthy control sequence is demonstrated for comparison. (**D).** *NFKBID* c.767delC leads to a frameshift at AA residue 256. There is a high degree of sequence homology of IκBNS between species, demonstrating the protein's conservation throughout evolution. **(E).** IκBNS protein is highly conserved in mammalian species, and moderately conserved in reptiles. (**F).** Wild type IκBNS contains a nuclear localisation signal, NLS, and 7 ankyrin repeat domains (ARDs). The *NFKBID* c.767delC variant introduces a frameshift at the 6th ankyrin repeat domain, leading to a truncated protein of 301 amino acids in length.

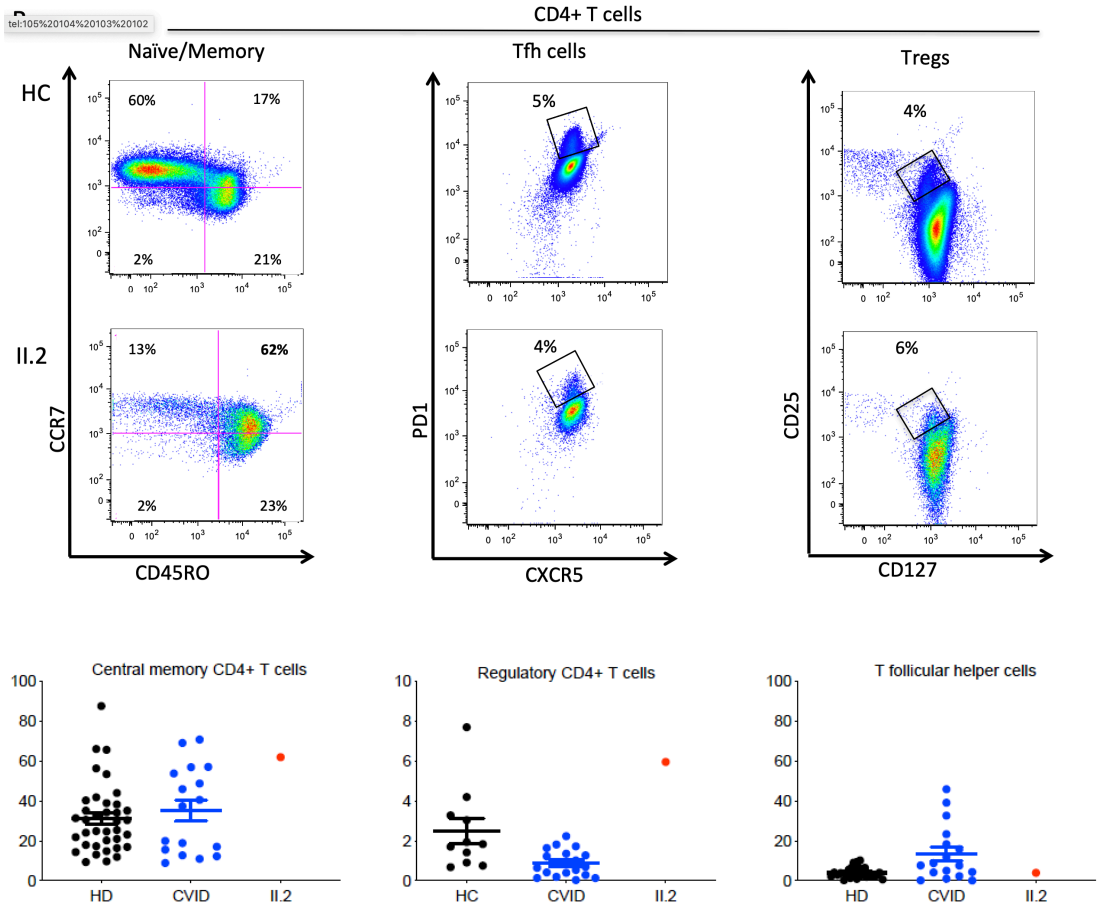
Familial segregation by Sanger sequencing of gDNA from all immediate family members was first performed. The presence of the mutation was confirmed in homozygous state in the proband (Figure 6.3C) and the proband's mother, father and siblings were all shown to be heterozygous for the mutation (Figure 6.3C). Although the other family members were all clinically well, the older brother (II.1) had reduced IgG2 and IgG4 levels, and all family members had an increase in the ratio of $CD4^+/CD8^+$ T cells (Table 6.1). Interestingly serum IgM levels were at the lower end of the normal reference range in the father (I.1) and sister (II.3) (Table 6.1), consistent with a report describing the phenotype of reduced IgM in mice heterozygous for a loss of function mutation in *Nfkbid* (Pedersen, Adori et al. 2016).

6.2.3 Immunophenotyping of PBMCs demonstrated a significant reduction in B and $CD8^+$ lymphocytes in the proband

To determine the proportions of lymphocyte populations in the proband (II.2), PBMCs were analysed by flow cytometry for expression of the following surface markers: CD20, CD27, CD3, CD4, CD8, CD45RO, CD25, CD127, CXCR5, CCR6, CXCR3, CCR7 and PD-1 (Methods, Chapter 2). This demonstrated significantly reduced proportions of B cells, and $CD8^+$ T cells in the proband compared to a healthy control (Figure 6.5A). The total number of B cells was too low to allow meaningful sub-classification of these cells. Of the $CD8^+$ T cells, there was a predominance of naïve cells in the proband compared to a healthy control. There was a reduction in naïve $CD4^+$ T cells; the majority of the $CD4^+$ population co-expressed CD45RO and CCR7, consistent with a central memory phenotype (Figure 6.5B). T regulatory cells, as determined by $CD127^{lo}/CD25^{hi}$ expression on $CD4^+$ T cells were present in normal

proportions (Figure 6.5B). Insufficient sample was available for intracellular labeling of FoxP3 in the proband, however, expression of FoxP3 has been shown to be reduced in *Nfkbid*$^{-/-}$ mice (Schuster, Glauben et al. 2012). CXCR5^{+}/PD1^{+} Tfh cells were present in normal proportions (Figure 6.5B). Although the immunophenotyping at diagnosis was not as extensive, the lymphocyte populations had clearly changed over time. In addition to the underlying genetic abnormality, it is likely that iatrogenic immunosuppression and chronic CMV infection contributed to the progression of the cellular immunophenotype.




CD4+ T cells
Naïve/Memory
Tfh cells
Tregs
HC
IL.2
60%
17%
2%
21%
13%
62%
2%
23%
5%
4%
4%
6%
CCR7
CD45RO
PD1
CXCR5
CD25
CD127

Central memory CD4+ T cells
Regulatory CD4+ T cells
T follicular helper cells
HD
CVID
IL.2
HC

FIGURE 6.4 IMMUNOPHENOTYPING OF PBMCS FROM PATIENT WITH IκBNS DEFICIENCY REVEALS A MARKED REDUCTION IN B AND CYTOTOXIC T CELLS, AND A PREDOMINANCE OF CENTRAL MEMORY CD4⁺ T CELLS.

A. Naïve ($CD20^+/CD27^-$) and memory ($CD20^+/CD27^+$) B cells (left panels), $CD8^+/CD3^+$ T cells (right panels), $CD4^+/CD3^+$ T cells (centre panels). Bottom row depicts values of total B cells, $CD4^+/CD3^+$ T cells and $CD8^+/CD3^+$ T cells in the patient compared to a cohort of CVID patients and healthy controls. **B**. $CD4^+$ T cells subsets in healthy control (HC, top) and patient, (II.2, bottom). From left to right: $CD4^+$ and $CD8^+$ cells; $CD4^+$ Central memory ($CCR7^+/CD45RO^+$) and effector memory ($CCR7^-/CD45RO^+$); T follicular helper cells ($CXCR5^+/PD1^+$), regulatory T cells ($CD25^+/CD127lo$). Bottom row depicts values of central memory $CD4^+/CD45RO^+/CCR7^+$ T cells, T follicular helper cells, and regulatory T cells (all shown as percent of $CD4^+$ T cells) in the patient compared to a cohort of CVID patients (age range: 28-78 years; median, 40 years) and healthy controls (age range: 23-63 years; median, 38 years).

6.2.4 IκBNS expression and NF-κB signaling is disturbed in PBMCs and monocytes, and is associated with increased inflammatory cytokine production

To determine the functional relevance of the gene variant on IκBNS protein expression and NF-κB signaling, total PBMCs were analysed by Western blot. First, total PBMCs were stimulated for 30 minutes with PMA/ionomycin, and lysates prepared as described (Chapter 2.4.6.3). In healthy control (HC) cells, a band was observed at the expected molecular weight of IκBNS (27 kDa) 30 minutes following PMA/ionomycin stimulation. However, no protein expression was detected in patient cells, suggesting the mutant protein was either not expressed, or was rapidly degraded (Figure 6.5A). Analysis of NF-κB1 signaling revealed a relative increase in phosphorylation of the canonical NF-κB protein, p105 in PMA/ionomycin-treated PBMCs from the proband compared with HC cells (Figure 6.5B), suggesting increased

activation of canonical NF-κB signaling.

Given the absence of B cells and significant reduction in $CD8^+$ and naïve $CD4^+$ T cells in peripheral blood, the observed differences may have been influenced by comparison of disparate cell types in the healthy donors and patient. To directly compare NF-κB1 signaling in comparable leukocyte populations and determine a cellular phenotype in the proband, functional studies were next performed in monocytes. Primary monocytes were purified from whole blood, using a $CD14^+$ cell positive selection kit (Miltenyi), as described (Chapter 2.5.1). Purity was confirmed by staining $CD14^+/CD45^+$ cells, with results of >95%.

Monocytes were stimulated with LPS (100 mcg/ml) to study activation of the canonical NF-κB pathway. In order to study short and delayed effects of stimulation, cells were treated for 10, 30, 60, 90 or 120 minutes. Levels of total p105 protein were similar between HC and patient cells. However, an increase in levels of phosphorylated p105 in the patient compared with the HC was observed after 30, 60 and 90 minutes, suggesting a loss of regulation of canonical signaling (Figure 6.5C). Total levels of p65 were also tested and similar expression was observed at all timepoints, as were levels of the phosphorylated p65 protein. Increased activation of canonical signaling was also demonstrated by a concomitant greater degradation of IκBα in the proband compared with HC (Figure 6.5C), at 15 minutes there was almost complete absence of detectable IκBα in the proband, and at all timepoints after stimulation, weaker expression of the protein than in the HC. Taken together these results suggest that loss of IκBNS leads to a partial loss of regulation of canonical NF-κB signaling in monocytes, although the effect on other cell types needs further investigation.

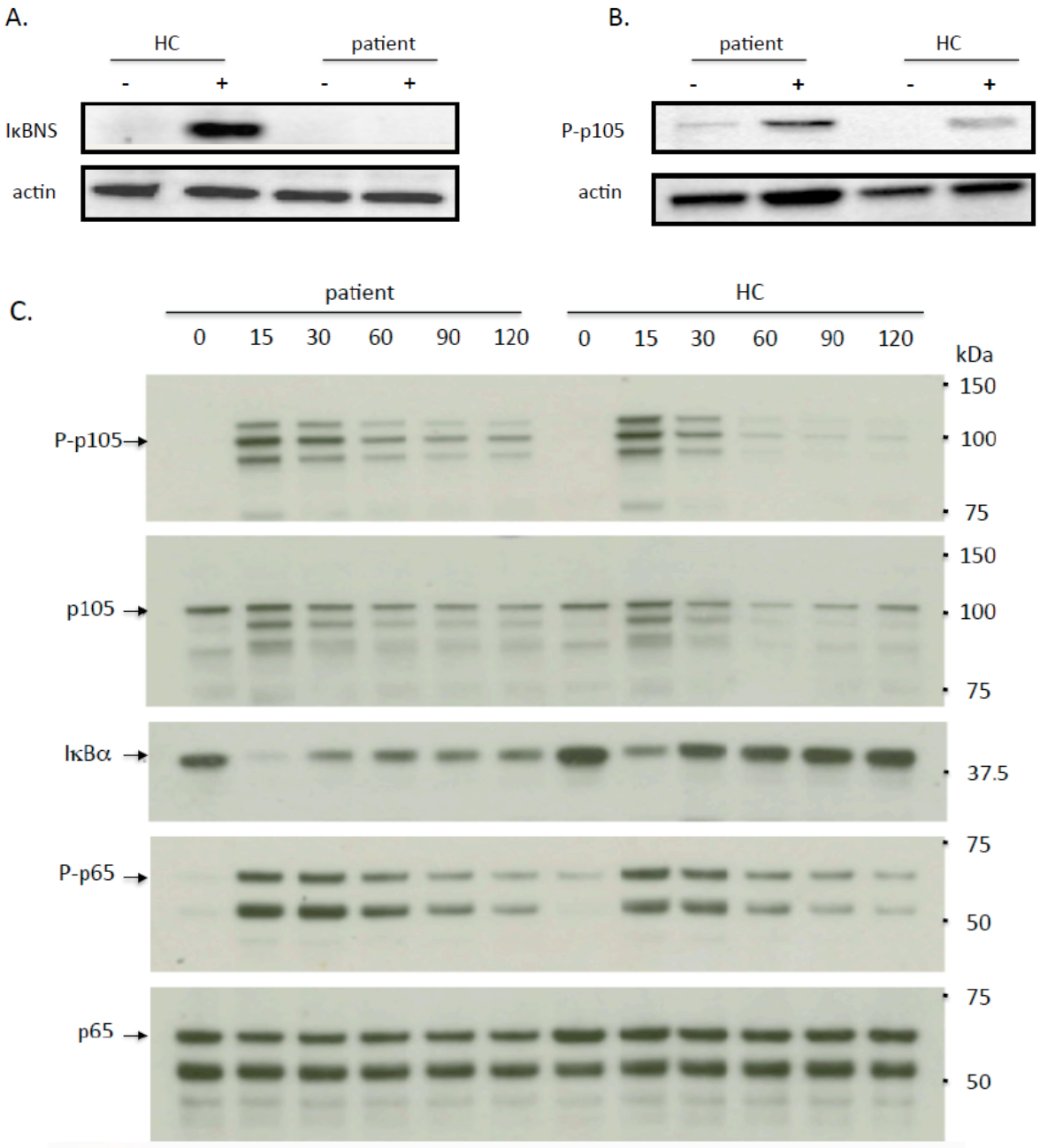

A.
HC
patient
- + - +
IκBNS
actin
B.
patient
HC
- + - +
P-p105
actin
C.
patient
HC
0 15 30 60 90 120 0 15 30 60 90 120
kDa
P-p105
150
100
75
p105
150
100
75
IκBα
37.5
P-p65
75
50
p65
75
50

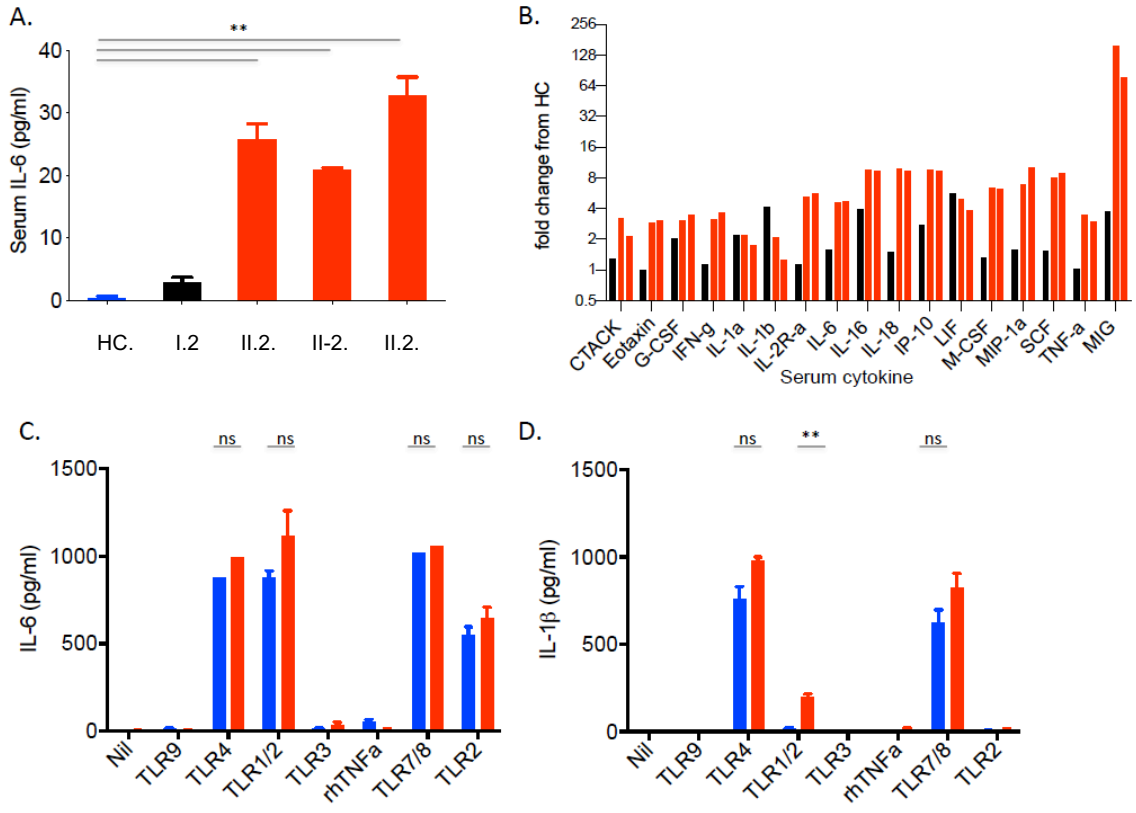
FIGURE 6.5 LACK OF EXPRESSION OF IκBNS, AND DISTURBED NF-κB SIGNALING IN PBMCS AND MONOCYTES FROM PROBAND.

(A). Western blot of lysates of total PBMCs stimulated with PMA/ionomycin demonstrates the presence of a band at 27 kDa after 30mins in HC (left), consistent with IκBNS. No band of equivalent size is seen in lysates from stimulated patient PBMCs (right). (**B).** Lysates of total PBMCs, unstimulated (-), and stimulated for 30 minutes with PMA/ionomycin (+) demonstrate an increase in phosphorylated-p105 expression in proband (left), compared with HC (right). Actin was used as a loading control. (**C).** Canonical NF-κB in LPS-stimulated monocytes from proband (left) and healthy control (right). In the proband, there is increased expression of phosphorylated p105 at later timepoints, corresponding to lower expression of IκBα. Expression of total p105, p65 and phosphorylated p65, are similar between the proband and HC. Ponceau staining was used as a loading control. (Data representative of 2 independent experiments.)

We next wanted to study the effect of mutant IκBNS on on inflammatory cytokine production. Firstly, we measured serum levels of IL-6 in the proband, his healthy mother and a HC. (Nb. samples were drawn from the proband immediately prior to IVIG infusion) IL6 levels were elevated in the patient on 3 separate occasions (Figure 6.6A). We also chose to measure a panel of cytokines as a tool to identify potential further targets for investigation. A broad range of cytokines and chemokines were elevated in the proband and his father, most notably the chemokines MIG (CXCL9) and IP10 (CXCL10) (Figure 6.6B). This has been performed only once, and will need to be followed up to determine the significance of these findings.

Again, due to the relative deficiency B and cytotoxic T cells, we first investigated if there were any differences in cytokine production from patient monocytes following direct stimulation with TLR agonists. To do this, monocytes were isolated from the proband and an unrelated HC and stimulated with a range of Toll-like receptor (TLR) agonists for 16 hours, to allow sufficient time for cytokine production, and also to avoid cell death, which occurs after this time (data not shown). Supernatants from these cultures were harvested and the levels of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α were measured by ELISA, as described in chapter 2.7. The level of IL-1β was significantly greater in the proband's monocyte cultures after stimulation with the TLR1/2 agonist, Pam3CysSerLys4 (Pam3CSK4; Figure 6.6C, D). Other observed differences were not significant, although there was a trend towards higher levels of IL-6 upon Pam3CSK4 stimulation. Similarly, significantly elevated IL-1β and IL-6 production was observed following stimulation with LPS from the patient's monocytes (Figure 6.6C, D). Given that both TLR1/2 and TLR4 trigger NF-κB signaling (Hayden and Ghosh 2008), these observations were consistent with loss of regulation of the pathway.

The differences in LPS sensitivity between the healthy control and patient cell were particularly interesting given the previous report of increased sensitivity to LPS and elevated production of IL-6 in *Nfkbid*$^{-/-}$ mice (Kuwata, Matsumoto et al. 2006). Next, we chose to study early cytokine secretion by monocytes from the patient, and a HC, to determine if the early kinetics of the inflammatory response were also different. Here, monocytes were stimulated with LPS (100 µg/ml) for two, four, six, or eight hours, and supernatants analyzed for IL-6 and IL1–β production (Figure 6.6E, F). Significantly elevated levels of IL-6 were observed in supernatant of stimulated patient monocytes after eight-hours, but were otherwise comparable with the HC at other timepoints (Figure 6.6E). Similarly, when IL-1β levels were tested, although low levels of Il-1β were detected early in the proband, significantly increased levels were detected at 6 and 8 hours following stimulation, suggesting that the kinetics of both IL-1β and IL-6 secretion are altered in the absence of functional IκBNS.



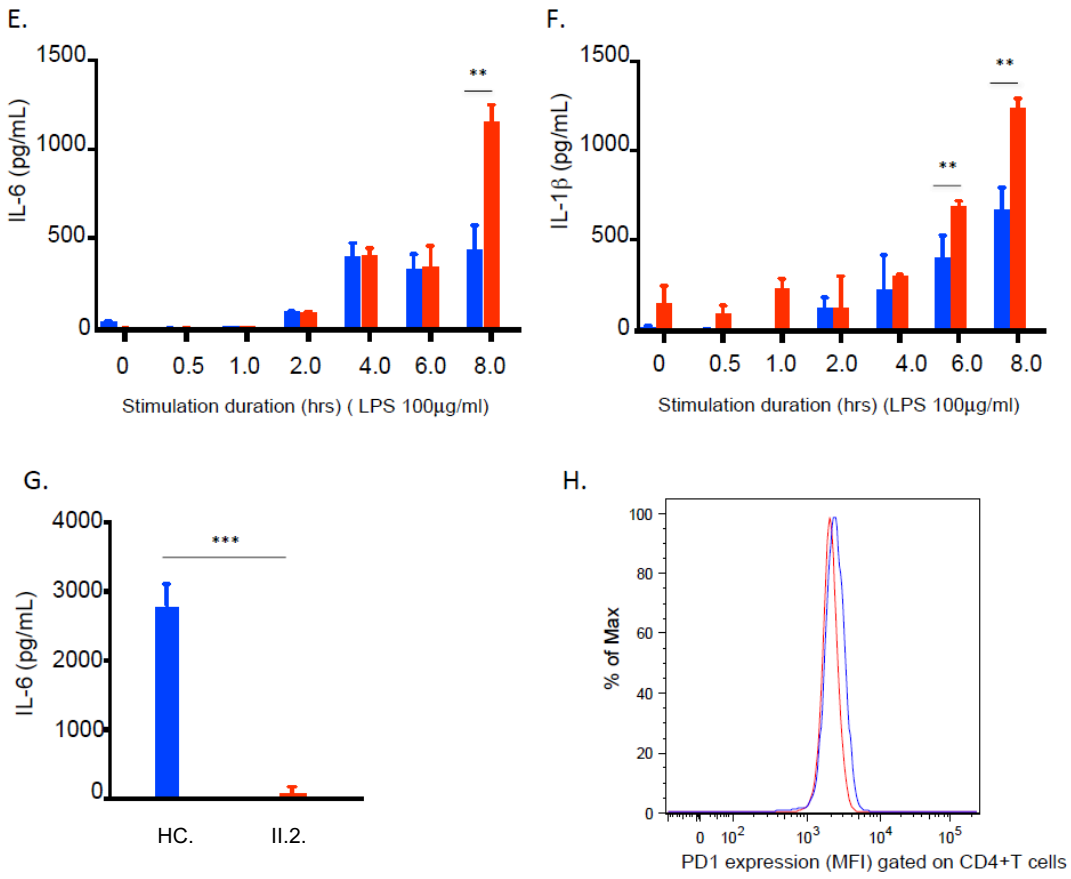


FIGURE 6.6 INCREASED LEVELS OF PRO-INFLAMMATORY CYTOKINES IN IκBNS DEFICIENCY.

(A). Serum levels of IL-6 in patient (II.2; 3 timepoints), his mother (I.1), and an unrelated healthy control. **(B).** Serum levels of multiple inflammatory cytokines were elevated in the proband, normalised to mean of 2 healthy control samples (red columns, serum samples from proband 2017 (left), 2018 (right); black columns, serum samples from father, I.2). C. Production of IL-6 from monocytes cultured with a range of TLR stimuli as indicated for 16 hours (HC, blue; patient, red). **(D)**. Production of IL-1β from monocytes cultured with TLR stimuli for 16 hours (HC, blue; patient, red). **(E).** Production of IL-6 from monocytes cultured with LPS (which one, 100 µg/ml) for indicated times (HC, blue; patient, red). **(F).** Production of IL-1β from monocytes cultured with LPS for indicated times (HC, blue; patient, red). **(G)**. Production of IL-6 from $CD4^+RO^+$ cells after 24 hours PMA/ionomycin stimulation. **(H).** Histogram of expression of PD1 on cell surface of total $CD3^+/CD4^+$ T cells in HC (blue) and proband (red). *(ns, not significant; **,0.001< p <0.01; ***, p <0.001 HC, healthy control)*

6.2.5 Memory CD4+ T cells from the patient demonstrate reduced pro-inflammatory cytokine production

Whilst studies of protein expression, and cytokine production from primary monocytes demonstrated subtle differences in canonical NF-κB signaling in the patient, compared with the healthy control, the relative abundance of central memory $CD4^+$ T cells in proband PBMCs (Figure 6.4B) raised the question of whether differences would be more marked when comparing the specific dominant lymphocyte population. To do this, first cytokine production was measured from isolated stimulated memory $CD4^+$ T cell cultures. In contrast to monocyte cultures, significantly less IL-6 was detected in supernatants from $CD4^+$/$CD45RO^+$ T cells stimulated with PMA/Ionomycin for 24 hours, suggesting that these cells were either dying, or exhausted (Figure 6.6E), although we did not have a measure of live cell numbers from those cultures after 24 hours. To assess a marker of exhaustion, PD-1 expression on $CD4^+$ T-cells was compared to a healthy control, and demonstrated no significant difference (Figure 6.6F). An alternative possibility is that IκBNS in memory $CD4^+$ T cells has a direct impact on IL6 production. The remaining questions regarding survival and cell function will be addressed further by functional studies of human monocyte (THP-1), B- (Daudi), and T- (Jurkat) cell lines, CRISPR/Cas9 deleted for *NFKBID* and transduced with *NFKBID* c.767delC.

6.2.6 Identification of a second patient with possible IκBNS deficiency (Kindred 2)

During the course of investigating the family described above, an additional patient was identified, harboring novel compound heterozygous variants in *NFKBID*. The proband in the second kindred was investigated through the clinical genomics study described

in Chapter 4. At the time of recruitment, she had been diagnosed with CVID, although there were some unusual manifestations, including atypical eczema, and neutropenia. She was the second child of non-consanguineous parents (Figure 6.8). The variants identified were a splice site variant at *NFKBID* c.259-2A>G, and a missense change at *NFKBID* c.473A>G; p. (His158Arg). Neither variant was present in any population database. The splice site variant was confirmed to have been maternally inherited, indicative that the variants are in compound heterozygous state in the proband of Kindred 2. Testing of the father and older brother is awaited. *In silico* predictions for each variant were damaging, and neither was present in any population database.

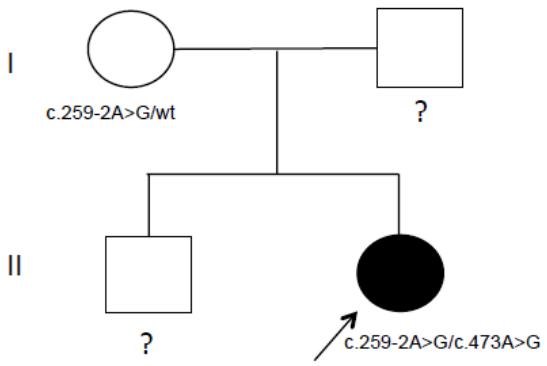


FIGURE 6.7 PEDIGREE OF KINDRED 2.

Circles represent females and squares, males. Arrow indicates proband. The proband has biallelic variants in *NFKBID*, as depicted. The mother is heterozygous for the splice site variant, c.259-2A>G. Both the father and brother have not yet had genetic testing completed.

Immunological investigations in this second patient confirmed some similarities with the proband from the first family, namely, very low IgM, low memory B cells, in particular marginal zone memory B cells, and a predominance of naïve $CD8^+$ T cells, although IgE levels were within normal range despite eczema (Table 6.3). Within the

$CD4^+$ T cell compartment she also demonstrated increased absolute numbers of central memory cells. Ongoing functional investigations are underway.

Table 6.3 Immunologic investigations in proband from Kindred 2

Investigation	At diagnosis	Current	Reference range
IgG	**3.5 g/L**	7 g/L*	(5.4-18.2 g/L)
IgA	**0.5 g/L**	**0.5 g/L**	(0.7-4.2 g/L)
IgM	**0.1 g/L**	**0.1 g/L**	(0.3-2.9 g/L)
IgE	59 kU/L	ND	(0-120 kU/L)
Lymphocytes ($x10^9$/L)	4.13	3.5	(2.0-8.0)
B cells (% of lymphocytes) Absolute ($x10^9$/L)	12.2% 0.5	12.2 0.43	(10.0-31.0) (0.2-0.6)
Naïve (% of B cells) **Total ($x10^9$/L)**	**93.2%** 0.47	**91.8%** 0.39	(59.7-88.4) (0.2-0.65)
Memory (% of B cells) **Total ($x10^9$/L)**	**3.9%** **0.02**	**3.5%** **0.01**	(8.1-33.3) (0.02-0.19)
MZ memory B cells (% of B) **Total ($x10^9$/L)**	3.5% 0.02	**2.1%** **0.01**	(3.1-18.0) (0.01-0.09)
T cells (% of lymphocytes) Total ($x10^9$/L)	74% 3.06	**81.3%** 2.85	(55.0-78.0) (0.7-4.2)
$CD4^+$ (% of T cells) Total ($x10^9$/L)	47% 1.94	49.4% 1.73	(27-53) (0.3-2.0)
$CD8^+$ (% of T cells) Total ($x10^9$/L)	22% 0.91	25.9% 0.91	(19-34) (0.3-1.8)
NK cells (% of lymphocytes) Total ($x10^9$/L)	4.0% 0.17	**3.8%** 0.13	(4.0-26.0) (0.09-0.9)
Naïve $CD4^+$ (% of $CD4^+$) Total ($x10^9$/L)	69% **1.59**	ND	(54-73) (0.46-1.49)
Central memory $CD4^+$ (% of $CD4^+$) **Total ($x10^9$/L)**	30% **0.69**	ND	(24.5-39.4) (0.26-0.61)
Effector memory $CD4^+$ (% of $CD4^+$) Total ($x10^9$/L)	**1.0%** 0.02	ND	(1.2-6.5%) (0.01-0.09)
Naïve $CD8^+$ (% of $CD8^+$) **Total ($x10^9$/L)**	**86.9%** **1.14**	ND	(37.9-78.9) (0.26-1.06)
Central memory $CD8^+$(% of $CD8^+$) **Total ($x10^9$/L)**	**12.4%** 0.16	ND	(13.1-36.8) (0.09-0.41)
Effector memory $CD8^+$ (% of $CD8^+$) **Total ($x10^9$/L)**	**0.2%** **0.0**	ND	(1.0-17.0) (0.01 – (0.17)

*(*indicates on IVIG therapy; bold values indicate outside of reference range)*

6.3 Discussion

The study of primary cells from an individual with a novel homozygous frameshift mutation in *NFKBID* resulting in complete loss of expression has provided important preliminary insights into the function of IκBNS in humans, which, to our knowledge, has not before been well-studied. We believe this case to be the first description of human IκBNS deficiency. The proband's initial presentation was consistent with a predominantly antibody deficiency, manifesting as recurrent pneumonia in the setting of low IgM, low IgG2 and impaired humoral immune responses to polyvalent polysaccharide pneumococcal antigen. Further progression of disease revealed features of a combined immunodeficiency, with susceptibility to EBV (prior to iatrogenic systemic immunosuppression), and CMV, after immunosuppression for associated systemic autoimmunity, which manifest as acute necrotizing glomerulonephritis and pulmonary vasculitis. In addition to the infectious susceptibility and systemic vasculitis, the patient had additional clinical features of dysregulated inflammatory responses, as demonstrated by lymphocytic infiltrates in the liver and bone marrow, as well as massive splenomegaly and intermittent lymphadenopathy. Immune dysregulation was also evident by persistent elevation of the inflammatory marker, CRP, in serum, and further demonstrated by increased serum levels of IL-6, on multiple occasions, as well as a number of cytokines and chemokines, including, but not limited to MIG (CXCL9) and IP-10 (CXCL10).

Investigations at the time of diagnosis of 'possible CVID' revealed normal numbers of B-, T- and NK cells, although over time the loss of B cells and $CD8^+$ T cells in the peripheral blood suggests that IκBNS is important for maintenance of these

populations. Skewing of $CD4^+$ T cells to a central memory phenotype, and the finding of aggregates of $CD3^+/CD4^+$ T cells in the marrow and liver are suggestive of dysregulated activation of $CD4^+$ T cells in the absence of functional IκBNS, although, chronic CMV may also be a contributing factor. Studies are ongoing to determine the functional effect of the homozygous c.767delC, mutation in transfected human B and T cell lines, particularly with respect to measurement of cell proliferation, cytokine secretion and survival. The additional identification of a second CVID patient with likely compound heterozygous variants in this gene, adds weight to the case for this to be a *bona fide* novel PID.

IκBNS was originally identified as a protein induced, and rapidly expressed, upon TCR signaling during negative selection of thymocytes (Fiorini, Schmitz et al. 2002), of relevance given the autoimmune phenotype observed in the proband; it is attractive to hypothesise that impaired negative selection led to the autoimmune cytopenias and vasculitis observed in the patient. Subsequently, the role of this protein in B cell development has been further examined. Mice with a targeted deletion of *Nfkbid*, or a truncating mutation generated by N-ethyl-N-nitrosourea (ENU) mutagenesis, demonstrated reduced levels of IgM, IgG2b and IgG3 subclasses, significantly reduced marginal zone and B1 B cells, and did not produce antibody to T-dependent or T-independent antigen (Touma, Keskin et al. 2011, Arnold, Pirie et al. 2012). Although it was not determined whether the proband had a specific absence of B1 B cells at diagnosis, the very low levels of IgM, and susceptibility to infection with encapsulated bacteria, suggests a possible deficiency of this subset, which is also important for secreting natural antibody in humans (Griffin, Holodick et al. 2011).

Further studies of *Nfkbid*-/- T cells have demonstrated a number of defects in aspects of T cell development and function, which need to be further explored. IκBNS is induced in DP thymocytes upon receipt of a strong TCR signal, demonstrating its role in negative selection. T cells from *Nfkbid-/-* mice produce diminished levels of IL-2 and IFNγ in response to a strong TCR stimulus (Touma, Antonini et al. 2007, Touma, Keskin et al. 2011). IκBNS-deficient T cells are also relatively insensitive to CD3 and CD28 stimulation, however this is partly overcome by addition of exogenous IL-2 (Touma, Antonini et al. 2007). These studies also incorporated analysis of differentially expressed genes in *Nfkbid*$^{-/-}$ T cells, and demonstrated a reduction of expression of several critical immune genes, including *Ctla4*. After stimulation with anti-CD3 and anti-CD28 *Nfkbid*$^{-/-}$ T cells expressed significantly lower levels of *IL-2, Irf8, Il-1, Map4k, Sh2d1a and Bcl2l1* (Touma, Antonini et al. 2007). Single-cell RNA-sequencing (ssRNA-Seq) of sorted lymphocyte populations from the proband and heterozygous relatives, will soon be performed to determine the effect of *NFKBID* c.767delC on the expression of other genes, and to address the question of whether the observed dysregulated NF-κB signaling is accompanied by a predominant gene signature that may guide rational therapeutic targeting of any patients identified in the future with loss of function of IκBNS.

Whilst the thymic defects, and impaired negative selection, described in the *Nfkbid*$^{-/-}$ mice may be a possible explanation for the development of autoimmune disease, other defects in T cell differentiation in the mouse model may protect against some autoimmune diseases: *Nfkbid*$^{-/-}$ mice are relatively protected against experimental autoimmune encephalitis, due to impaired Th17 cell differentiation (Kobayashi, Hara et al. 2014). Together these defects in T cell function could be consistent with some

aspects of the immune dysfunction in the proband, however the limited numbers of naïve T cells have made the study of primary T cell responses challenging, and further work will be carried out in transfected cell lines. This will enable the study of the effect of loss of IκBNS on T lymphocyte survival and function, without the potential for non-genetic influences, such as infection and therapies.

Defects of innate immunity have also been demonstrated in an animal model of IκBNS deficiency, and parallel features observed in the clinical manifestations of the patient reported here. *Nfkbid* deficient mice have a heightened inflammatory response to endotoxin-induced sepsis (Kuwata, Matsumoto et al. 2006). This response is driven by elevated activity of NF-κB signaling, and enhanced TLR4 and IL-6 expression (Kuwata, Matsumoto et al. 2006, Niida, Isoda et al. 2012). Strikingly, the proband suffered a severe inflammatory response within hours of insertion of a Tenckoff catheter, which was performed with a view to commence peritoneal dialysis. This procedure in fact mimics a commonly used model of LPS induced sepsis in animals. Taken together, the complex immunologic features in the mouse models of IκBNS deficiency of prominent B cell defects, accompanied by deranged T cell function and dysregulated innate immune responses, fit well with the homozygous frameshift *NFKBID* mutation being the cause of this novel immunodeficiency.

CHAPTER 7 GENERAL DISCUSSION

The work presented within this thesis has led to novel insights into the genetic causes of PID, with a major focus on the heterogeneous group of PADs. In Chapter 1, I introduced the humoral immune response, and the evolution of our understanding of its relevance in human disease. Incremental gains in understanding the normal immune response have come about from decades of studying the humoral and cellular immune responses in healthy humans and animal models. Specific examples of so-called 'human experiments of nature' have also led to a major increase in our understanding of the immune system, albeit with the revelation that many more questions remain to be answered. Over time, the application of genomics and complimentary 'omics', will undoubtedly lead to further advances in our understanding of the immune response, whereby the pathogenesis of complex, polygenic immune disease may be better elucidated.

Chapter 3 sets the scene from a clinical perspective; presenting a retrospective study of a large cohort of adult individuals with predominantly antibody deficiencies, in Victoria, Australia. Analysis of that group of patients confirmed similarities with international cohorts, as well as some notable differences. Strikingly, the patients in this cohort suffered more disease complications, and longer diagnostic delays than other reported cohorts. We also observed increased mortality in patients with complications of bronchiectasis and immune dysregulation, defined as autoimmune disease, inflammatory complications and malignancy. At the time of reporting, only a small proportion of non-XLA patients had received genetic testing, largely through the 'Novel gene discovery project' (Chapter 2.4.1).

For some patients, the uncertainty of their condition being monogenic caused a level of anxiety; some patients have indicated this has led to the decision not to have children, whilst others have been more fatalistic in their approach. This is exemplified in the Dutch-Australian kindred described in the paper, *Haploinsufficiency of the NF-κB1 Subunit p50 in Common Variable Immunodeficiency.* (Appendix 5). At the time of this discovery, which was made as part of the 'Novel gene discovery project' described in Chapter 2.4.1, one of the Australian affected family members was pregnant; when she received the result, she was not interested in pursuing prenatal testing. The 'roll of the die' had become accepted in that large kindred with affected members over four generations. Nevertheless, genetic testing of other family members has provided the opportunity for informed genetic counselling, early institution of treatment and monitoring, or reassurance, and 'release' from frequent monitoring. In fact, the first born child of the patient described above, has been found to harbor the mutation; now regular monitoring is performed for early identification of infections, and other potential disease manifestations. Furthermore, there may be potential targeted therapies or preventative therapies to trial in the future, as greater numbers of patients are identified.

The findings from the 'Novel gene discovery project' spearheaded a larger project, described in detail in Chapter 4. The 'Clinical genomics project' aimed to determine the diagnostic and clinical utility of genomic sequencing in a heterogeneous population of individuals with immune diseases and enable equality of access to testing. In order to achieve this, broad inclusion criteria were implemented, and thus this study included a large proportion of adults with sporadic disease. This resulted in a comparatively low

rate of definitive diagnostic results, however for some patients there were very significant impacts on clinical care; three individuals who had somewhat atypical clinical presentations of the identified genetic conditions, IPEX and GATA2 deficiency, underwent successful HSCT on the basis of a genomic result. Additional patients were able to receive targeted therapies, including two siblings with CTLA4 deficiency. These findings also have implications for genetic counselling and family planning in the extended families.

Approximately 20% of patients had identifiable pathogenic variants in single genes, confirming the original diagnosis, or providing a new, more precise diagnosis. In many more patients VUSs were identified. Whilst these variants may be related to the clinical phenotype, further functional validation is required to determine their functional effect. Together, these findings point to the complexity of the pathogenesis of immunodeficiencies, and the challenges associated with performing clinical genomics in patients with complex disease. Furthermore, giving such information to patients may generate a degree of anxiety, due to the uncertainty about the effect of the genetic variant and with regard to the potential relevance for their relatives.

Work from this thesis identified and described a novel *NFKB2* mutation as a cause of CVID (Chapter 5.2.1). A family with two affected sisters, and an ostensibly healthy carrier father, is an example of monogenic CVID as a disease of variable penetrance. Even between the affected sisters there were striking differences in their clinical manifestations; one sister suffered more infective complications, and passed away due to enteroviral encephalitis, whereas her sister was afflicted by autoimmune cytopenias and endocrine disease. Similarly, a divergent immunological phenotype was observed

in these siblings, with complete absence of B cells in one, and absent memory, but present naïve B cells in the other. This led to the question of how these different phenotypes may be attributed to different cellular behaviours. This prompted a series of experiments in an animal model of NF-κB2 deficiency, to determine the specific differences in behavior of immune cells in that model. NF-κB2 deficient naïve B cells responded to the TLR9 agonist, CpG, identically to wild-type, but were, effectively non-responsive to T-cell help via CD40 ligation. Mirroring the defect in CD40 stimulation, proliferation of NF-κB2 deficient $CD8^+$ T cells in response to stimulation with IL-2 was normal, but when anti-CD3/CD28 co-stimulation was used (without the effect of IL-2 by blocking antibody) the proliferative response was significantly blunted. Interestingly, this effect appeared to be gene-dosage dependent, since haploinsufficiency for NF-κB2 essentially halved the proliferative response (Chapter 5.2.2, Figure).

The types of quantitative assay described in Chapter 5, are now being established in the human system, and will be applied to individuals with monogenic and polygenic disease, in order to dissect the mechanisms of immune deficiency, in more detail. It is likely that studies of this nature will aid in the understanding of variable disease penetrance, and variability in disease severity. The application of mathematical modeling to these findings, offers potential to discern more information about these systems, and may also lead to tools to predict responsiveness to therapies, by *in vitro* studies.

Finally, I have described a new NF-κB-related immunodeficiency, due to dysregulated NF-κB activity, that led to susceptibility to pneumococcal and viral infections, and a

predisposition to autoimmune disease. The proband presented initially with features suggestive of an antibody deficiency, however the development of significant complications over time changed the nature of the immunodeficiency. Autoimmunity and inflammation were prominent features, leading to end-stage renal failure, liver disease, and portal hypertension. Autoimmune cytopenias initially responded to corticosteroids, however the more intensive immunosuppression for glomerulonephritis was probably in part related to the subsequent loss of circulating B cells and $CD8^+$ T cells over time. Chronic CMV viraemia developed with 3 episodes of invasive disease, causing pneumonitis and colitis. Additionally, T cell infiltrates in BM and lymph nodes; and massive splenomegaly (no tissue unfortunately), and intermittent lymphadenopathy. Together, the evolving clinical manifestations presented enormous challenges for the treatment and management of his disease, not unusual for individuals with CVID and CID with complications of immune dysregulation.

Exploratory investigations performed as part of this project suggested immune dysregulation that may be IL-6 dependent, since the proband had consistently elevated levels of IL-6, however immunomodulation with the anti-IL6R, tocilizumab, was unsuccessful, due to the complication of staphylococcal infection. Since the time of writing this thesis, IL-6R deficiency has been reported in 2 patients, who manifest recurrent staphylococcal skin infections. Other inflammatory cytokines were found to be elevated in the patient's serum, in particular, IP10 (CXCR3) and MIG, but more investigations are required to determine the cellular source of these cytokines. Ongoing studies are underway to confirm the pathogenicity of the mutation in human B cell, T cell and monocyte lines, as well as functional validation of a second patient identified that shares features with patient 1.

In conclusion, genomic investigations are widely changing the way PIDs are diagnosed, and frequently these findings may lead to targeted therapies. However, there is still much progress to be made in applying these discoveries for better outcomes in patients. Some of the key challenges, and opportunities for discovery, lie in identifying factors that impact the penetrance of genetic variants on disease. Furthermore, many cases are still unsolved despite germline genetic studies. This is likely to come about with additional, complementary studies, such as transcript-, proteo- and epigenomics. The real challenge will be in interpretation of these data in the context of each individual patient, and using that to guide treatment and prevention of disease.

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Comparison of diagnostic criteria for common variable immunodeficiency disorder

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Common variable immunodeficiency disorders (CVIDs) are the most frequent symptomatic primary immune deficiency condition in adults. The genetic basis for the condition is not known and no single clinical feature or laboratory test can establish the diagnosis; it has been a diagnosis of exclusion. In areas of uncertainty, diagnostic criteria can provide valuable clinical information. Here, we compare the revised European society of immune deficiencies (ESID) registry (2014) criteria with the diagnostic criteria of Ameratunga et al. (2013) and the original ESID/pan American group for immune deficiency (ESID/PAGID 1999) criteria. The ESID/PAGID (1999) criteria either require absent isohemagglutinins or impaired vaccine responses to establish the diagnosis in patients with primary hypogammaglobulinemia. Although commonly encountered, infective and autoimmune sequelae of CVID were not part of the original ESID/PAGID (1999) criteria. Also excluded were a series of characteristic laboratory and histological abnormalities, which are useful when making the diagnosis. The diagnostic criteria of Ameratunga et al. (2013) for CVID are based on these markers. The revised ESID registry (2014) criteria for CVID require the presence of symptoms as well as laboratory abnormalities to establish the diagnosis. Once validated, criteria for CVID will improve diagnostic precision and will result in more equitable and judicious use of intravenous or subcutaneous immunoglobulin therapy.

Keywords: common variable immunodeficiency, diagnostic criteria, HGUS

INTRODUCTION

Common variable immunodeficiency disorders (CVIDs) are the most frequent symptomatic primary immune deficiency disorder in adults. While most patients suffer recurrent infections (1–5), there is also an increased risk of autoimmune disorders and malignancy because of immune dysregulation. CVID is likely to represent a heterogeneous group of polygenic disorders (6).

Although some patients have symptoms dating back to early childhood (7), the hallmark of CVID is primary hypogammaglobulinemia, which is a consequence of late onset antibody failure (LOAF). The majority of adult patients with CVID have IgG levels below 5 g/l (5). Most patients also have reduced or undetectable IgA and/or IgM levels (1, 8). Other laboratory features may include reduced switched memory B cells and/or increased numbers of CD21 low B cells in the periphery (9). A subgroup of patients with severe T-cell defects, originally included within the spectrum of CVID, are now defined separately as late onset combined immune deficiency (LOCID) (10).

In addition to the laboratory features outlined above, many patients have characteristic histological lesions including a sarcoidosis-like granulomatous disorder, nodular lymphoid hyperplasia of the gut, nodular regenerative hyperplasia of the liver, or lymphoid interstitial pneumonitis of the lungs (11–16). Absence of plasma cells in gastrointestinal biopsies is another characteristic finding in the majority of CVID patients (17–19).

DIAGNOSIS OF CVID

It can be very difficult to determine which patients are suffering from CVID and require intravenous immunoglobulin (IVIG) or subcutaneous immunoglobulin (SCIG) replacement. IVIG/SCIG treatment can substantially improve both quality of life (20) and longevity in patients with CVID. This underscores the importance of accurate diagnostic criteria for CVID (21).

Here, we compare the original (1999) European society of immune deficiencies (ESID)/pan American group for immune deficiency (PAGID) criteria, the Ameratunga et al. (2013) criteria and the revised ESID registry (2014) criteria (**Tables 1–3**). We have previously described in detail the evidence base for the Ameratunga et al. (2013) criteria (21). It is necessary to provide an abbreviated version of these criteria here to allow comparison with the revised ESID registry (2014) criteria. We have devoted similar space to each of these criteria.

REVIEW OF THE ESID/PAGID (1999) CRITERIA FOR CVID

According to the ESID/PAGID (1999) criteria, CVID is a diagnosis of exclusion (**Table 1**) (33). Patients were required to have an IgG level below 7–8 g/l [2 SD below the mean, or more accurately, below the 97.5th percentile, as immunoglobulin levels are not normally distributed (34)], as well as impaired vaccine challenge responses or absent isohemagglutinins and other secondary causes of hypogammaglobulinemia were to be excluded. The

Table 1 | The original ESID/PAGID (1999) criteria for probable and possible CVID.

Probable

Probable CVID

Male or female patient who has a marked decrease of IgG (at least 2 SD below the mean for age) and a marked decrease in at least one of the isotypes IgM or IgA, and fulfils all of the following criteria:

- Onset of immunodeficiency at >2 years of age
- Absent isohemagglutinins and/or poor response to vaccines
- Defined causes of hypogammaglobulinemia have been excluded

Possible CVID

Male or female patient who has a marked decrease (at least 2 SD below the mean for age) in at least one of the major isotypes (IgM, IgG, and IgA) and fulfils all of the following criteria:

1. Onset of immunodeficiency at >2 years of age
2. Absent isohemagglutinins and/or poor response to vaccines
3. Defined causes of hypogammaglobulinemia have been excluded

Table 2 | New diagnostic criteria (Ameratunga et al., 2013) for CVID.

A Must meet all major criteria
- Hypogammaglobulinemia IgG < 5 g/l (5)
- No other cause identified for immune defect (22)
- Age > 4 years (23)

B Sequelae directly attributable to immune system failure (ISF) (one or more)
- Recurrent, severe, or unusual infections
- Poor response to antibiotics
- Breakthrough infections in spite of prophylactic antibiotics
- Infections in spite of appropriate vaccination, e.g., HPV disease
- Bronchiectasis and/or chronic sinus disease
- Inflammatory disorders or autoimmunity (24)

C Supportive laboratory evidence (three or more criteria)
- Concomitant reduction or deficiency of IgA (<0.8 g/l) and/or IgM (0.4 g/l) (1, 4)
- Presence of B cells but reduced memory B cell subsets and/or increased CD21 low subsets by flow cytometry (9)
- IgG3 deficiency (<0.2 g/l) (25, 26)
- Impaired vaccine responses compared to age-matched controls (27)
- Transient vaccine responses compared with age-matched controls (28, 29)
- Absent isohemagglutinins (if not blood group AB) (30)
- Serological evidence of significant autoimmunity, e.g., Coombs test
- Sequence variations of genes predisposing to CVID, e.g., *TACI*, *BAFFR*, *MSH5*, etc. (31, 32)

D Presence of relatively specific histological markers of CVID (not required for diagnosis but presence increases diagnostic certainty, in the context of category A and B criteria)
- Lymphoid interstitial pneumonitis (11)
- Granulomatous disorder (12, 13)
- Nodular regenerative hyperplasia of the liver (14, 15)
- Nodular lymphoid hyperplasia of the gut (16)
- Absence of plasma cells on gut biopsy (17, 18)

*Meeting criteria in categories ABC or ABD indicates probable CVID. Patients meeting criteria ABC and ABD should be treated with IVIG/SCIG (**Figure 1**). Patients meeting criteria A alone, AB or AC or AD but not B, are termed possible CVID. Some of these patients may need to be treated with IVIG/SCIG. Patients with levels of IgG > 5 g/l, not meeting any other criteria are termed hypogammaglobulinemia of uncertain significance (HGUS) (21). These diagnostic criteria must be applied sequentially as none are specific individually.*

criteria did not include the characteristic histological features of the disorder or clinical sequelae resulting from the immune system failure (ISF).

Since that time, there have been major advances in the understanding of CVID. The majority of CVID patients have impaired memory B cell function with a reduction in switched memory B cells (9). In the last decade, there have been several genetic discoveries in patients with CVID-like conditions. Rare patients with monogenic defects of *CD19*, *CD20*, *CD21*, *CD81*, and *ICOS* have been identified (35–38). If identified by molecular diagnostic studies (39), these patients are, however, no longer classified as having CVID and are removed from further consideration of the disorder (40, 41). Genetic alterations from genome wide association studies, including copy number variations (42) and sequence variations in genes such as *TACI*, *BAFF* receptor, and *MSH5* may predispose to CVID. Mutations of *TACI*, *BAFF* receptor, and *MSH5* are also found in healthy individuals, but at lower frequency (28, 31, 43).

The ESID/PAGID (1999) criteria require IgG levels to be below 2 SD of the mean (**Table 1**). This means that 2.5% of the general population would meet this criterion (23). There is general agreement with the third ESID/PAGID (1999) criterion that other secondary causes of hypogammaglobulinemia including drug-induced disorders need to be excluded (22, 44, 45).

Perhaps the greatest difficulty with the ESID/PAGID (1999) criteria is the requirement for poor responses to vaccines. The ESID/PAGID (1999) criteria do not specify which vaccines should be used and there are significant variations in vaccine protocols in different studies (46, 47). Therefore, patients with trivial hypogammaglobulinemia with mildly impaired diphtheria antibody responses could be classified as having CVID. Poor responses to the diphtheria vaccine are common, even in normal persons, particularly with increasing age (23).

It is likely many patients with CVID have already generated vaccine-specific memory B cells following childhood immunization prior to LOAF. Therefore, assessing booster responses to childhood vaccines may be diagnostically misleading. This may explain why a significant minority of patients with presumed CVID have protective responses to tetanus toxoid and Pneumovax® (48, 49). It is also debatable if the response to highly immunogenic proteins such as tetanus toxoid, administered with adjuvant, is a valid and reliable predictor of a protective response to pathogens *in vivo* (21). Specific concerns about using vaccines to assess the immune response are shown in **Table 4**.

The use of neoantigens such as rabies vaccine, typhoid vaccine, and experimental vaccines such as ψX174 to assess LOAF may be more predictive of an immune defect as patients are unlikely to

Table 3 | Revised ESID (2014) diagnostic criteria for CVID.

At least one of the following:

- Increased susceptibility to infection
- Autoimmune manifestations
- Granulomatous disease
- Unexplained polyclonal lymphoproliferation
- Affected family member with antibody deficiency

AND marked decrease of IgG and marked decrease of IgA with or without low IgM levels (measured at least twice; <2 SD of the normal levels for their age);

AND at least one of the following:

- Poor antibody response to vaccines (and/or absent isohemagglutinins); i.e., absence of protective levels despite vaccination where defined
- Low switched memory B cells (<70% of age-related normal value)

AND secondary causes of hypogammaglobulinemia have been excluded (see separate list)

AND diagnosis is established after the fourth year of life (but symptoms may be present before)

AND no evidence of profound T-cell deficiency, defined as two out of the following (y = year of life):

- CD4 numbers/microliter: 2–6 y < 300, 6–12 y < 250, >12 y < 200
- % Naive CD4: 2–6 y < 25%, 6–16 y < 20%, >16 y < 10%
- T-cell proliferation absent

http://esid.org/Working-Parties/Registry/Diagnosis-criteria.

have previously encountered these antigens (54). However, there have been concerns about risks associated with the rabies vaccine (54) and the typhoid vaccine is not yet widely used. The ψx174 vaccine has not been registered by the FDA and cannot be used in routine clinical practice (67).

The difficulty with diagnosis is illustrated in a recent study in which a new category of idiopathic primary hypogammaglobulinemia was proposed for symptomatic patients who did not meet the ESID/PAGID (1999) criteria for CVID (68). In spite of not meeting the ESID/PAGID (1999) criteria, many of these patients were treated with immunoglobulin. There is thus a discord between diagnosis and treatment in many patients with hypogammaglobulinemia/CVID.

The ESID/PAGID (1999) criteria do not specify the need for symptoms to establish the diagnosis. Therefore, important clinical manifestations and complications may not be obvious from different parts of the world, when using these criteria. This was illustrated in recent CVID studies, where there were wide variations in complications leading to different clinical phenotypes as well as bronchiectasis in countries within the European Union as well as the United States (4, 7, 69).

It is accepted that these criteria are relatively simple and could be used to diagnose patients in developing countries. The major difficulty is the interpretation of vaccine responses (**Table 4**), which is a pivotal component of these criteria. We are also concerned that the application of relatively simple criteria to a complex set of disorders will result in inaccuracies, as evidenced by the need for a new category of disorders, idiopathic primary hypogammaglobulinemia, described above. We are thus left with criteria that can be difficult to measure and interpret. Several eminent authors have expressed concern about the need for revised diagnostic criteria for CVID (70, 71).

NEW DIAGNOSTIC CRITERIA FOR CVID

We recently proposed new diagnostic criteria for CVID (**Table 2**) (21). Treatment recommendations are closely linked to these diagnostic criteria (**Figure 1**). The Ameratunga et al. (2013) criteria emphasize both the clinical and laboratory sequelae of ISF in these patients (72).

In order to qualify as having probable CVID, patients must meet criteria in categories A, B, and C or D (**Table 2**). It is important for these criteria to be applied sequentially, as each subsequent category confers increasing specificity (**Figure 1**). A threshold of 5 g/l for IgG has been set for adults as this is the cut-off used in the French DEFI study (5). Patients must be older than 4 years of age and must not have a secondary cause for their hypogammaglobulinemia. If patients do not meet category A criteria, other criteria do not apply and a diagnosis of CVID cannot be entertained (**Figure 1**).

The most important feature of these criteria is clinical evidence of ISF (category B). Patients must have predisposition to infections and/or autoimmune disease, as a direct result of their immune defect. We have not been prescriptive about the numbers of upper or lower respiratory tract infections, as these will be influenced by the patient's socio-demographic circumstances. While there may be debate about the specificity of each criterion in category B, the intention is to distinguish symptomatic patients from those who are well. These symptomatic patients must have laboratory evidence of immune dysfunction (category C) or the characteristic histological findings (category D) associated with CVID (**Table 2**).

None of the category C criteria are specific but in combination support the diagnosis of CVID. The majority of CVID patients will have a reduction of IgA and/or IgM (1, 4). Most will have impaired vaccine responses (49). It is, however, important to compare antibody responses to those of normal controls, as many CVID patients who have received their primary immunization series are likely to have developed memory B cells and are able to generate protective antibody levels. Using protective antibody levels as eligibility criteria for IVIG/SCIG is unacceptable in symptomatic patients as it will exclude a significant proportion of patients who will benefit from IVIG/SCIG treatment (48, 49).

Vaccine responses in normal controls have been published (50, 52, 53). Patients receiving a single booster dose of tetanus toxoid should achieve an antibody response of at least 1 IU/ml (50). Although a response of 1 IU/ml has been suggested following diphtheria immunization, our experience and original immunization studies show that this vaccine is much less immunogenic than the tetanus vaccine (50). Even otherwise healthy individuals may fail to reach this level, particularly persons over 50 years of age. The inclusion of diphtheria vaccine responses is thus problematic.

Adults and children receiving the *Haemophilus influenzae* type B (HIB) vaccine should reach antibody levels of at least 1.0 μg/ml rather than the protective level of 0.15 μg/ml (52, 53). Adults receiving the Pneumovax® should achieve a protective level of

Table 4 | Difficulties interpreting vaccine responses in CVID.

Tetanus toxoid	Excellent immunogen (48)
	Presence of memory B cells from childhood tetanus vaccination can make responses difficult to interpret in CVID patients (21)
	Results should be compared to normal individuals (50)
	Uncertain validity of using simple antigens with adjuvant to gauge response to pathogens *in vivo* (28)
Diphtheria toxoid	Poor immunogen (23)
	Response should be compared with normal individuals (50)
	Questionable validity of using simple antigens to gauge response to pathogens *in vivo* (28)
H. influenzae type B (HIB)	There may be major differences between those who have not been immunized vs. those who have had this as part of their routine vaccines (51)
	Protective levels may not be logical: need to compare response with normal persons (52, 53)
Pneumovax® (PPV)	Poor response in infants <2 years (54)
	Differences in responses between middle aged and elderly adults (55)
	Risk of unresponsiveness with repeated doses of PPV (56)
	Difficulties in measuring antibody responses
	Assays not standardized (8)
	Cross-reactive carbohydrates can interfere with the assay (57, 58)
	Some serotypes (serotype 3) are more immunogenic than others (6B and 23F) (54)
	No external quality assurance program for the assay
	Different platforms for pneumococcal antibody measurement may not be comparable (59)
	Disagreement about protective antibody levels (60–62)
	Mucosal protection may require higher antibody levels cf sepsis (63)
	Diagnostic criteria have not been defined: at least five different criteria in the literature (64, 65)
	Vaccine quality may vary: stability of conjugated vaccines. Lot to lot variation (54)
	Up to 18% of CVID patients respond to PPV (49)
	Use of Prevnar13® as part of routine vaccines will make is difficult to measure responses to carbohydrates
Other vaccines	Not widely used, e.g., typhoid vaccine (54)
	Many CVID patients may respond, e.g., meningococcal vaccine (66)
	Experimental vaccines not approved by FDA ψ174 (67)
	Risk of adverse reactions: e.g. rabies vaccines (54)

PPV, pneumococcal polysaccharide vaccine. PPV is administered to test response to T-cell independent carbohydrate antigens, while conjugated vaccines such as HIB and toxoids test responses to T-cell dependent antigens.

1.3 μg/ml for at least 70% of serotypes while children should reach 50% (64, 65). Adequate but transient vaccine responses are also included in category C criteria (21, 29). This may reflect *in vivo* failure of B cell memory in some patients.

We have included absent isohemagglutinins in these criteria providing the patient is not blood group AB (30). Although absent isohemagglutinins are part of the PAGID/ESID (1999) criteria, in our experience it is rare for a diagnosis of CVID to be made or for IVIG/SCIG to be prescribed, purely on the basis of absent isohemagglutinins in a patient with reduced IgG levels.

Most patients with CVID have impaired memory B cells and these constitute a diagnostic criterion in category C (9). It is, however, important for memory B cell subsets to be measured on at least two occasions, as we have shown the numbers can vary on repeat testing (73). Genetic predisposition (mutations of *TACI*, *BAFF* receptor, *MSH5*, etc.) to CVID is included in category C

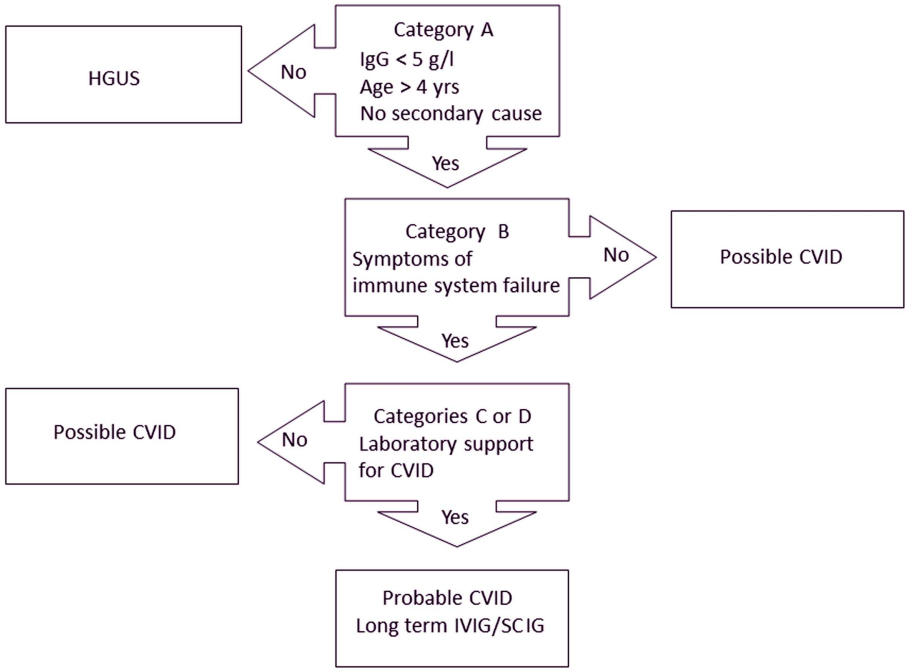


FIGURE 1 | Treatment algorithm for CVID (21). Patients must meet all major criteria in category A for consideration of CVID. Category B confirms the presence of symptoms indicating immune system failure (ISF). To have probable CVID, patients must also have supportive laboratory evidence of immune system dysfunction (category C) or characteristic histological lesions of CVID (category D). Patients with mild hypogammaglobulinemia (IgG > 5 g/l) are termed hypogammaglobulinemia of uncertain significance (HGUS). Patients meeting category A criteria but not other criteria are deemed to have possible CVID. Most patients with probable CVID are likely to require IVIG/SCIG. Some patients with possible CVID who have profound hypogammaglobulinemia will require IVIG/SCIG but most patients with HGUS are unlikely to need IVIG/SCIG replacement.

diagnostic criteria (42). This criterion is primarily intended for patients who may have been enrolled in clinical research studies. In the interim, we strongly discourage routine sequencing of these genes for diagnostic purposes until their utility is established.

Reduction or deficiency of IgG3 has been included in the new diagnostic criteria. There is considerable peer-reviewed literature suggesting IgG3 deficiency should be considered an important biomarker for a humoral immune defect (25, 26). We have included serological manifestations of significant autoimmunity in category C. This would include positive tests for lupus anticoagulant, Coombs test, etc. In most large series of CVID patients there are approximately 15% of patients who have severe autoimmunity and some have minimal infections in spite of their profound hypogammaglobulinemia. These patients will qualify as having CVID according to the Ameratunga et al. (2013) criteria, providing they have not been treated with rituximab.

Finally, some CVID patients have characteristic histological findings, which have been included in the Ameratunga et al. (2013) criteria (category D). These require biopsy for confirmation. In the context of primary (category A), symptomatic (category B) hypogammaglobulinemia, the presence of these relatively specific histological markers obviates the need to undertake vaccine responses, measure memory B cells, etc. Most of the histological criteria described in category D can occur with other disorders. Because category D will only apply if patients have already met category A and B criteria (**Figure 1**), this will confer specificity for CVID. Category C and D criteria may serve as an useful check list when assessing patients with hypogammaglobulinemia (21). We have shown that careful review of category C and D criteria may help to distinguish CVID from secondary causes of hypogammaglobulinemia (46, 74, 75).

As in the past, a diagnostic rectal biopsy could be undertaken to confirm the absence of plasma cells (19). Although a minority of CVID patients have plasma cells, the absence of these cells in a gut biopsy is a characteristic feature of CVID (21). Incorporating histological features in the diagnostic criteria may be useful where patients have already commenced IVIG/SCIG as there are risks in stopping treatment to assess vaccine responses.

We have designated a category of possible CVID for those patients meeting category A criteria (or AC or AD) but not category B criteria (**Figure 1**). Some asymptomatic patients with profound hypogammaglobulinemia may need to be treated with IVIG/SCIG as they may be at risk of severe viral infections or bacterial sepsis (28).

There are other patients who have mild hypogammaglobulinemia (IgG > 5 g/l) who are otherwise well or have only mild symptoms. We have termed these patients hypogammaglobulinemia of uncertain significance (HGUS) (21). In the future, it may be useful to sub classify HGUS patients depending on whether (sHGUS) or not (aHGUS) they have symptoms attributable to ISF. Prospective studies will indicate if the prognosis for these two sub groups is different.

The Ameratunga et al. (2013) criteria are intended primarily for clinical use and we therefore felt it was important to link them closely to treatment (**Figure 1**) (21). As with the Jones criteria for acute rheumatic fever, CVID no longer needs to be a diagnosis of exclusion.

REVISED ESID REGISTRY (2014) CRITERIA FOR CVID

The revised ESID registry (2014) criteria for probable CVID have been recently released (http://esid.org/Working-Parties/Registry/Diagnosis-criteria) and are shown in **Table 3**. These are structured in a similar way to the Ameratunga et al. (2013) criteria but have not been given named categories. In contrast to the previous ESID/PAGID (1999) criteria, patients are required to have symptoms of their immune deficiency or a family history of antibody deficiency to be eligible for a diagnosis of CVID. Increased susceptibility to infection along with autoimmunity, unexplained polyclonal lymphoproliferation, or granulomatous disease qualifies patients for further consideration of CVID. This is similar to the Ameratunga et al. (2013) category B criteria, where a diagnosis of CVID cannot be made in the absence of symptoms (21).

Symptomatic patients are required to have a marked decrease of IgG as well as IgA and/or IgM. Again immunoglobulin levels 2 SD below mean is required for the relevant population. Immunoglobulin levels need to be repeated to confirm persistent reduction. This would exclude transient reductions in immunoglobulins that can sometimes be seen following viral infections or use of medications (44). Although a large number of patients with mild hypogammaglobulinemia will qualify for further investigation, the subsequent criteria will increase the specificity of the diagnosis.

Patients with hypogammaglobulinemia must then have either impaired antibody responses to vaccines and/or absent isohemagglutinins or reduced numbers of switched memory B cells for further consideration of CVID. Unlike the previous ESID/PAGID (1999) criteria, protective antibody levels are deemed to be required.

Like the Ameratunga et al. (2013) criteria, the revised ESID registry (2014) criteria do not specify which vaccines should be used. It is likely vaccine protocols will evolve with the availability and experience with new vaccines. As stated in **Table 4**, the increasing use of Prevnar13® in routine childhood vaccine schedules will make assessing pneumococcal polysaccharide responses increasingly problematic. In contrast to the revised ESID registry (2014) criteria, the Ameratunga et al. (2013) criteria require vaccine responses to be compared to the normal population, as a significant proportion of presumed CVID patients have protective antibody responses to tetanus and Pneumovax® (48, 49).

In contrast to the previous ESID/PAGID (1999) criteria, vaccine responses do not play such a pivotal role in the revised ESID registry (2014) criteria for CVID. Like the Ameratunga et al. (2013) criteria, it is therefore possible for patients to qualify as having CVID with normal vaccine challenge responses if they have either absent isohemagglutinins or reduced switched memory B cell numbers (**Table 3**). The requirement for reduced memory B cells in the revised ESID registry (2014) criteria is not mandatory and patients with absent B cells may still qualify for the diagnosis if they have impaired vaccine responses and/or absent isohemagglutinins.

As with all criteria described here, secondary causes of hypogammaglobulinemia must be excluded. The previous ESID/PAGID (1999) criteria specified 2 years as the eligible age for diagnosis, while the Ameratunga et al. (2013) and revised ESID registry criteria specify 4 years. The older age for diagnosis will help exclude monogenic defects as well as many cases of transient hypogammaglobulinemia of infancy. Unlike the ESID/PAGID (1999) and Ameratunga et al. (2013) criteria, the revised ESID registry (2014) criteria excludes severe T-cell defects from the spectrum of CVID, since these patients are deemed to have a combined immune deficiency. It is likely the genetic defect will differ from those with a largely humoral deficiency.

Granulomatous disease and lymphoproliferation are included in the initial set of clinical criteria in the revised ESID registry (2014) criteria (**Table 3**). This will require histological confirmation. Although not explicitly stated, our interpretation is that lymphoproliferation will include nodular lymphoid hyperplasia of the gut, nodular regenerative hyperplasia of the liver and lymphoid interstitial pneumonitis. These characteristic histological features and are included in category D of the Ameratunga et al. (2013) criteria. We assume an increase in CD21 low B cells may be included in the lymphoproliferation criterion of the revised ESID (2014) criteria. Like the Ameratunga et al. (2013) criteria, secondary causes for the histological lesions will be excluded by subsequent criteria. Nodular regenerative hyperplasia of the liver for example can occur with azathioprine treatment.

Like the Ameratunga et al. (2013) criteria, the revised ESID registry (2014) criteria may allow the diagnosis of CVID in patients who have already commenced IVIG/SCIG treatment. The presence of lymphoproliferation or reduced switched memory B cells will allow the diagnosis as long as other criteria are satisfied. This may obviate the need to stop IVIG/SCIG treatment to assess vaccine responses. Neither the Ameratunga et al. (2013) criteria nor the revised ESID (2014) registry criteria attempt to address the complex situation of hypogammaglobulinemia/CVID associated with malignancy. It can be very difficult to determine if hypogammaglobulinemia/CVID is the cause or the effect of malignancy.

Unlike the Ameratunga et al. (2013) criteria, the absence of plasma cells is not included in the revised ESID registry (2014) criteria. As indicated above, this is a useful feature in patients who have already commenced IVIG/SCIG replacement and may be identified by a diagnostic rectal biopsy. This is perhaps the most useful histological marker of CVID. The Ameratunga et al. (2013) criteria may be less useful if there are no characteristic histological lesions in patients who have already commenced IVIG/SCIG treatment. The emphasis on reduced switched memory B cells in the revised ESID registry (2014) criteria may allow the diagnosis in patients who have already commenced IVIG/SCIG. The revised ESID registry (2014) criteria require switched memory B cells to be below 70% of the normal population. Reference intervals will therefore have to be established for each laboratory. Given the variability in these cells, the Ameratunga et al. (2013) criteria require the assay to be repeated (73).

In comparison with the Ameratunga et al. (2013) criteria, transient vaccine responses are not included in the ESID registry (2014) criteria or breakthrough infections in spite of prophylactic

antibiotics. Failure of vaccines to prevent infections, e.g., human papillomavirus is also not included in the revised criteria. The latter two may, however, be covered in the initial criterion of increased susceptibility to infection in the revised ESID registry (2013) criteria. Sequence variations in genes (*TACI*, *BAFF* receptor, *MSH5*, etc.) predisposing to CVID and IgG3 deficiency are not included in the revised ESID registry criteria (2014). Prospective studies will determine the value of these criteria (21).

The previous ESID/PAGID (1999) criteria had a category of possible CVID for patients not meeting the complete criteria for CVID (**Table 1**). The revised ESID registry (2014) criteria have a category of unclassified hypogammaglobulinemia for patients who do not meet all the criteria for CVID. Studies of cohorts of hypogammaglobulinemia patients will indicate if these categories are equivalent to patients with HGUS in the Ameratunga et al. (2013) criteria.

Like the previous ESID/PAGID (1999) criteria, diagnosis has not been linked to eligibility to IVIG/SCIG treatment in the revised ESID registry (2014) criteria, as these are intended for clinical research rather than therapy. However, it is inevitable these criteria will be used by clinicians to determine which patients will qualify for immunoglobulin treatment in the absence of specific national guidelines. Cohorts such as the NZ CVID/hypogammaglobulinemia study will be important in determining if the revised ESID registry (2014) criteria can be used to determine eligibility for treatment. It will be important to undertake prospective head to head comparisons of these criteria in hypogammaglobulinemic patients as we have done for memory B cells in CVID (73).

The latter two (Ameratunga et al. 2013 and the revised ESID registry 2014) criteria are based on the framework established by the ESID/PAGID 1999 diagnostic criteria. Most patients diagnosed with CVID will have substantially reduced IgG, reduction in other isotypes, impaired memory B cells, and impaired vaccine responses. The majority will thus qualify as having CVID by all three criteria. It should be noted that none of these criteria address partial antibody deficiency syndromes. This is an area that will need to be addressed in future studies.

It will be important to determine how these criteria perform in real-life clinical situations in ethnically diverse populations across the globe. We expect most if not all patients deemed to have idiopathic primary hypogammaglobulinemia, will qualify as having CVID, which will secure their eligibility for treatment with IVIG/SCIG (45, 68). Equally, it is hoped fewer asymptomatic patients with mild hypogammaglobulinemia will be treated with IVIG/SCIG.

Regardless of which criteria are used, it is essential that sound clinical judgment is exercised when diagnosing and treating these patients. It will be important to offer IVIG/SCIG for patients with bronchiectasis with higher levels of IgG for example (sHGUS) (76). Similarly, when assessing the cause of recurrent infections, there may be other contributing factors, which could be potentially treated in patients with hypogammaglobulinemia. The immune defect may not be the dominant predisposing factor for infections in some such individuals. Correcting functional or anatomical predisposing factors such as chronic sinus disease may reduce the number of infections and the patient may not need to be treated with IVIG/SCIG in spite of the hypogammaglobulinemia. This is why it is critical for these patients to be under the care of experienced clinical immunologists.

AUTHOR CONTRIBUTIONS

Rohan Ameratunga conceived the idea of the new diagnostic and treatment criteria for CVID. Maia Brewerton, Charlotte Slade, Anthony Jordan, David Gillis, Richard Steele, Wikke Koopmans, and See-Tarn Woon contributed references and made editorial changes necessary to support the manuscript.

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Appendix 2. Immunology gene lists

Combined Immunodeficiency

ADA, AK2, ATM, B2M, BCL10, BLM, CARD11, CCBE1, CD27, CD3G, CD40LG, CD8A, CHD7, CIITA, CTPS1, DCLRE1B, DCLRE1C, DKC1, DNMT3B, DOCK2, DOCK8, EPG5, FOXN1, ICOS, IKBKB, IKBKG, IKZF1, IL21, IL21R, IL2RG, IL7R, ITK, LCK, LIG4, LRBA, MAGT1, MALT1, MAP3K14, MCM4, MTHFD1, NFKB1, NFKB2, NFKBIA, NFKBID, NHP2, NOP10, ORAI1, PARN, PGM3, PNP, POLE, RAC2, RAG1, RAG2, RBCK1, RFX5, RFXANK, RFXAP, RHOH, RMRP, RNF168, RNF31, RTEL1, SEMA3E, SLC46A1, SMARCAL1, SP110, SPINK5, STAT3, STAT5B, STIM1, STK4, TAP1, TAP2, TAPBP, TBX1, TCN2, TERC, TERT, TINF2, TNFRSF4, TPP1, TTC7A, WAS, WIPF1, ZAP70, ZBTB24

Common Variable Immunodeficiency

CD19, CD27, CD81, CR2, CTLA4, CXCR4, ICOS, ICOSLG, IKZF1, MAP3K14, MS4A1, MSH6, NFKB1, NFKB2, NFKBID, PIK3CD, PRKCD, TCF3, TNFRSF13B, TNFRSF13C, TNFSF12, TRNT1, TTC37

Complement Ceficiencies

C1QA, C1QB, C1QC, C1R, C1S, C2, C3, C4A, C4B, C5, C6, C7, C8A, C8B, C8G, C9, CD46, CD59, CFB, CFD, CFH, CFHR1, CFHR2, CFHR3, CFHR4, CFHR5, CFI, CFP, FCN3, ITGAM, MASP2, SERPING1, THBD

Defects of Innate Immunity

APOL1, CARD9, CXCR4, CYBB, FCGR3A, IFNGR1, IFNGR2, IL12B, IL12RB1, IL17F, IL17RA, IL17RC, IRAK4, IRF7, IRF8, ISG15, MYD88, RORC, RPSA, STAT1, STAT2, TBK1, TICAM1, TLR3, TMC6, TMC8, TRAF3, TRAF3IP2, TYK2, UNC93B1

Disorders of Immune Dysregulation

ACP5, ADAR, AIRE, AP3B1, BLOC1S6, CASP10, CASP8, CECR1, CTLA4, FADD, FAS, FASLG, FOXP3, IFIH1, IL10, IL10RA, IL10RB, IL2RA, ITCH, LYST, NFAT5, PRF1, PRKCD, RAB27A, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, SH2D1A, STAT3, STX11, STXBP2, TMEM173, TPP2, TREX1, UNC13D, XIAP

Ectodermal Dysplasia

CDH3, CHD1, CTNND1, EDA, EDAR, EDARADD, GJB6, IFT122, IFT43, IKBKG, KRT85, MSX1, NFKBIA, PKP1, PVRL1, PVRL4, TP63, WDR35, WNT10A

Epidermolysis Bullosa

COL17A1, COL7A1, DSP, DST, EXPH5, FERMT1, ITGA3, ITGA6, ITGB4, JUP, KLHL24, KRT14, KRT5, LAMA3, LAMB3, LAMC2, PKP1, PLEC, TGM5

Hereditary Angioedema

F12, SERPING1

Hyper-IgE Syndrome

DOCK8, ERCC2, ERCC3, GTF2H5, NFKBID, PGM3, SPINK5, STAT3, TYK2, WAS, WIPF1

Immunological Disorders

ACP5, ACTB, ADA, ADAM17, ADAR, AICDA, AIRE, AK2, AP3B1, APOL1, ATM, B2M, BCL10, BLM, BLNK, BLOC1S6, BTK, C1QA, C1QB, C1QC, C1R, C1S, C2, C3, C4A, C4B, C5, C6, C7, C8A, C8B, C8G, C9, CARD11, CARD14, CARD9, CASP10, CASP8, CCBE1, CD19, CD247, CD27, CD3D, CD3E, CD3G, CD40LG, CD46, CD59, CD79A, CD79B, CD81, CD8A, CEBPE, CECR1, CFB, CFD, CFH, CFHR1, CFHR2, CFHR3, CFHR4, CFHR5, CFI, CFP, CHD7, CIITA, CLPB, COPA, CORO1A, CR2, CSF2RA, CSF3R, CTLA4, CTPS1, CTSC, CXCR4, CYBA, CYBB, DCLRE1B, DCLRE1C, DKC1, DNMT3B, DOCK2, DOCK8, ELANE, EPG5, ERCC2, ERCC3, F12, FADD, FAS, FASLG, FAT4, FBF1, FCGR3A, FCN3, FERMT3, FOXN1, FOXP3, FPR1, G6PC3, GATA2, GFI1, GTF2H5, HAX1, ICOS, ICOSLG, IFIH1, IFNGR1, IFNGR2, IGLL1, IKBKB, IKBKG, IKZF1, IL10, IL10RA, IL10RB, IL12B, IL12RB1, IL17F, IL17RA, IL17RC, IL1RN, IL21, IL21R, IL2RA, IL2RG, IL36RN, IL7R, INO80, IRAK4, IRF7, IRF8, ISG15, ITCH, ITGAM, ITGB2, ITK, JAGN1, JAK3, LAMTOR2, LCK, LIG4, LPIN2, LRBA, LYST, MAGT1, MALT1, MAP3K14, MASP2, MCM4, MEFV, MOGS, MPI, MS4A1, MSH2, MSH6, MTHFD1, MVK, MYD88, NCF1, NCF2, NCF4, NFAT5, NFKB1, NFKB2, NFKBIA, NFKBID, NHEJ1, NHP2, NLRC4, NLRP12, NLRP3, NOD2, NOP10, ORAI1, PARN, PGM3, PIK3CD, PIK3R1, PLCG2, PNP, POLE, PRF1, PRKCD, PRKDC, PSMB8, PSTPIP1, PTPRC, RAB27A, RAC2, RAG1, RAG2, RBCK1, RFX5, RFXANK, RFXAP, RHOH, RMRP, RNASEH2A, RNASEH2B, RNASEH2C, RNF168, RNF31, RORC, RPSA, RTEL1, SAMHD1, SBDS, SEMA3E, SERPING1, SH2D1A, SH3BP2, SLC29A3, SLC35C1, SLC37A4, SLC46A1, SMARCAL1, SP110, SPINK5, STAT1, STAT2, STAT3, STAT5B, STIM1, STK4, STX11, STXBP2, TAP1, TAP2, TAPBP, TAZ, TBK1, TBX1, TCF3, TCN2, TERC, TERT, THBD, TICAM1, TINF2, TLR3, TMC6, TMC8, TMEM173, TNFRSF13B, TNFRSF13C, TNFRSF1A, TNFRSF4, TNFSF12, TPP1, TPP2, TRAF3, TRAF3IP2, TREX1, TRNT1, TTC37, TTC7A, TYK2, UNC13D, UNC93B1, UNG, USB1, VPS13B, VPS45, WAS, WIPF1, XIAP, ZAP70, ZBTB24

Neutrophil Defects

ACTB, CEBPE, CLPB, CSF2RA, CSF3R, CTSC, CYBA, CYBB, ELANE, FERMT3, FPR1, G6PC3, GATA2, GFI1, HAX1, ITGB2, JAGN1, LAMTOR2, NCF1, NCF2, NCF4, RAC2, SBDS, SLC35C1, SLC37A4, TAZ, USB1, VPS13B, VPS45

Phagocyte Defects

ACTB, CEBPE, CLPB, CSF3R, CTSC, CXCR4, CYBA, CYBB, ELANE, FERMT3, FPR1, G6PC3, GATA2, GFI1, HAX1, ITGB2, JAGN1, LAMTOR2, NCF1, NCF2, NCF4, RAC2, SBDS, SLC35C1, SLC37A4, TAZ, USB1, VPS13B, VPS45, WAS, WIPF1

Predominantly Antibody Deficiency

AICDA, BLNK, BTK, CARD11, CD19, CD79A, CD79B, CD81, CR2, CXCR4, DNMT3B, IGLL1, INO80, MOGS, MS4A1, MSH2, MSH6, NFKB1, NFKB2, NFKBID, PIK3CD, PIK3R1, SH2D1A, SP110, TCF3, TNFRSF13B, TNFRSF13C, TNFSF12, TRNT1, TTC37, UNG, WAS, WIPF1, XIAP

Severe Combined Immunodeficiency (Absent T, Absent B Cells)

ADA, AK2, DCLRE1C, LIG4, NHEJ1, PRKDC, RAG1, RAG2

Severe Combined Immunodeficiency (Absent T, Present B Cells)

CD247, CD3D, CD3E, CORO1A, IL2RG, IL7R, JAK3, PTPRC

Susceptibility to Fungal Infections

CARD9, IL17F, IL17RA, IL17RC, RORC, STAT1, TRAF3IP2

Susceptibility to Viral Infections

CXCR4, FCGR3A, IRF7, STAT1, STAT2, TBK1, TICAM1, TLR3, TMC6, TMC8, TRAF3, UNC93B1

Systemic Autoinflammatory Disease

ADAM17, CARD14, COPA, IL1RN, IL36RN, LPIN2, MEFV, MVK, NLRC4, NLRP12, NLRP3, NOD2, PLCG2, PSMB8, PSTPIP1, SH3BP2, SLC29A3, TNFRSF1A

Appendix 3. Scoring matrix for variant classification

Table 1: Classification of PTV and Null variants

Evidence (NOTE: Each evidence block as signified by continuous color can only have ONE yes checked)	Cat.	Yes	No	Not available	Not applicable
1.1.1 Protein truncating (PTV) or Null Variant in a gene where LOF is known mechanism of pathogenicity (e.g. through functional assay or if truncating variants have been shown to segregate in multiple families) - E.g. frameshift, initiator codon disruption, premature stop codon (nonsense), canonical splice site mutation - IF Canonical splice site (intronic 1-2bp upstream/downstream of exon-intron boundary), functional data required on protein or RNA level - IF initiator codon (ATG) mutation, no known alternative transcription start sites allowing functional protein production AND next available initiator codon (if any) results in frameshift - Affects critical functional domains - Novel Stop codon is not in the last exon or the last 50bp of the second to last exon - Region harboring the variant is not known to be subject to alternative splicing/missing in alternative RefSeq transcripts	PVS				
1.1.2 Affects canonical splice site (within 2bp of exon-intron boundary) WITHOUT functional data supporting splice effect on protein or RNA level	PS				
1.1.3 PTV/Null Variant where alternative splicing of the exon occurs BUT there is strong evidence for the exon to be PRESENT in the transcript relevant for the disease of interest (e.g. cardiac specific transcrpt in cardiomyopathy) AND where LOF is known mechanism of pathogenicity	PS				
1.1.4 PTV/Null Variant where alternative splicing of the exon occurs AND there is strong evidence for the exon to be ABSENT in the transcript relevant for the disease of interest (e.g. cardiac specific transcrpt in cardiomyopathy)	BS				
1.1.5 PTV/Null Variant where alternative splicing is proposed to occur BUT supporting evidence for alternative splicing of the exon is not well established AND in a gene where LOF is known mechanism of pathogenicity	PM				
1.1.6 Novel Protein truncating (PTV) or Null Variant in the last exon or the last 50bp of the second to last exon in a gene with less than 4 exons AND where LOF is known mechanism of pathogenicity	PM				
1.2.1 MAF >=0.05 in at least one sufficiently large subpopulation	BA				
1.2.2 MAF >=0.03 and <0.05 in at least one sufficiently large subpopulation OR MAF is out of keeping with known disease frequency. CAUTION: if similar truncating mutations are common in the same gene (~>0.005, e.g. TITAN, APC), to be used as supporting evidence (BP) only.	BS				
1.2.3 IF dominant disorder MAF >0.01 in at least one sufficiently large subpopulation	BS				
1.3.1 In-silico predicted effect on splicing by at least two tools (NNSplice, HSF3) in gene where splice defects are known pathogenic mechanism and nucleotide position is conserved (NOT FOR CANONICAL splice site intronic 1-2bp upstream/downstream of exon-intron boundary)	PP				
1.3.2 In-silico predicted effect on splicing by at least two tools (NNSplice, HSF3) in gene where LOF is known mechanism of pathogenicity and alternative splicing would cause a frame-shift mutation (NOT FOR CANONICAL splice site intronic 1-2bp upstream/downstream of exon-intron boundary)	PM				
1.3.3 In-silico predicted effect on splicing by at least two tools (NNSplice, HSF3) where affected exon is missing in alternative transcripts (NOT FOR CANONICAL splice site intronic 1-2bp upstream/downstream of exon-intron boundary)	BP				
1.4.1 Variant affects at least one well established (essential) functional domain	PM				
1.5.1 In-frame insertion or deletion in repetitive region without known function or not conserved	BP				
1.5.2 In-frame insertion or deletion in non-repetitive region that is conserved in at least mammals and birds	PM				
1.6.1 Same variant type in affected codon/region has previously been shown to be pathogenic (IF in-frame Insertion/Deletion mutation, affecting same region; IF initiator codon mutation, different nucleotide change has been shown to be pathogenic; IF Premature stop with NMD: haploinsufficiency or complete loss of protein established pathogenic mechanism (beware of exceptions such as collagen disorders); IF Premature stop w/o NMD: premature stop downstream of variant is known pathogenic)	PS				
1.7.1 Heterozygous variant detected in *trans* with a second, known pathogenic, heterozygous variant in a recessive disease (Variant phasing may need to be confirmed by testing of parents)	PM				
1.7.2 Heterozygous variant observed in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in *cis* with a pathogenic variant in any inheritance pattern (Variant phasing may need to be confirmed by testing of parents)	BP				
1.8.1 Functional consequence of variant is not consistent with expected spectrum of pathogenic variants in the affected gene	BP				
1.9.1 Existing classification of pathogenicity (Variant has previously been classified by experts) NOTE: Can be seen as PVS if expert panel is known to have considered extensive additional information that is not available to the lab (i.e. InSight database, some BRCA gene classifications)	PS				
1.9.2 Existing classification of neutrality (Variant has previously been classified by experts) NOTE: Can be seen as BA if expert panel is known to have considered extensive additional information that is not available to the lab (i.e. InSight database, some BRCA gene classifications)	BS				
1.9.3 Previous description of pathogenicity (Variant has previously been described as pathogenic in databases or literature in independent patients)	PP				
1.9.4 Previous description of neutrality (Variant has previously been described as benign in databases or literature in independent patients)	BP				
1.10.1 Strong functional evidence supporting ABNORMAL protein function of the variant (eg. endogenous tissue)	PS				
1.10.2 Moderate functional evidence supporting ABNORMAL protein function of the variant (eg. exogenous cell line)	PM				
1.10.3 Strong functional evidence supporting NORMAL protein function of the variant (eg. endogenous tissue)	BS				
1.10.4 Moderate functional evidence supporting NORMAL protein function of the variant (eg. exogenous cell line)	BP				
1.11.1 Significant Segregation: Variant segregates with the same phenotype as the patient in 3 or more independent families or 1 large family to at least a third degree relative (LOD >= 3)	PS				
1.11.2 Moderate Segregation: Variant segregates with the same phenotype as the patient in 2 independent families only (LOD >=1.5 and <3)	PM				
1.11.3 Low Segregation: Variant segregates with the same phenotype as the patient in 1 family only OR homozygous variant in a recessive condition where both parents are confirmed to be carriers	PP				
1.11.4 Non-segregation has been clearly demonstrated	BS				
1.12.1 De Novo Variant (Parental status confirmed for both mother and father)	PS				
1.12.2 De Novo Variant (Parental status not tested but assumed)	PM				
1.13.1 Variant in a gene that is known to be causative for a well defined syndrome with unambigous clinical presentation. Gene has to have low tolerance for variation (ExAC Constraint LOF pLi >=0.9) and inheritance pattern of the variant has to be consistent with suspected disease.	PP				

Table 2: Classification of Missense Variants

Evidence (NOTE: Each evidence block as signified by continuous color can only have ONE yes checked)	Cat.	Yes	No	Not available	Not applicable
2.1.1 MAF for dominant indication <0.0002 or absent in databases with sufficient coverage	PS				
2.1.2 MAF for dominant indication >=0.0002 and <0.001	PM				
2.1.3 MAF for recessive indication <0.01 (use caution with minority ethnicities)	PM				
2.1.4 MAF for recessive indication <0.03 and >=0.01 (use caution with minority ethnicities)	PP				
2.1.5 MAF >=0.05 in at least one sufficiently large subpopulation	BA				
2.1.6 MAF >=0.03 and <0.05 in at least one sufficiently large subpopulation OR MAF is out of keeping with known disease frequency	BS				
2.1.7 IF dominant disorder MAF >=0.01 in at least one sufficiently large subpopulation	BS				
2.1.8 MAF is out of keeping with known disease frequency OR Homozygous mutations are present in ExAc at a higher frequency than expected for the disease (e.g. homozygous variant present in ExAc when investigating lethal recessive early onset childhood condition)	BS				
2.2.1 Variant region conserved in at least mammals and birds with Grantham score > 100	PM				
2.2.2 Variant region conserved in at least mammals and birds with Grantham score > 65	PP				
2.2.3 Variant region not conserved in at least mammals and birds with Grantham score < 65	BP				
2.3.1 In-silico consistently supports pathogenicity (SIFT, PolyPhen2, Mutation Taster, CADD)	PP				
2.3.2 In-silico consistently supports neutrality (SIFT, PolyPhen2, Mutation Taster, CADD)	BP				
2.4.1 Variant is located in well established, critical functional region without benign variation	PP				
2.5.1 Variant is located in exon with strong evidence for alternative splicing where the exon is known NOT to be present in tissue relevant transcript for the disease of interest	BS				
2.6.1 Heterozygous variant detected in *trans* with a second, known pathogenic, heterozygous variant in a recessive disease (Variant phasing may need to be confirmed by testing of parents)	PM				
2.6.2 Heterozygous variant observed in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in *cis* with a pathogenic variant in any inheritance pattern (Variant phasing may need to be confirmed by testing of parents)	BP				
2.7.1 Novel missense change in a codon where a different amino acid change has previously been described as pathogenic	PP				
2.8.1 Functional consequence of variant is not consistent with expected spectrum of pathogenic variants in the affected gene. NOTE: Can be seen as BS depending on level of evidence	BP				
2.9.1 Existing classification of pathogenicity (Variant has previously been classified by experts) NOTE: Can be seen as PVS if expert panel is known to have considered extensive additional information that is not available to the lab (i.e. InSight database, some BRCA gene classifications)	PS				
2.9.2 Existing classification of neutrality (Variant has previously been classified by experts) NOTE: Can be seen as BA if expert panel is known to have considered extensive additional information that is not available to the lab (i.e. InSight database, some BRCA gene classifications)	BS				
2.9.3 Previous description of pathogenicity (Variant has previously been described as pathogenic in databases or literature in independent patients)	PP				
2.9.4 Previous description of neutrality (Variant has previously been described as benign in databases or literature in independent patients)	BP				
2.10.1 Strong functional evidence supporting ABNORMAL protein function of the variant (eg. endogenous tissue)	PS				
2.10.2 Moderate functional evidence supporting ABNORMAL protein function of the variant (eg. exogenous cell line)	PM				
2.10.3 Strong functional evidence supporting NORMAL protein function of the variant (eg. endogenous tissue)	BS				
2.10.4 Moderate functional evidence supporting NORMAL protein function of the variant (eg. exogenous cell line)	BP				
2.11.1 Significant Segregation: Variant segregates with the same phenotype as the patient in 3 or more families or 1 large family to at least a third degree relative (LOD >= 3)	PS				
2.11.2 Moderate Segregation: Variant segregates with the same phenotype as the patient in 2 families only (LOD >=1.5 and <3)	PM				
2.11.3 Low Segregation: Variant segregates with the same phenotype as the patient in 1 family only OR homozygous variant in a recessive condition where both parents are confirmed to be carriers	PP				
2.11.4 Non-segregation has been clearly demonstrated	BS				
2.12.1 De Novo Variant (Parental status confirmed for both mother and father)	PS				
2.12.2 De Novo Variant (Parental status not tested but assumed)	PP				
2.13.1 Variant in a gene that is known to be causative for a well defined syndrome with unambigous clinical presentation. Gene has to have low tolerance for variation (ExAC Constraint Missense z >= 2) and inheritance pattern of the variant has to be consistent with suspected disease.	PP				

Count Evidence Levels (Categories):

Category	Count
PVS	
PS	
PM	
PP	
BA	
BS	
BP	

Worksheet classification:

Reason for change of Classification weight

NOTE: The classification formulas below represent a MINIMUM requirement for a given class. The curator has to decide if conflicting evidence is sufficient to justify VUS classification. E.g. 1BS + 3BP + 1PM = 2 - Likely benign, 1BS + 2BP + 1PS + 2PP = 3b - VUS

5- Pathogenic:	4 - Likely Pathogenic:	3a - VUS (potentially pathogenic)	3b - VUS	3c - VUS (potentially benign)	2 - Likely Benign	1 - Benign:
2 PVS	1 PVS and >= 1 PM	Not unambigously classifiable with predominantly pathogenic evidence	Not unambigously classifiable	Not unambigously classifiable with predominantly benign evidence	1 BS + >= 1 BP	>= 1 BA
1 PVS and > 1 PS	1 PVS and >= 4 PP				>= 2 BP	>= 2 BS
1 PVS and >= 2 PM	1 PS and >2 PM					
1 PVS and 1 PM and >= 2 PP	1 PS and 1 PM and > 2 PP					
>= 2 PS	1 PS and >= 3 PP					
1 PS and >=3 PM	>= 3 PM					
1 PS and 2 PM and >= 2 PP	2 PM and >= 2 PP					
1 PS and 1 PM and >= 4 PP	1 PM and >= 4 PP					

Category	Description
PVS	Pathogenic very strong
PS	Pathogenic strong
PM	Pathogenic moderate
PP	Pathogenic supporting
BS	Benign strong
BP	Benign supporting
BA	Benign stand alone

Appendix 4. Supplementary Table: Genetic causes of non-XLA PADs

Supplementary table: *Symptom onset and diagnostic delay of patients with genetic causes of PADs*

Patient no.	Clinical diagnosis	Gene affected	Age at onset (years)	Age at diagnosis (years)	Diagnostic delay (years)	Total no. patients	Median diagnostic delay
30	XLA	*BTK*	0	0	0		
38	XLA	*BTK*	0	0	0		
39	XLA	*BTK*	0	0	0		
40	XLA	*BTK*	0	0	0		
41	XLA	*BTK*	1	0	0		
116	XLA	*BTK*	0	1	1		
120	XLA	*BTK*	0	4	4		
122	XLA	*BTK*	0	0	0		
122	XLA	*BTK*	0	0	0		
125	XLA	*BTK*	0	0	0		
126	XLA	*BTK*	0	3	3		
132	XLA	*BTK*	0	0	0		
133	XLA	*BTK*	0	3	3		
159	XLA	*BTK*	?	?	?		
161	XLA	*BTK*	0	15	15		
163	XLA	*BTK*	0	0	0		
164	XLA	*BTK*	0	0	0		
165	XLA	*BTK*	?	?	?		
164	XLA	*BTK*	?	?	?		
165	XLA	*BTK*	1	3	2		
169	XLA	*BTK*	?	?	?	**21**	**2**
46	IGSCD	*NFKB2*	2	9	7		
47	IGSCD	*NFKB2*	2	7	5		
48	CVID	*NFKB2*	27	38	11		
49	CVID	*NFKB2*	1	5	4		

59	CVID	*NFKB2*	13	17	4	**5**	**8**
45	CVID	*NFKB1*	14	27	13		
112	CVID	*NFKB1*	31	39	8		
113	CVID	*NFKB1*	54	67	13		
114	CVID	*NFKB1*	27	28	1	**4**	**7**
84	CVID[#]	*CXCR4*	2	27	25		
85	CVID[#]	*CXCR4*	3	4	1	**2**	**13**
103	CVID	*TCF3**	1	2	1	**1**	**N/A**
56	CVID	*TACI*‡	36	41	5		
83	CVID	*TACI*‡	44	46	2	**3**	**2.5**
119	CVID	*CTLA4*	6	16	10		
141	CVID	*CTLA4*	15	19	4		
120	CVID	*CTLA4*	7	13	6	**3**	**7**
19	CVID	*STAT1*	16	35	19	**1**	**N/A**
125	CVID	*PI3KCD*	28	47	16	**1**	**N/A**

*(CVID, Common Variable Immunodeficiency; XLA, X-linked agammaglobulinemia; ?, indicates unknown; IGSCD, Immunoglobulin subclass deficiency; #, diagnosis changed to WHIM syndrome after genetic testing; *, patient also harbors TACI variant; ‡, C104R variant; TACI, encoded by gene TNFRSF13B)*

ARTICLE

Haploinsufficiency of the NF-κB1 Subunit p50 in Common Variable Immunodeficiency

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Common variable immunodeficiency (CVID), characterized by recurrent infections, is the most prevalent symptomatic antibody deficiency. In ~90% of CVID-affected individuals, no genetic cause of the disease has been identified. In a Dutch-Australian CVID-affected family, we identified a *NFKB1* heterozygous splice-donor-site mutation (c.730+4A>G), causing in-frame skipping of exon 8. *NFKB1* encodes the transcription-factor precursor p105, which is processed to p50 (canonical NF-κB pathway). The altered protein bearing an internal deletion (p.Asp191_Lys244delinsGlu; p105ΔEx8) is degraded, but is not processed to p50ΔEx8. Altered NF-κB1 proteins were also undetectable in a German CVID-affected family with a heterozygous in-frame exon 9 skipping mutation (c.835+2T>G) and in a CVID-affected family from New Zealand with a heterozygous frameshift mutation (c.465dupA) in exon 7. Given that residual p105 and p50—translated from the non-mutated alleles—were normal, and altered p50 proteins were absent, we conclude that the CVID phenotype in these families is caused by NF-κB1 p50 haploinsufficiency.

Introduction

Common variable immunodeficiency (CVID1 [MIM: 607594], see this entry for additional MIM numbers for CVID2–CVID11) is a clinically and genetically heterogeneous disorder with an estimated incidence of 1:10,000–1:50,000.[1] CVID is the most frequent symptomatic primary immunodeficiency and is characterized by recurrent infections due to hypogammaglobulinemia and by deficiency of IgA and/or IgM. Recurrent pneumonia due to bacterial infection is the most common feature at initial presentation, and specific antibody responses are severely impaired. There is an increased prevalence of autoimmune disorders in CVID, and lymphoproliferative conditions are frequently observed.[2,3]

The milder disorder, selective IgA deficiency (sIgAD), has an incidence of 1:600–1:800, which is higher than that of CVID. Approximately 10% of CVID-affected family members will also have a relative with sIgAD,[4,5] which shares common susceptibility loci[6] with, and can progress to, CVID.[7–9] Similarly, within CVID-affected families, individual members might be diagnosed with hypogammaglobulinemia, a CVID-like disorder, or "possible" or "probable CVID" when they do not fulfill the diagnostic criteria for CVID.[2,10,11] Because diagnosis of CVID is established largely by exclusion— ruling out alternative causes for hypogammaglobulinemia— the CVID cohort is heterogeneous, comprising monogenic as well as polygenic disorders of variable origin.[2]

Although abnormal B and T cell functions are commonly observed in CVID, no genetic basis of these immune defects has yet been identified in the majority of these cases. Most CVID-affected individuals have reduced numbers of isotype-switched memory B cells, a severe reduction in post-germinal center B cells, and relative preservation of pre-germinal center B cells.[12] A potential role for regulatory T cells (Treg) in the pathogenesis of CVID has been suggested, given that affected individuals collectively have significantly lower Treg counts than unaffected control individuals do,[13,14] and a defect in Treg function has been demonstrated in some CVID-affected persons with mutations in *CTLA4*[15] (MIM: 123890). A genetic defect has been identified in only ~10% of CVID-affected individuals;[16,17] these defects include mutations in *ICOS* (inducible costimulator [MIM: 607594]), *TNFRSF13B* (which encodes TACI, a transmembrane activator and calcium-modulator and cyclophilin ligand interactor [MIM: 240500]), *CD19* (MIM: 613493), *BAFF-R* (B cell activating factor receptor; *TNFRSF13C* [MIM: 613494]), and several others. However, the molecular mechanisms leading to cellular defects and insufficient antibody production are poorly understood.

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Signaling via NF-κB (nuclear factor of kappa light polypeptide gene enhancer in B cells) is involved in a multitude of biological processes, including immune responses during inflammation, cell development and survival, and stress responses.[18–20] In B cell differentiation and function, both the canonical and the non-canonical NF-κB signaling pathways play pivotal roles.[21–23] The NF-κB transcription-factor family comprises five members, including NF-κB1 (the mature p50 and its precursor, p105), NF-κB2 (the mature p52 and its precursor, p100), RelA (p65), RelB, and c-Rel. These assemble into various homo- and heterodimeric combinations to regulate the expression of a large number (>500) of target genes, including those of cytokines, chemokines, growth factors, apoptosis regulators, cell surface receptors, and other transcription factors.

The five NF-κB proteins share in their sequences a Rel homology domain (RHD) in their N-terminal portions. The RHD mediates dimerization, interaction with each protein's specific inhibitors, and DNA binding. Following phosphorylation and poly-ubiquitination within the C-terminal portion of the protein, p105 and p100 undergo co- and/or posttranslational proteasomal processing into p50 and p52 subunits, respectively, and the processed subunits retain the RHD (Figure S1). In unstimulated cells, both the inactive NF-κB precursors and the NF-κB dimers that are associated with inhibitory proteins (IκBα, IκBβ, and IκBγ) are sequestered within the cytoplasm and therefore remain transcriptionally inactive.[18,21,24] Rapid activation of NF-κB occurs in response to numerous stimuli, typically with proteasomal degradation of IkBα (NF-kappa-B inhibitor alpha) in the canonical NF-κB pathway and partial proteolysis of p100 to p52 in the non-canonical NF-κB pathway.

NFKB1 (MIM: 164011) encodes p105 (969 amino acids), which is processed to the active subunit p50 (433 amino acids). In the canonical pathway, p50 assembles a heteromeric transcription factor, predominantly with RelA, that is complexed with IκBα. Upon stimulation of the canonical pathway by cytokines such as TNFα and IL-1, bacterial products (e.g., lipopolysaccharide), antigen receptors, or CD40L, the inhibitory κB kinase (IKK) of the NEMO-IKK complex becomes activated, which subsequently leads to phosphorylation of the inhibitory IκBα protein, poly-ubiquitination, and degradation by the 26S proteasome. The active p50-RelA dimer is then released, translocates to the nucleus, and binds to the promoters of its target genes.[24] For transcriptional activation, p50 is required to heterodimerize with RelA, which contains a transactivation domain. In contrast, homodimers of p50 lack a transactivation domain and act as transcriptional repressors. Transcriptional activation via the canonical pathway is characterized by instant and reversible expression of target genes encoding inflammatory cytokines, such as IL-6, and immune response genes.

Excessive or constitutive NF-κB activation has been implicated in various human malignancies, including solid tumors, acute and chronic leukemia, and B and T cell lymphomas.[25,26] Genetic lesions that directly cause impaired regulation of NF-κB-dependent signaling and are associated with one or more immunodeficiencies include those on genes *NFKBIA*[27,28] (*IKBA* [MIM: 164008]), *IKBKB*[29–31] (MIM: 603258), *IKBKG*[32,33] (*NEMO* [MIM: 300248]), *CARD9*[34] (MIM: 607212), *CARD11*[35] (MIM: 607210), *NOD2*[36,37] (*CARD15* [MIM: 605956]), *NLRP3*[38] (*CIAS1* [MIM: 606416]), and others.[19,20,39] A promoter polymorphism in *NFKB1* (−94 in/del ATTG [rs28362491]) has been associated with increased susceptibility to inflammatory bowel disease and/or ulcerative colitis (MIM: 266600).[40,41] B cell maturation, survival, differentiation, and T-cell-independent antibody class switching are associated with NF-κB-mediated signaling.[42,43] Furthermore, known disease-associated CVID genes, e.g., *TNFRSF13B* (encodes TACI [MIM: 604907]) and *TNFRSF13C* (encodes BAFF-R [MIM: 606269]), encode receptors that signal via the NF-κB pathways.[44,45]

In B cells, TACI signaling is a potent inducer of the canonical NF-κB1 pathway, whereas BAFF-R signaling mainly activates the non-canonical NF-κB2 pathway. Recent studies have identified *NFKB2* mutations (MIM: 164012) in CVID (MIM: 615577);[46–51] however, mutations in *NFKB1* have not yet been reported.

We have previously described a five-generation Dutch-Australian family with autosomal-dominant inheritance of CVID[52] and established an 18.7-Mbp linkage interval on chromosome 4q. However, in a candidate gene analysis (that included *NFKB1*), no mutations were identified by Sanger sequencing in coding exons within the linkage region. In the present study, we identified, by whole-exome sequencing (WES), a heterozygous splice-donor-site mutation in intron 8 of *NFKB1* that segregates with disease status in this CVID-affected family. The altered protein, p105ΔEx8, which results from in-frame skipping of exon 8, is rapidly degraded, and processing to p50ΔEx8 was undetectable. Subsequent targeted next-generation sequencing (NGS) identified a heterozygous splice-donor-site mutation in intron 9 of *NFKB1* in a German family affected by CVID. Analogously, in-frame skipping of exon 9 does not lead to the production of detectable amounts of an altered p50ΔEx9 protein. Furthermore, by WES, we identified a heterozygous single-base-pair duplication in exon 7 of *NFKB1* in a CVID-affected family from New Zealand. A severely truncated, non-functional protein, which might have been predicted and delineated from the mutant sequence, was undetectable. Our observations demonstrate that haploinsufficiency of the NF-κB1 subunit p50 is associated with a CVID phenotype.

Material and Methods

This study was conducted under protocols for human subjects, and samples were collected with the written consent of all study participants and/or their parental guardians after formal ethical approval by the local ethics committees at the University of Freiburg, the Radboud University, Melbourne Health, the University of Auckland, and collaborating institutions.

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Cell Culture and Transfection

Peripheral blood mononuclear cells (PBMCs) were isolated and immortalized and Epstein-Barr virus (EBV)-transformed B-lymphocyte cultures were prepared according to standard procedures. Cells were cultured in RPMI 1640 Medium and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). For stimulation experiments, phorbol 12-myristate 13-acetate (PMA; 125 ng/μl in dimethyl sulfoxide; Sigma) and ionomycin (500 ng/μl in ethanol; Sigma) were added directly to the cell cultures (final concentrations: 50 ng/ml PMA and 1 μg/ml ionomycin) or CD40L (100 ng/ml MegaCD40L; Enzo Life Sciences, Sapphire Bioscience) and cells were harvested after incubation at 37°C as indicated. HEK293T and mouse fibroblasts (NIH 3T3 cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. Cells were plated onto collagen-coated coverslips and transfected with X-tremeGENE 9HP reagent (Roche), according to the suppliers' recommendations.

Sequence Analysis

Genomic DNA was isolated from blood samples with QIAamp Kits (QIAGEN). For mutational analysis in family FamNL1, WES was performed at BGI– Shenzhen and the Australian Genome Research Facility. Variants were annotated with an in-house annotation software system. Within the previously specified linkage region on chromosome 4q, a single unknown single-nucleotide variant (SNV) was identified, including a splice-donor-site mutation of intron 8 in *NFKB1*. A 582-bp genomic fragment of *NFKB1* comprising exon 8 and both intron and exon boundaries was amplified by PCR with the primers 5′-GAGGGA GGCGATC TGATACA-3′ (forward) and 5′-CTGGACATCAACCTTTCAAGC-3′ (reverse). In family FamNZ, a 370-bp amplicon encompassing the mutation in exon 7 was amplified by PCR with primers 5′-AAT TTA CAAGTGAAGCCAGAT-3′ and 5′-AAAAGGTGACAGCAGT GTG-3′. In family Fam089, a 281-bp genomic fragment spanning exon 9 was amplified by PCR with the primers 5′-AGGGGA GGGTCCTACCTAAAG-3′ (forward) and 5′-CTTGTCCACTGCTGA AATGTG-3′ (reverse). PCR primers were used for Sanger sequencing according to standard techniques.

cDNA Sequencing

RNA was isolated from EBV-transformed B cells with an RNeasy Mini Kit (QIAGEN) and reverse transcribed with Omniscript (QIAGEN) or Superscript III (Invitrogen). In FamNL1, a 649-bp cDNA fragment spanning exons 7–10 was amplified by PCR and sequenced with the primers 5′-GTGAGGATGGGATCTGC ACT-3′ (forward; exon 6) and 5′-CGAAGCTGGACAAACACAGA-3′ (reverse; exon 11). In Fam089, a 682-bp cDNA fragment spanning exons 8–11 was amplified and sequenced with the primers 5′-AAC ACTGGAAGCACG AATGAC-3′ (forward; exon 7) and 5′-ACT ACC ACC GCC GAA ACT ATC-3′ (reverse; exon 12).

Western Blotting

Cells were washed with PBS and lysed on ice in radioimmunoprecipitation assay buffer (50 mM Tris pH 8, 1% Igepal, 0.5% sodium-deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.1% SDS, 1% protease inhibitor cocktail). Supernatants were separated on 4%–12% NuPAGE Bis-Tris gels (Invitrogen) and transferred onto polyvinylidene-difluoride membranes (Roche). p105 and p50 were detected with a rabbit antibody raised against the N terminus of NF-κB1 (#3035; Cell Signaling; NEB). Phosphorylated p105 was detected with a monoclonal rabbit antibody (#4806, Cell Signaling). Signals were detected with horseradish-peroxidase-coupled anti-rabbit secondary antibodies (NEB) via enhanced chemiluminescence (LumiGlo or SignalFire; NEB).

Preparation of Nuclear Extracts

Protein extracts enriched for nuclear proteins were prepared as previously described.[53] Cells were washed with PBS, resuspended in 400 μl of Buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 1% protease inhibitor cocktail; Sigma), and incubated on ice for 10 min. After brief vortexing and centrifugation, pellets were resuspended in 20–100 μl of Buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1% protease inhibitor cocktail) and incubated on ice for 20 min. Supernatants were collected and subjected to western blotting.

cDNA Expression Vectors and GFP-Fusion Constructs

The cDNAs encoding the full-length p105 (p105-FL) and the truncated p105ΔEx8 were both cloned by RT-PCR from EBV-transformed B lymphocytes derived from an affected individual harboring the heterozygous c.730+4A>G mutation. Restriction sites (XhoI and EcoRI) were introduced with the PCR primers. After sequence verification, the cDNAs were subcloned into pEGFP-C1 (Clontech, Takara) to generate GFP-fusion constructs. The cDNAs encoding p50 and p50ΔEx8 were generated by PCR from the p105-FL and truncated p105ΔEx8 versions, respectively. Non-fusion versions were obtained by subcloning into pEGFP-N1.

Fluorescence Staining and Confocal Imaging

Glass coverslips with transfected cells expressing GFP-p50, GFP-p50ΔEx8, GFP-p105-FL, or GFP-p105ΔEx8 fusion constructs were rinsed with PBS, and nuclei were stained with Hoechst 33342 (Sigma). Samples were mounted onto glass slides with fluorescence mounting medium (Dako) for microscopy. Confocal fluorescence images were taken on a Zeiss laser scanning microscope, LSM710, with an Axiovert 200M equipped with a 63× water immersion objective (Carl Zeiss). Images were evaluated and processed with the Zeiss ZEN blue-ZEN black software.

Identification of a Splice-Donor-Site Mutation in Intron 9 of *NFKB1* in a German Family by Targeted NGS

Genomic DNA was extracted from PBMCs of the proband (Fam089-II2). For target enrichment, commercial reagent kits were used according to the manufacturer's instructions (Agilent). DNA samples were treated with a restriction-enzyme master mix and the products were hybridized to the HaloPlex probe capture library including the indexing primer cassettes. The target DNA was captured by a biotin-streptavidin system with HaloPlex magnetic beads, and the circular fragments were closed in a ligation reaction. The captured target libraries were amplified by PCR, and the amplified target libraries were purified with AMPure XP beads (Beckman Coulter) and washed in ethanol. Enrichment was validated on a BioAnalyzer or TapeStation (Agilent). Subsequently, samples were pooled in equimolar amounts for multiplexed sequencing on an Illumina MiSeq system. Libraries were denatured and diluted to a final concentration of 8–12 pM. For sequencing, an Illumina Reagent Kit v.2 was used.

Identification of a *NFKB1* Frameshift Mutation in a CVID-Affected Family from New Zealand by Exome Sequencing

Genomic DNA was extracted from PBMCs of the male proband (FamNZ-II1), his mother (FamNZ-I2), and his affected sister (FamNZ-II2). Library preparation, exome enrichment (Nextera Rapid Capture Exome, Illumina), and exome sequencing (Illumina HiSeq 2000) was performed by New Zealand Genomics Limited. Approximately 59 million, 100-nucleotide paired-end reads were aligned to the 1000 Genomes Project reference genome (GRCh37.p13) with the Burrows-Wheeler aligner.[54] Duplicates were identified with Picard, followed by indel realignment and base-quality-score recalibration with the Genome Analysis Toolkit (GATK).[55] Final coverage of targeted intervals was 49-fold (49.2-, 49.2-, and 49.7-fold for the male proband, his mother, and his sister,respectively). Exome alignments from 44 unrelated individuals were added to support variant identification with GATK's HaplotypeCaller in "joint calling" mode and to empower variant quality-score recalibration.[56] A total of 63,486 high-confidence variants and 21,041 shared genotypes were detected in the three individuals. 6,018 shared variants were predicted to cause gain or loss of translation of start or stop codons, a frameshift, a codon insertion or deletion, or a non-synonymous amino-acid substitution or were located in consensus splice motifs or 5′- or 3′-untranslated regions. We removed variants exceeding an alternate allele frequency of 0.02 in the European subsets of the HapMap, 1000 Genomes Project, NHLBI GO Exome Sequencing Project, or Exome Aggregation Consortium (December 2014 version) cohorts, as well as variants with genotypes observed more than once in 125 in-house exomes (individuals with non-immunological phenotypes) and variants located in olfactory receptor genes. 79 variants were retained and analyzed for their disease-causing potential on the basis of the known biological roles, disease associations, and tissue expression of their genes, together with the predicted functional consequences of the individual mutations.

Results

Autosomal-Dominant Inheritance of a Heterogeneous Form of CVID in a Large Five-Generation Family

The index family (FamNL1, of Dutch origin) with multiplex CVID-sIgAD-hypogammaglobuliemia over five generations has previously been described.[52,57] The disease status, an overt late-onset hypogammaglobulinemia, segregated with an autosomal-dominant inheritance pattern. In the present study, additional family members have been included (Figure 1A), particularly one branch of the family, which migrated to Australia. FamNL1 is therefore referred to as a Dutch-Australian family. Clinical records have been reevaluated and follow-up examinations have been conducted, when possible. All study participants signed research informed consent forms after approval of the study by local research ethics committees. For those individuals who participated in both studies, the subject IDs are identical. The revised pedigree includes eleven individuals with CVID and nine with hypogammaglobulinemia. An update on the clinical phenotype of the affected family members is summarized in Table 1 and described in detail in the Supplemental Data case reports. The clinical histories are remarkably variable, ranging from moderate to severe hypogammaglobulinemia and from recurrent sinus infections to severe CVID characteristics, such as progressing pulmonary disease.

To localize the genetic defect in this family, a genetic linkage study was conducted in 2005, establishing an 18.7-Mbp linkage interval on chromosome 4q24.[52] The linkage interval was robust to various assumptions about whether individuals with milder phenotypes should be considered as "affected." Although the coding exons of several genes in the candidate region, including *NFKB1* (which spans ~116 kbp and comprises 23 coding exons plus a non-coding first exon [MIM: 164011]), were analyzed by Sanger sequencing, a disease-causing mutation was not identified. In light of our findings in the present study, the previous results might be attributed to an underestimation of sequence variants adjacent to the coding exons, such as the non-coding intronic splice sites, which were not exhaustively studied at that time.

Identification of a Splice-Donor-Site Mutation in Intron 8 of *NFKB1* by WES

Because a previous candidate-gene approach failed to identify disease-associated coding-sequence variants, we performed WES on two affected individuals (FamNL1-34 and FamNL1-49). In an independent approach, mutational analysis by exome sequencing was conducted in three additional family members (FamNL1-57, FamNL1-62, and FamNL1-64). Within the minimal linkage region (flanked by the microsatellite markers D4S2361 and D4S1572), only one single SNV was identified, namely an intronic sequence variant in *NFKB1* (g.103500200A>G; GRCh37). The sequence variant was not listed in the dbSNP, the Human Gene Mutation Database, the 1000 Genomes catalog, or the Exome Aggregation Consortium browser.

NFKB1 comprises 24 exons (Figure S1) and encodes two variants of the precursor protein p105 with 969 amino acids (variant 1 [GenBank: NM_003998.3]) and 968 amino acids (variant 2 [GenBank: NM_001165412.1]). The precursor undergoes proteasomal processing to produce p50 (433 amino acids). The identified sequence variant, c.730+4A>G (GenBank: NM_003998.3), affects the splice donor site of intron 8, suggesting that defective mRNA and/or protein synthesis might occur.

Segregation of the Heterozygous *NFKB1* Intron 8 Splice-Site Mutation with CVID Disease Status in FamNL1

To confirm that the identified *NFKB1* sequence variant segregates with disease status in the Dutch-Australian CVID-affected family, we analyzed genomic DNA from 35 affected and non-affected family members by Sanger sequencing of exon 8, including both exon and intron boundaries (Figure 1B). We found that all ten analyzed CVID-affected individuals and three family members with hypogammaglobulinemia (FamNL1-38, FamNL1-42,

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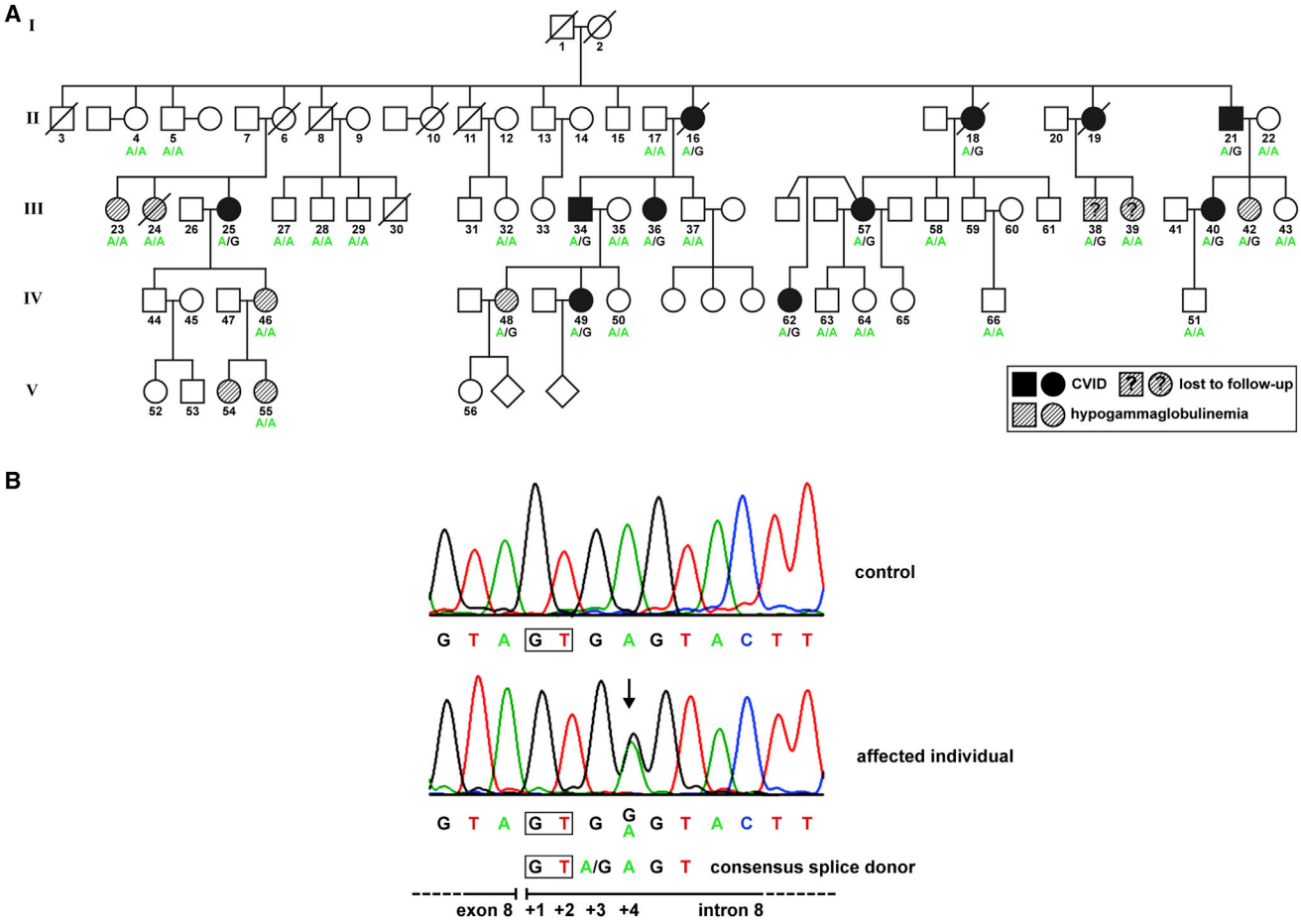


Figure 1. Autosomal-Dominant Inheritance of a Heterozygous *NFKB1* Mutation in a Large CVID-Affected Family
(A) The pedigree of FamNL1[52] was completed and revised after re-evaluation of clinical records and follow-up examinations. Circles, female; squares, male; filled symbols, affected individual with CVID; hatched symbols, affected individual with hypogammaglobulinemia; open symbol, healthy member with wild-type *NFKB1*; slash, deceased individual.
(B) The mutation (c.730+4A>G) affects the intron 8 splice donor site, which perfectly aligns to the consensus splice site.

and FamNL1-48) harbored the mutation (Figure 1A). An inconsistency was observed in one individual with an ambiguous medical history (FamNL1-39) who does not carry the mutation and who was hence classified as a "phenocopy." The presence of a phenocopy is not surprising in light of the high incidence of hypogammaglobulinemia in this family. The possibilities of phenocopies and non-penetrance have been included in the previous genetic linkage analysis.[52] In four of the tested individuals with hypogammaglobulinemia (FamNL1-23, FamNL1-24, FamNL1-46, and FamNL1-55,), the mutation was also undetectable. These family members are together on one branch of the pedigree, suggesting the presence of other genetic factors associated with hypogammaglobulinemia in this branch.

The *NFKB1* Intron 8 Splice-Donor-Site Mutation Causes In-Frame Skipping of Exon 8

Because the identified sequence variant c.730+4A>G affects the splice donor site of intron 8, we next tested whether NF-κB1 mRNA splicing was affected. We analyzed the mRNA expression in EBV-transformed B lymphocytes derived from affected individuals (n = 3) and a healthy control individual by RT-PCR (Figure 2A). Using primers spanning exons 7 to 10, we observed a single band with the expected size; this band was in the control sample. In contrast, in samples from affected individuals, an additional shorter product was obtained, indicating skipping or mis-splicing of either exon 7, 8, or 9.

In the control sample, sequencing of the cDNA product was normal. In contrast, the shortened product in the mutant samples lacked exon 8 (159 bp). Thus, the mutation of the splice donor site in intron 8 most likely renders exon 8 unrecognizable to the splicing machinery and thus causes in-frame splicing of exon 7 to exon 9 (Figure 2B).

A Deletion within the NF-κB1 RHD in p105ΔEx8 Leads to Rapid Degradation and p50 Haploinsufficiency

As a consequence of the *NFKB1* splice-donor-site mutation, in-frame skipping of exon 8 predicts the deletion of an internal 53-amino-acid fragment from the N-terminal RHD with insertion of a glutamic acid residue due to the fusion

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Table 1. Clinical Information of 20 Individuals Harboring *NFKB1* Mutant Alleles

Individual	Year of Birth	Year of Diagnosis	Diagnosis	IgG (g/L)	IgA (g/L)	IgM (g/L)	Clinical Phenotype
FamNL1							
16	1935	1964	CVID	2.76	0.30	0.16	COPD, pyoderma gangrenosum, pansinusitis, atrophic gastritis, squamous cell carcinoma, respiratory tract infections, pneumonia; died due to pulmonary insufficiency
18	1936	2000	CVID	2.49	0.13	0.53	sinopulmonary infections, splenomegaly, lymphadenopathy, mild thrombocytopenia, ischemic heart disease, lung adenocarcinoma
21	1940	1997 (2000)[a]	CVID	6.0	1.26 (0.93)[a]	0.4 (0.17)[a]	COPD, pneumonia, recurrent sinusitis, bronchiectasis, lung fibrosis, respiratory insufficiency
25	1948	uk	CVID	4.6	1.0	0.4	COPD, pulmonary infections, respiratory insufficiency, cor pulmonale
34	1960	uk	CVID	8.0	0.83	0.25	autoimmune hypothyroidism, alopecia areata, recurrent respiratory infections, pyoderma gangrenosum
36	1961	1991	CVID	1.81	0.06	0.48	recurrent sinusitis, pneumonia
40	1966	uk	CVID	5.1	<0.5	0.65	COPD, recurrent upper airway infections
42	1968	uk	HGG	uk	uk	uk	recurrent paronychia and superficial skin infections, minor laboratory abnormalities
48	1985	uk	HGG	3.72	0.59	0.74	aortic stenosis, furunculosis, folliculitis, autoimmune thyroiditis
49	1986	1988	CVID	9.1[b]	1.41	0.11	enteritis, recurrent otitis media, giardiasis, recurrent respiratory infections
57	1961	2000	CVID	1.3	<0.04	0.3	LIP, NRH, recurrent sinopulmonary infections
62	1985	2015	CVID	4.7	0.4	0.3	mild recurrent sinus infections
Fam089							
II1	1946	2010	CVID	1.42	0.08	0.4	severe pneumonia, pleural empyema, multiple liver hemangiomas
II2	1979	1995 (2006)[a]	CVID	6.29 (4.41)[a]	<0.3 (<0.07)	<0.3 (0.2)[a]	ITP, recurrent pulmonary infections, autoimmune hemolytic anemia, hepatomegaly, lymphadenopathy
III2	2009	2014	HGG	1.94	<0.07	0.29	transient HGG
III4	2013	–	–	–	–	–	healthy
FamNZ							
I2	1941	–	–	9.9	0.58	0.8	alopecia, thrombocytopenia, normal immunoglobulins
II1	1966	1968	CVID	5.17	0.07	0.5	thrombocytopenia, neutropenia, autoimmune hemolytic anemia
II2	1968	1988	CVID	–	–	–	alopecia, bronchiectasis, NRH, marginal-zone non-Hodgkin lymphoma
II3	1971	–	–	7.78	1.5	0.56	healthy

CVID, common variable immunodeficiency; HGG, hypogammaglobulinemia; COPD, chronic obstructive pulmonary disease; LIP, lymphocytic interstitial pneumonitis; NRH, nodular regenerative hyperplasia; ITP, idiopathic thrombocytopenic purpura; uk, unknown.
[a]Additional data from follow-up exam.
[b]IgG levels after IVIG.

of exons 7 and 9 (p.Asp191_Lys244delinsGlu). Because p105 is processed to p50 by limited proteolysis of its C-terminal half, the deletion is predicted to affect both the precursor and mature NF-κB1 proteins (Figure S1).

To test whether a shortened protein (with a molecular weight reduced by ~5.8 kDa) is produced from the mutant mRNA, we analyzed protein extracts from EBV B cell lines from affected individuals (n = 3), healthy family members (n = 3), and healthy control individuals (n = 2) by western blotting. Using an antibody raised against the N-terminal end of NF-κB1, we detected both subunits p105 and p50 in crude lysates in all analyzed samples (Figure 2C). Yet, in the heterozygous affected individuals, both p105 and p50 protein amounts were uniformly lower (reduced to ~50%) than those in non-affected family members and control individuals, consistent with expression exclusively from the non-mutant allele. As predicted, a smaller additional band was detected in all samples from affected

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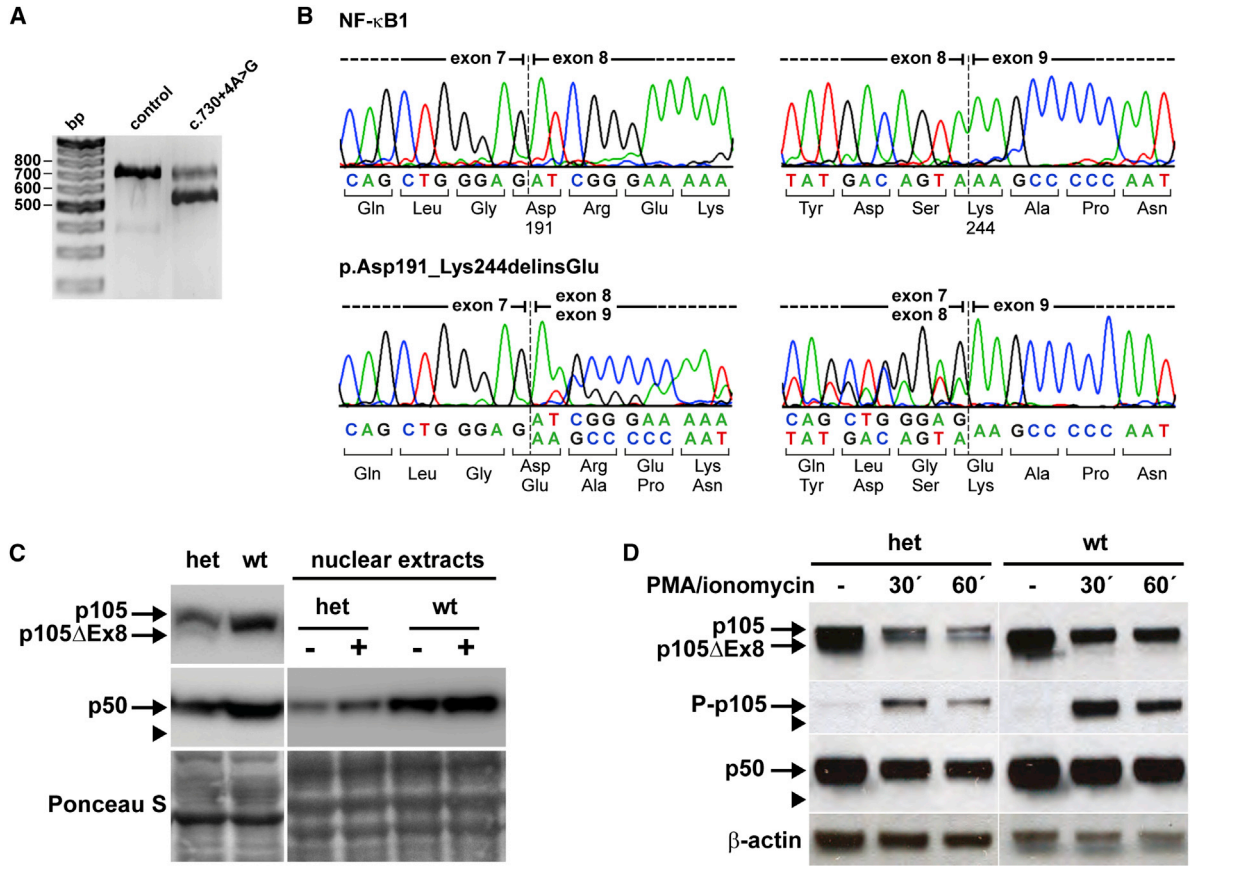


Figure 2. The Heterozygous Splice-Donor-Site Mutation in Intron 8 of *NFKB1* Causes In-Frame Skipping of Exon 8 and Haploinsufficiency of the p50 Subunit

(A) EBV-transformed B cell lines derived from a healthy control individual and affected individuals (n = 3) were analyzed by RT-PCR for correct *NFKB1* mRNA splicing with primers located in exons 6 and 11. In addition to the expected band (649 bp) a shorter product is observed in affected individuals, which indicates the loss of either exon 7 (164 bp), 8 (159 bp), or 9 (105 bp). Representative results are shown.

(B) Sequencing of the RT-PCR products shows normal splicing in the control individual (upper panels). The heterozygous mutation (c.730+4A>G) causes skipping of exon 8 and in-frame fusion of exon 7 to exon 9 (lower panels: left, forward; right, reverse), indicated by the double peaks at the exon borders. The mutant mRNA predicts the translation of a shortened NF-κB1 p105 (p105ΔEx8) with a 53-amino-acid internal deletion (p.Asp191_Lys244delinsGlu).

(C) EBV B cell lines from affected persons (n = 3) harboring the heterozygous *NFKB1* mutation (het), from unaffected (wt) family members (n = 4) and healthy control cells (n = 2) were stimulated with PMA plus ionomycin as indicated (−/+) and analyzed by western blot for NF-κB1 protein amounts with antibodies raised against the N terminus of both p150 and the processed form, p50. Representative results are shown. As depicted in the left panel, the altered protein p105ΔEx8 is only marginally preserved and processing to p50ΔEx8 is not detectable (expected size is indicated by the arrowhead). Overall amounts of both p105 and p50 are reduced to ~50% in EBV B cells from affected individuals (het). As depicted in the right panel, activation and nuclear translocation of residual p50 (derived only from the non-mutant allele) in affected individuals and normal nuclear translocation of p50 in controls after PMA plus ionomycin stimulation for 45 min. Ponceau S staining was used to demonstrate equal loading.

(D) PBMCs from affected family members (n = 2) and healthy control individuals (n = 4) were stimulated with PMA plus ionomycin as indicated, and cell lysates were analyzed for p105 and p50 amounts and for phosphorylation of p105 (Ser933, P-p105) by western blotting (representative results). In samples derived from affected individuals, both p105 and the altered p105ΔEx8 are detectable. Only the non-altered p105 is phosphorylated. Phosphorylation of p105ΔEx8 and processing to p50ΔEx8 is undetectable (arrowheads). Whether stimulated or not, p50 is sustained at reduced amounts in comparison to that in controls. β-actin was used as a loading control.

individuals. The molecular weight of this protein corresponds to the truncated p105, which is translated from the mutant allele and lacks the sequences encoded by exon 8 (p105ΔEx8). Unexpectedly, however, the altered protein band was evidently less intense than its wild-type counterpart.

The marginal abundance of p105ΔEx8 indicated that the mutant mRNA or altered protein was either only weakly transcribed or translated, respectively, or rapidly degraded. Most importantly, an additional smaller band corresponding to p50ΔEx8 (~44 kDa) was never detected. Thus, in contrast to p105, which was processed to the active p50 subunit, the mutant p105ΔEx8 was not processed to a corresponding truncated form (p50ΔEx8). We concluded that, in EBV B cell lines from affected individuals, the amount of p105ΔEx8 is not sustained, and instead, the altered protein is rapidly degraded. The p105 and p50 proteins, translated from the non-mutated allele, are normal.

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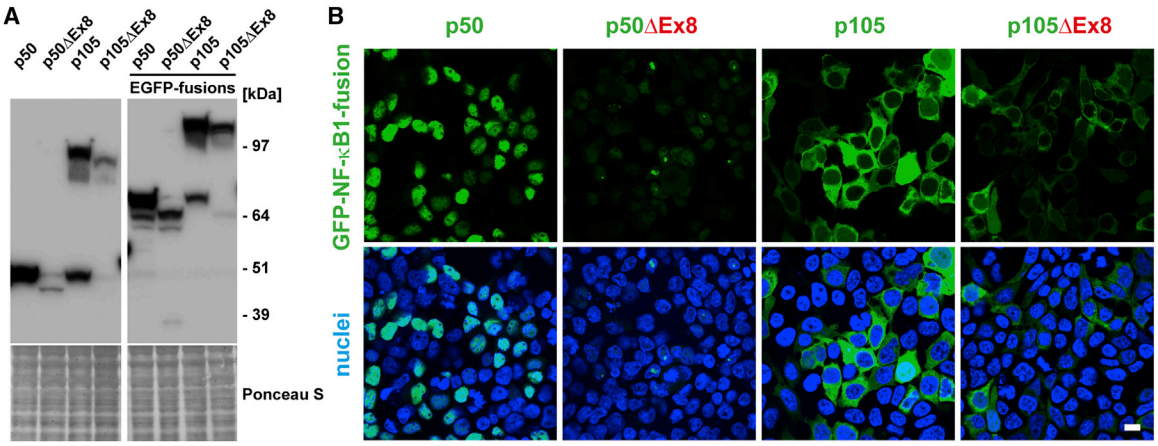


Figure 3. Rapid Elimination of Altered p50ΔEx8 and p105ΔEx8 in Transfected Cells
(A) HEK293T cells were transiently transfected with the indicated constructs, and the amounts of the ectopic proteins were analyzed in crude lysates by western blot. The altered proteins gain only weak signals and processing of p105ΔEx8 to p50ΔEx8 is almost undetectable. The faint band at 50 kDa in all lanes is the endogeneous p50. Molecular weight markers are indicated on the right side. Ponceau S staining of the blotting membranes confirm equal loading.
(B) Fluorescence imaging shows nuclear localization of GFP-p50 and cytoplasmic localization of GFP-p105. Only aberrant signals are detected with the mutant proteins. Green, GFP-fusion proteins as indicated; blue, nuclei. Scale bar represents 10 μm.

The internal deletion is predicted to affect the RHD, which mediates dimerization, nuclear translocation, and DNA binding. Thus, the altered proteins (p105ΔEx8 and/or p50ΔEx8) potentially could interfere with NF-κB1-mediated signaling. We wanted to confirm that no mutant p50ΔEx8, although undetectable in crude lysates, is translocated to the nucleus after NF-κB pathway stimulation. We therefore treated EBV-transformed B cells derived from affected individuals (n = 3), unaffected family members who do not carry the mutation (n = 4), and healthy control individuals (n = 2) with non-specific NF-κB1 activators (PMA plus ionomycin) and analyzed the amount of p50 translocation in nuclear protein extracts (Figure 2C). In western blot analyses, the "active" nuclear p50 was also detected only at severely reduced amounts in unstimulated samples derived from affected individuals in comparison to levels in control samples. After stimulation, p50 was increased in samples from affected individuals but only reached approximately half the amount observed in the control individuals. Again, the hypothetical altered form, p50ΔEx8, was not detected.

These findings were further confirmed by western blot analysis of lysates from PBMCs isolated from affected family members and healthy control individuals (Figure 2D). In the samples from affected family members, the precursors produced from both the wild-type allele (p105) and the mutant allele (p105ΔEx8) were detectable. Upon stimulation of the cells with PMA plus ionomycin, only the wild-type p105 was phosphorylated (Ser933). Thus, the total amount of phosphorylated p105 (phospho-p105) was reduced to ~50% of the amount seen in control samples. Consistently, only the wild-type p105 was further processed to p50, whereas processing of p105ΔEx8 to a mutant p50ΔEx8 was not detectable. Similar results were obtained when cells were treated with CD40L (data not shown).

In summary, these observations suggest that the heterozygous c.730+4A>G splice-donor-site mutation leads to rapid degradation of an aberrant protein, p105ΔEx8, which is not further processed to p50ΔEx8 and thus causes a functional haploinsufficiency of NF-κB1 subunit p50.

Rapid Elimination of Altered p50ΔEx8 and p105ΔEx8 in Transfected Cells

To verify that the existence of both the p105ΔEx8 and hypothetical p50ΔEx8 proteins are not tolerated in viable cells and thus are degraded, we generated cytomegalovirus promoter-driven vectors for ectopic expression in cell-culture models. We transiently transfected p50, p50ΔEx8, p105, and p105ΔEx8, as well as N-terminal GFP-fusion constructs (GFP-p50 and GFP-p50ΔEx8, GFP-p105 and GFP-p105ΔEx8), in human embryonic kidney cells (HEK293T) and/or murine NIH 3T3 fibroblasts (Figure 3 and data not shown). Western blot analyses showed that the non-mutant proteins p50 and p105, as well as their GFP-fused derivatives, were highly abundant upon transient transfection. In contrast, the protein amounts of the truncated p50ΔEx8 and p105ΔEx8 were only modest, whether fused to GFP or not (Figure 3A). Furthermore, both ectopic p105 variants were readily processed to p50 and GFP-p50, respectively, whereas proteolytically produced p50ΔEx8 and GFP-p50ΔEx8 were almost undetectable (Figure 3A).

Consistently, confocal fluorescence microscopy demonstrated that GFP-p50 localized to the nucleus in transfected cells, whereas GFP-p50ΔEx8 showed only speckled fluorescence signals in individual cells (Figure 3B). In contrast to transiently transfected GFP-p105, which was detected at high levels, mainly within the cytoplasm, only very weak GFP-p105ΔEx8 signals were detected. These observations indicate that the altered proteins (p105ΔEx8 and

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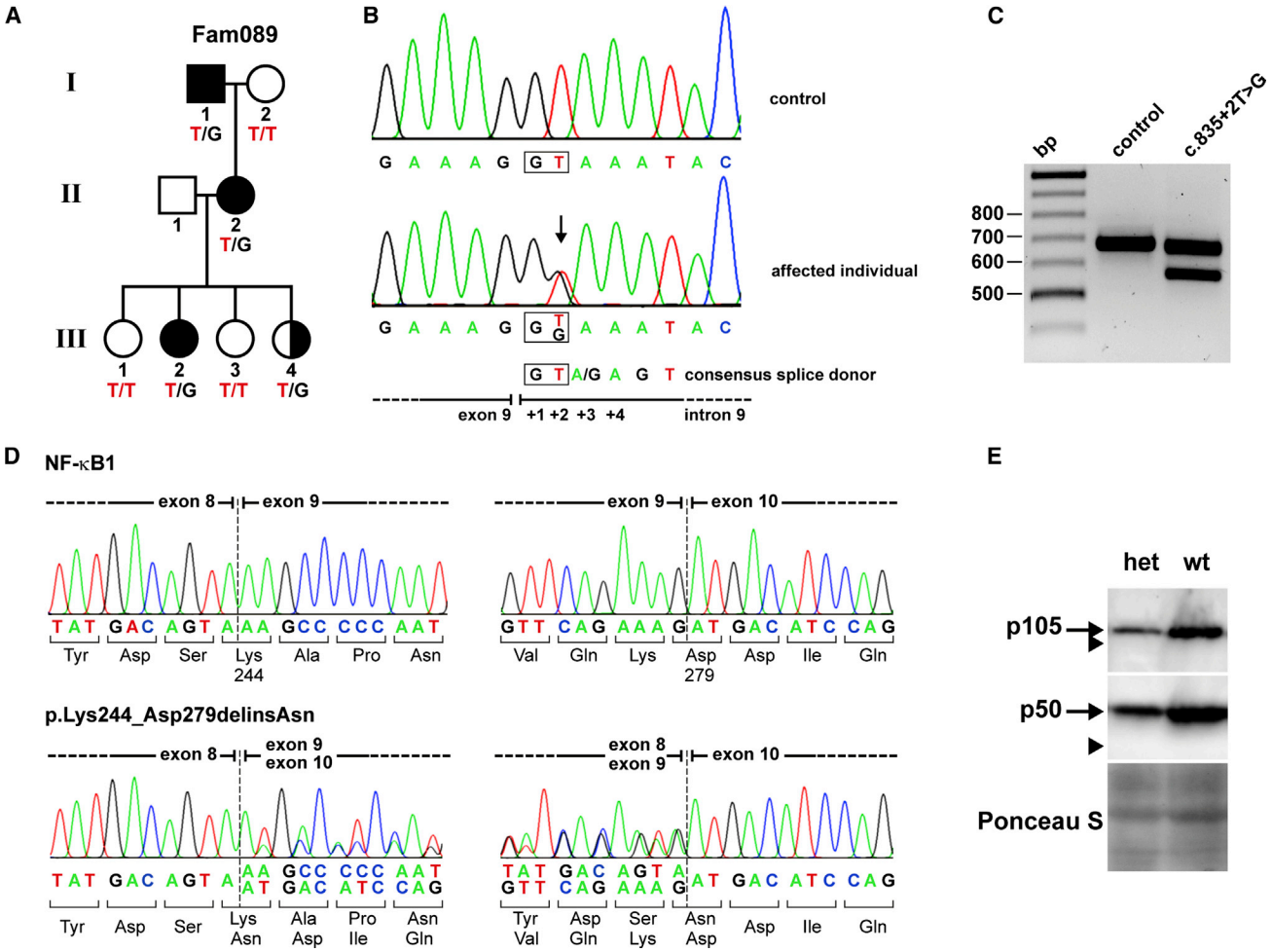


Figure 4. Haploinsufficiency of p50 Due to a Heterozygous Splice-Donor-Site Mutation in Intron 9 of *NFKB1*
(A) Autosomal-dominant inheritance of a heterozygous *NFKB1* mutation in family Fam089. Shaded symbols, affected members; half-shaded symbol, healthy members harboring the mutation.
(B) The mutation (c.835+2T>G) affects the splice-donor-site mutation in intron 9.
(C) RT-PCR shows an additional shorter band in EBV-transformed B cells from the proband, indicating exon skipping.
(D) cDNA sequencing demonstrates skipping of exon 9. In-frame fusion of exon 8 to exon 10 predicts a shortened NF-κB1 p105 (p105ΔEx9) with a 35-amino-acid internal deletion (p.Lys244_Asp279delinsAsn).
(E) Western blotting shows reduced amounts of p105 and p50 in EBV B cell lines from affected individuals (n = 2) in comparison to those in unaffected family members (n = 2) and healthy control cells (n = 2). The predicted altered proteins p105ΔEx9 and p50ΔEx9 are undetectable (arrowheads), indicating rapid elimination or mRNA decay. Representative results are shown.

p50ΔEx8) are highly unstable and probably non-functional, thus allowing NF-κB1-mediated transcriptional activation (via p105 and p50, translated from the non-mutant allele) to be sustained.

Identification of a Splice-Donor-Site Mutation in Intron 9 of *NFKB1* by Targeted NGS in a German CVID-Affected Family

To identify disease-causing mutations in a cohort of CVID-affected families, we screened candidate genes, including *NFKB1*, by targeted NGS. In one family, Fam089 (Figure 4A), we identified a second heterozygous splice-donor-site mutation (Figure 4B). In contrast to the mutation in FamNL1, this mutation affected intron 9 (c.835+2T>G [GenBank: NM_003998.3]). This sequence variant caused in-frame skipping of exon 9 (Figures 4C and 4D) and also resulted in haploinsufficiency of p50 (Figure 4E). Variant p50ΔEx9 (p.Lys244_Asp279delinsAsn) was not detected (Figure 4E and Figure S1).

Identification of a Frameshift Mutation in *NFKB1* by WES in a CVID-Affected Family from New Zealand

To identify the disease-causing mutation in a family from New Zealand with European descent (FamNZ; Figure 5A), genomic DNA was isolated from PBMCs of the male proband (FamNZ-II1), his mother (FamNZ- I2), and his affected sister (FamNZ-II2). Samples were then subjected to WES. We identified a total of 79 variants that were shared between the three sequenced exomes and which are rare in the European population (allele frequency ≤ 0.02), and are likely to affect the protein function. One of these variants was a *NFKB1* heterozygous frameshift

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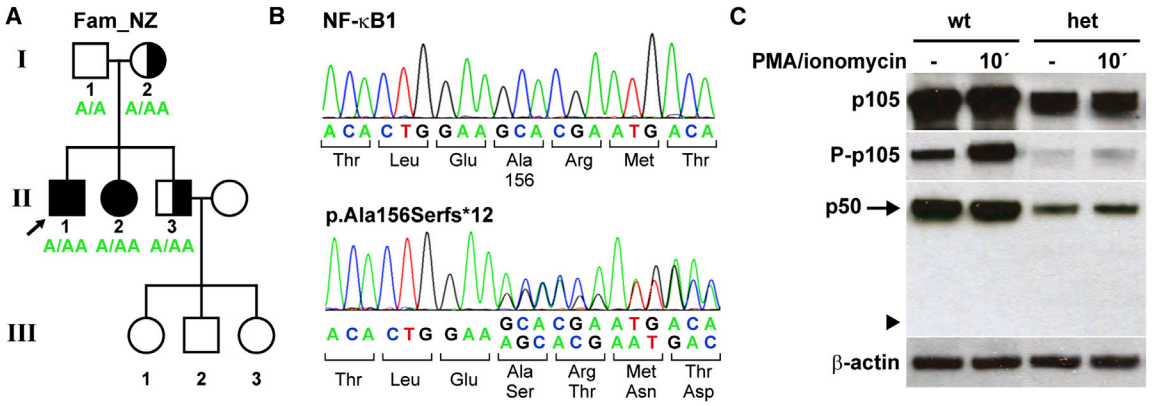


Figure 5. A Frameshift Mutation in *NFKB1* Causes p50 Haploinsufficiency in a CVID-Affected Family from New Zealand
(A) A heterozygous one-base-pair duplication (c.465dupA) in exon 7 was identified in the indicated family members. The three children in the third generation were not tested. Shaded symbols, affected members; half-shaded symbols, healthy members harboring the mutation.
(B) The single-base-pair insertion predicts a shift of the reading frame and the premature termination of translation (p.Ala156Serfs*12).
(C) PBMCs from affected family members (n = 3) and healthy control individuals (n = 3) were stimulated with PMA plus ionomycin as indicated and analyzed by western blotting. Representative results are shown. In affected individuals, normal p105 can only be derived from the healthy allele. Thus, p105 amounts, phosphorylation of p105, and processing to p50 are severely reduced in comparison to controls. The mutation predicts the translation of a non-functional severely truncated protein (~18 kDa in size), which is not detectable (arrowhead). The β-actin control confirms equal loading.

mutation, which was confirmed by Sanger sequencing in the proband, his mother, and his siblings (Figures 5A and 5B). The one base-pair duplication (c.465dupA [GenBank: NM_003998.3]) in exon 7 causes a premature termination of translation and predicts the translation of a non-functional, severely truncated protein (p.Ala156Serfs*12, ~18 kDa) that retains only the N-terminal part of the RHD and lacks all other functional domains (Figure S1B). This variant is absent from the 61,000 exomes of the Exome Aggregation Consortium cohorts and has not been reported previously.

To test whether a truncated 166-amino-acid protein is produced from the mutant allele, we analyzed PBMCs from affected family members and healthy control individuals by western blotting (Figure 5C). In heterozygous affected individuals, overall amounts and phosphorylation of p105, as well as p50 amounts, were severely reduced in comparison to those in the control individuals. Phospho-p105 was increased following activation of NF-κB-signaling in these cells, indicating that residual p105 translated from the non-mutant allele was normal. However, when we used the antibodies directed against the N-terminal amino acids, no truncated protein was detectable. Hence, it appears unlikely that NF-κB1-dependent signaling can be impaired by the presence of a remnant protein. We therefore conclude that, in the CVID-affected family FamNZ, the heterozygous c.465dupA mutation in *NFKB1* also causes a functional p50 haploinsufficiency.

Discussion

In this study, we demonstrated that two heterozygous splice-site mutations and a frameshift mutation affecting the RHD of *NFKB1* are associated with CVID in three unrelated families from diverse geographical regions. The mutations cause in-frame skipping of exon 8 or exon 9 or premature termination of translation, respectively. In each case, the altered protein (p105ΔEx8; p105ΔEx9, p.Ala156Serfs*12) is rapidly degraded, and no further processing to the truncated active protein (p50ΔEx8 or p50ΔEx9) occurs. Because p105 and p50 translated from the non-mutant alleles were apparently normal in all three pedigrees (with an overall amount of ~50% of that in healthy control individuals), we concluded that NF-κB1 haploinsufficiency accounts for the CVID phenotypes in the affected family members.

Maintenance of Residual NF-κB1 Function through Decay of the Altered Proteins

The in-frame deletions of either amino-acid residues 191–244 (corresponding to exon 8) or of amino-acid residues 244–279 (corresponding to exon 9) partially delete the N-terminal RHD (Figure S1B). Conversely, the C-terminal portion of p105, which determines the processing to p50 (via phosphorylation at S928 and S933, subsequent poly-ubiquitination at multiple sites, and proteasomal cleavage at amino-acid 433), is not affected by these mutations.

Because in-frame skipping can theoretically lead to aberrant proteins, we initially expected that the altered proteins p50ΔEx8 and p50ΔEx9 would be detectable in affected individuals and would interfere with NF-κB1-mediated signaling by impairing homo- or heterodimerization, nuclear translocation, and/or DNA binding. It is also conceivable that the altered p105ΔEx8 and p105ΔEx9 block the proteasome, causing false or delayed processing of p105 translated from the non-mutant allele, and thus exert a dominant negative effect on NF-κB1-mediated signaling. In contradiction to both hypotheses, we never

detected p50ΔEx8 or p50ΔEx9 in primary cells or EBV-transformed B lymphocytes derived from affected individuals by western blotting (Figures 2C and 2D) and only marginal amounts after forced transient transfection of p105ΔEx8 or even p50ΔEx8 in HEK293T cells (Figure 3A). Remarkably, fusion of GFP to their N termini did not substantially stabilize the altered p105ΔEx8 or p50ΔEx8 and only slightly delayed their degradation (Figures 3A and 3B). These observations are consistent with a previous report, demonstrating that an internal-deletion variant, p105Δ(356–498), was completely degraded by the 20S proteasome in vitro,[58] whereas deletion of the N-terminal 1–245 amino acids of p105 had no effect on p50 processing. Thus, the presence of mutant p105ΔEx8 or p105ΔEx9, which both undergo rapid protein decay, is most likely irrelevant to the disease etiology. This observation is supported by the third *NFKB1* mutation identified in this study. The frameshift mutation c.465dupA, if translated, would yield a 166-amino-acid protein (p.Ala156Serfs*12; including the N-terminal 155-amino-acid residues of p105 and p50, plus 11 amino acids encoded by the frameshifted sequence). However, no evidence was found for persistent amounts of a remnant protein.

Our results further suggest that NF-κB1 amounts are tightly regulated, and the heterozygous loss of p50 cannot be balanced, e.g., through increased expression from the non-mutant allele. It remains to be tested whether the ratio of cytoplasmic versus nuclear p50-RelA, before and after pathway stimulation, is also abnormal in cells derived from affected individuals. Such analyses (e.g., by methods based on confocal imaging) might be useful in future routine screenings of immunodeficiencies with NF-κB1 haploinsufficiency.

A functional haploinsufficiency of NF-κB1 p50 associated with immunodeficiency has also been reported in affected individuals with mutations in *NFKBIA* (*IKBA*).[59] In addition, NFKB2 haploinsufficiency has been acknowledged as the disease-causing mechanism of CVID-associated *NFKB2* mutations.[46–51] Furthermore, several reports have supported a functional haploinsufficiency as the predominant disease mechanism associated with various primary immunodeficiencies and autoimmune phenotypes caused by mutations in *TACI*,[60,61] *CTLA4*,[15,62] *NFAT5*[63] (MIM: 04708), *VAV1*[64] (MIM: 164875), *GATA2*[65] (MIM: 137295), and others. Thus haploinsufficiency, frequently associated with incomplete penetrance, appears as a recurrent paradigm and emphasizes the requirement for accurate gene regulation within the immune system.[66]

Other Possible Splicing Errors

The consensus splice donor site (see Figure 1B) predicts frequencies of 100% for both the guanine and the thymidine at the positions +1 and +2, respectively, but only of 70% for an adenine at position +4. Thus, the mutation c.835+2T>G in Fam089 probably completely inactivates the splice donor site in intron 9, whereas the mutant allele in FamNL1 (c.730+4A>G) could potentially retain residual splice-donor function. We cannot exclude, though, that alternative splice sites are used instead of the mutant sites or that other errors occur, thereby leaving the intron or parts thereof un-spliced. Accordingly, weak bands with aberrantly increased sizes were detectable under certain RT-PCR conditions (data not shown). However, these have not been further investigated because additional aberrant proteins have not been observed.

Variable Clinical Phenotype of CVID-Affected Individuals with p50 Haploinsufficiency

In addition to the adaptive immune response, the canonical NF-κB pathway plays many essential roles in innate immune responses such as toll-like receptor (TLR)-mediated signaling, cytokine production in response to microbial stimulation, and inflammatory responses. Upon initiation of the canonical NF-κB cascade, NEMO-containing IKK complexes lead to degradation of IκBα and release of NF-κB dimers. Thus, defects in NF-κB1 would also be expected to impair innate immune functions and, for instance, cause increased susceptibility to skin or airway infections. Conversely, individuals affected with innate immune system defects mostly have intact adaptive immune systems with normal B- and T-cell function and antibody amounts. The affected family members in our study were initially diagnosed with CVID, mainly based on laboratory findings, commonly with low immunoglobulin levels. Phenotypic observations of these family members were variable, comprising an increased susceptibility to infections, skin lesions, lymphoma, and inflammatory bowel disease. However, characteristic defects of the innate immune system were not directly obvious. Similarly, mutations in upstream components of NF-κB1, such as *TLR3* (MIM: 603029), *MYD88* (MIM: 602170), *IRAK4* (MIM: 606883), *HOIL1* (MIM: 610924), *IKBKG (NEMO)*, and *NFKBIA (IKBA)*, all lead to specific defects of the innate immune system.[67] A possible NF-κB1 dosage effect due to a promoter polymorphism might be associated with ulcerative colitis, given that reporter constructs with the −94delATTG allele showed less activity than constructs with the −94insATTG allele.[41] Thus, although variable, NF-κB1 p50 haploinsufficiency characterizes a unique disease entity.

Both, the Dutch-Australian family and the German family showed a highly variable age of onset of the CVID phenotype. For instance, individuals 18 and 21 in FamNL1 were only diagnosed at ages 65 and 57, respectively, whereas individual 62 in FamNL1 appears to be developing progressing CVID symptoms at the age of 30 years. She was diagnosed with CVID on the basis of the genetic result of the current study and is not yet on intravenous immunoglobulin (IVIG) replacement therapy. Similarly, individual I1 in Fam089 has only now been diagnosed with CVID at the age of 64 years. In contrast, individuals 49 from FamNL1 and II2 from Fam089 both presented with initial symptoms in early childhood. Additionally, the severity of

the disease varies considerably between individual members of each family. II2 from Fam089 shows a profound CVID phenotype associated with lymphoproliferation, lung disease, autoimmune cytopenias, and enteropathy, whereas her father (Fam089-I1) has an infection-only disease phenotype. In this family, the youngest family member, (Fam089-III4, 2 years of age) also harbors the mutation and has not developed any signs of disease to date but could develop disease later in her life. However, the penetrance of the various disease phenotypes at the different decades of life is currently not known.

In the New Zealand family, the heterozygous frameshift mutation c.465dupA is also associated with highly divergent disease phenotypes varying from asymptomatic to severe manifestations with malignancy and autoimmunity in addition to bronchiectasis. The reasons for the phenotypic variations, as well as for the variable age of disease onset, associated with p50 haploinsufficiency in this family remain speculative. It is possible that modifier genes, which are yet to be identified, account for the observed variability of penetrance and expressivity. Remarkably, both affected female members of this family, as well as the affected individual 34 in FamNL1, have alopecia, a phenotypic observation that has previously been associated with mutations in *NFKB2* (MIM: 164012).[48] However, we did not find evidence for additional mutations in other genes implicated in CVID when analyzing the index subjects by WES or NGS.

Abnormal B Cell Physiology in *Nfkb1*-Deficient Mice

Nfkb1$^{-/-}$ mice, which lack the homologous p50 subunit of NF-κB1, exhibit no developmental or lethal abnormalities and reproduce normally.[68] However, they show multifocal defects in immune responses involving B lymphocytes and nonspecific responses to infection. B cell dysfunction in these mice includes decreased proliferation, abnormal class-switch recombination, decreased marginal-zone B cell numbers, abnormal humoral immune response, and decreased immunoglobulin levels, with defective basal and specific antibody production.[21,22,60] Their phenotypic abnormalities also include increased susceptibility to infection with *S. pneumoniae*, large intestinal inflammation, abnormal CD4-positive T cell physiology, and abnormal secretion of cytokines. Interestingly, late-onset alopecia has recently been reported in *Nfkb1*$^{-/-}$ mice,[69] similar to the phenotype observed in FamNZ and FamNL1 in this study. With regard to the disease symptoms in CVID-affected persons with *NFKB1* mutations, analyzing the effects of p50 haploinsufficiency in *Nfkb1*$^{+/-}$ mice will be an interesting task of future investigation.

In this study, we employed NGS technologies to identify three distinct mutations in *NFKB1* in three CVID-affected families. The identification of mutations in this central regulator of inducible gene expression in the immune system could advance our understanding of the molecular pathomechanisms that lead to primary immunodeficiencies.

Supplemental Data

Supplemental Data include case reports and one figure and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2015.07.008.

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Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, http://www.1000genomes.org/
Burrows-Wheeler Aligner, http://bio-bwa.sourceforge.net
Ensembl Genome Browser, http://www.ensembl.org/index.html
ExAC Browser, http://exac.broadinstitute.org/
IGV, http://www.broadinstitute.org/igv/
NF-κB target genes, www.bu.edu/nf-kb/gene-resources/target-genes
OMIM, http://www.omim.org/
Picard, http://broadinstitute.github.io/picard
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq

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