Disease mechanism in two novel primary immunodeficiencies characterized by increased viral susceptibility and immune dysregulation

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Abstract

Primary Immunodeficiency Diseases (PIDs) are a heterogeneous group of genetic disorders characterised by malfunctioning of the immune system that predisposes to different patterns of infections, allergy, autoimmunity and cancer. To date, more than 300 gene defects have been identified to cause PIDs. PIDs are considered to be 'experiments of nature' as they provide in vivo assessment of the functional consequences when specific genes are defective and help our understanding of the basic cellular pathways and mechanisms of host defence in the human immune system. For this reason, many clinical and molecular studies have focused on the forward genetic potential of PIDs.

In my study, I identified two novel PIDs characterised by increased viral susceptibility and immune dysregulation. Firstly, I investigated a patient who developed fatal illness after routine immunization with live attenuated vaccine measles, mumps and rubella (MMR) vaccine. By investigating type I interferon (IFN) signalling and performing targeted sequencing, I identified a homozygous mutation in the IFNα receptor 2 (*IFNAR2*) as the causative variant in the patient as well as in a newborn sibling. This phenotype emphasises the important role played by type I IFN signalling in antiviral immunity.

Secondly, I investigated two siblings from a consanguineous marriage who presented with immune dysregulation characterised by lymphoproliferative disease (chronic lymphadenopathy, hepatosplenomegaly progressing to lymphoma) and susceptibility to herpesviruses. In addition, both patients developed autoimmunity. A clinical diagnosis of autoimmune lymphoproliferative syndrome (ALPS) was made, supported by laboratory findings that showed impaired FAS-mediated apoptosis and increased double negative (DN) T cells. By using the approach of combined whole exome sequencing and homozygosity mapping, I identified a homozygous missense mutation of Ten Eleven Translocation 2 (*TET2)* as the disease causing variant. TET2 is involved in epigenetic regulation of gene expression by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in the DNA demethylation process, amongst other mechanisms.

The identification of these two novel gene defects in the two different PIDs provides new understanding and broadens our knowledge about physiological and pathophysiological pathways in the human immune system. Furthermore, knowledge of these genetic defects can help in providing better care and treatment to future patients.

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Declaration

The candidate confirms that the work submitted is her own work under the guidance of supervisors, Professor Sophie Hambleton, Dr Karin Engelhardt and Professor Matthew Collin. Except for commonly held concepts, and where specific reference is made to other work, the content of the thesis is original. The work in this thesis was performed from December 2011 to December 2015. Most work was carried out in Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, UK and minor work was performed in Richard Cornall's lab, The Wellcome Trust Human Genetics Lab, Oxford University, UK. No part of this thesis has been submitted for the award of any other degree.

Contents

Abstract i

Acknowledgement iii

Attributed work iv

Declaration v

Contents vi

List of Figures x

List of Tables xiii

List of Abbreviations xv

Chapter 1: Introduction 1

1.1 Primary Immunodeficiency Diseases (PIDs) 1

1.1.1 Overview 1

1.2 PIDs involving innate antiviral immunity 3

1.2.1 Overview 3

1.2.2 Innate Antiviral immunity 4

1.3 PIDs involving adaptive Immunity 18

1.3.1 Overview 18

1.3.2 T lymphocyte biology and function 18

1.3.3 B lymphocyte biology and function 30

1.4 PIDs involving immune dysregulation 37

1.4.1 Overview 37

1.4.2 Autoimmune lymphoproliferative syndrome (ALPS) 38

1.4.3 Haemophagocytic Lymphohistiocytosis (HLH) 43

1.5 Approaches to the identification of defective genes of PIDs 48

1.5.1 Overview 48

1.5.2 Investigating known signalling pathways 48

1.5.3 Unbiased genetic approaches 49

1.5.4 Similarity of the clinical phenotypes to mouse models 52

1.5.5 The importance of molecular diagnosis for the patient and family members 53

1.6 Aims of the study 54

Chapter 2: Materials and Methods 55

2.1 Study subjects 55

2.2 Materials 55

2.3 Methods 62

2.3.1 Preparation and culture of human cells 62

2.3.2 DNA methods 66

2.3.3 RNA methods 69

2.3.4 PCR based methods 71

2.3.5 Western blot 75

2.3.6 Immunofluorescence Microscopy 77

2.3.7 TET2 wild type plasmid cloning 78

2.3.8 Site Directed Mutagenesis 80

2.3.9 Fas-mediated apoptosis assay for mouse B cells 82

Chapter 3: A novel defect in the Type 1 IFN signalling pathway 85

3.1 Introduction 85

3.2 Hypothesis 87

3.3 Aims 87

3.4 Results 87

3.4.1 Clinical case 87

3.4.2 Increased susceptibility to viral infection and lack of protection from IFNα in patient fibroblasts 91

3.4.3 Failure of ISG upregulation in response to IFNα in patient fibroblasts, despite preserved expression of STAT1 and STAT2. 93

3.4.4 Absence of antiviral transcriptional responses to IFNα and IFNβ but preserved transcriptional responses to IFNγ in patient cells 94

3.4.5 Absent phosphorylation of STAT1, STAT2 and STAT3 in response to IFNα but intact STAT1 phosphorylation in response to IFNγ............ 98
3.4.6 Homozygous deletion in exon 5 of IFNAR2............................... 101
3.4.7 Absence of IFNAR2 protein expression in patient fibroblasts..... 104
3.4.8 Reconstitution of IFNAR2 into patient fibroblasts restores IFNα/β responses... 105
3.4.9 Homozygous deletion in IFNAR2 in exon 5 in newborn sibling of the index patient.. 107
3.5 Discussion 108
Chapter 4: A novel autoimmune lymphoproliferative disorder.................... 113
4.1 Identification of the disease causing variant 113
4.1.1 Introduction.. 113
4.1.2 Hypothesis... 115
4.1.3 Aims 115
4.1.4 Results 115
Chapter 4: A novel autoimmune lymphoproliferative disorder.................... 131
4.2 : Effect of TET2 p.H1382 variant on its functional activity................ 131
4.2.1 Introduction.. 131
4.2.2 Summary 145
4.2.3 Hypothesis... 145
4.2.4 Aims 145
4.2.5 Results 146
4.2.6 Discussion 158
Chapter 4: A novel autoimmune lymphoproliferative disorder.................... 164
4.3 Effect of TET2 p.H1382R variant on lymphocyte apoptosis............. 164
4.3.1 Introduction.. 164
4.3.3 Aims 169
4.3.2 Hypothesis... 170
4.3.4 Results 170

4.3.5 Discussion 187
Chapter 5: Discussion and future work 191
Clinical impact 191
Scientific implications.. 192
References.. 197
Appendix.. 221
Oral presentation 226
Publication arising from this study.. 227

List of Figures

Figure 1.1: Pathways of type I Interferon (IFNα/β) induction………… 8

Figure 1.2: Signalling pathways activated by IFNα/β………………….. 10

Figure 1.3: Known mutations affecting the IFN pathway……………… 17

Figure 1.4: Overview of T cell development in the thymus……………. 20

Figure 1.5: TCR signalling cascade……………………………………... 24

Figure 1.6: BCR signalling cascade……………………………………... 33

Figure 1.7: Overview of B-cell development…………………………… 36

Figure 2.1 : A sandwich-based ELISA format………………………….. 68

Figure 3.1 : Type I and Type II IFN signalling pathway……………….. 86

Figure 3.2 : Brain biopsy showed the histological appearances of meningoencephalitis……………………………………………………… 91

Figure 3.3 Increased susceptibility to viral infection in patient fibroblasts……………………………………………………………… 92

Figure 3.4: Lack of protection from IFNα in patient fibroblasts……….. 93

Figure 3.5 : Failure of ISG upregulation in response to IFNα in patient fibroblasts despite preserved expression of STAT1 and STAT2…………………………………………………………………… 94

Figure 3.6 : Absence of transcriptional responses to IFNα and IFNβ but preserved transcriptional response to IFNγ by microarray……….. 95

Figure 3.7 : Absence of STAT1, STAT2 and STAT3 phosphorylation in response to IFNα in patient fibroblasts……………………………….. 99

Figure 3.8 : Preserved STAT1 phosphorylation in response to IFNγ in patient fibroblasts…………………………………………………….. 100

Figure 3.9 : Localization of the causative genetic lesion to IFNAR1, IFNAR2 or TYK2…………………………………………………………… 101

Figure 3.10: Deletion of A311 (c.A311del) in exon 5 in IFNAR2 gene using patient cDNA……………………………………………………... 102

Figure 3.11 : Homozygous deletion of A311 in exon 5 of IFNAR2 gene in patient and heterozygous deletion of A311 in both father and mother genomic DNA (gDNA)…………………………………………… 103

Figure 3.12: Absence of IFNAR2 protein expression in patient fibroblasts……………………………………………………………… 104

Figure 3.13 : Complementation of IFNAR2 in patient fibroblasts

restores responsiveness to IFNα…………………………………………… 105
Figure 3.14: Complementation of IFNAR2 protects patient fibroblasts against viral infection…………………………………………………… 106
Figure 3.15: Homozygous deletion in IFNAR2 in exon 5 in newborn baby………………………………………………………………………. 107
Figure 3.16 : Absence of IFNAR2 expression and MxA induction in newborn PBMCs……………………………………………………………………… 108
Figure 4.1.1 : Fas-mediated apoptosis patient 1 and patient 2………. 121
Figure 4.1.2 : Haemoglobin, platelets, total lymphocytes, total white cells, monocytes and neutrophil absolute counts in peripheral blood of patient 1 and patient 2………………………………………………. 122
Figure 4.1.3 : Absolute numbers of indicated lymphocyte subsets (CD4, CD3, CD8, CD19 and CD16+56+) and percentages of activated T cells……………………………………………………......... 123
Figure 4.1.4 : Whole exome sequencing and homozygosity mapping of patient 1 and patient 2………………………………………………. 125
Figure 4.1.5: Homozygous TET2 mutation in Patient 1……………….. 128
Figure 4.1.6 : PCR gel electrophoresis, pedigree of the family and sequencing of patients and family members…………………………… 129
Figure 4.2.1 : Structure of TET2 protein and DSBH Core…………….. 135
Figure 4.2.2 : Mechanisms of DNA demethylation involving TET proteins……………………………………………………………………... 136
Figure 4.2.3: The localization and types of TET2 mutations that had been discovered in acute myeloid cancers patients………………….. 138
Figure 4.2.3 : TET2 protein expression in patients and control fibroblasts…………………………………………………………………… 146
Figure 4.2.5 : Three TET2 plasmids for the recombinant system……. 148
Figure 4.2.6 : The catalytic activity of TET2 is compromised by TET2 p.H1382R mutation……………………………………………………….. 150
Figure 4.2.7 : 5hmC level in a human embryonic stem cell line (H9), in iPSCS from healthy control and in patient iPSCs analysed by Immunofluorescence…………………………………………………… 152
Figure 4.2.8 : Optimisation of the dot blot assay for measuring the

5hmC level in control DNA………………………………………………….. 154
Figure 4.2.9 : Percentage of 5hmC in patients and controls iPScs/hESCs quantified by ELISA………………………………………. 155
Figure 4.2.10 : Percentages of global 5hmC in DNA from patient and control B cells quantified by ELISA……………………………………… 156
Figure 4.2.11 : TET1, TET2 and TET3 expression levels in control B cells………………………………………………………………………. 157
Figure 4.3.1 : The intrinsic and extrinsic pathways of apoptosis…….. 167
Figure 4.3.2 : Principal Component Analysis (PCA) plot……………… 172
Figure 4.3.3 Principal Component Analysis (PCA) plot after batch correction……………………………………………………………… 174
Figure 4.3.4 : Volcano plot of differentially expressed genes in patient vs control………………………………………………………... 175
Figure 4.3.5: 15 dysregulated genes from the microarray analysis that regulate apoptosis identified by Gene Ontology (GO) Terms....... 176
Figure 4.3.6 : Validation of the microarray data by qRT-PCR………… 178
Figure 4.3.7 : Annexin V vs Propidium Iodide (PI) staining analysed by flow cytometry……………………………………………………... 180
Figure 4.3.8 : B cell apoptosis assay for wild type (WT) and TET2 Knock out (KO) mice…………………………………………………....... 182
Figure 4.3.9 : T cell apoptosis assay in wild type mice………………... 183
Figure 4.3.10 : T cell apoptosis assay in three wild type (WT) and three Tet2 knock out (KO) mice………………………………………….. 184
Figure 4.3.11 : CD4+ T cell apoptosis assay with wild type mouse T cells………………………………………………………………………. 185

List of Tables

Table 1.1. 10 warning signs of immune deficiency in paediatric suggested by 'The Jeffrey Modell Foundation'………………………… 2
Table 1.2. Diagnostic criteria for ALPS………………………………… 40
Table 1.3: Revised classification ALPS and ALPS-related disorders.. 41
Table 1.4: 2004 Diagnostic Guideline for HLH……………………… 47
Table 2.1 : Cell culture reagents and medium used for the research.. 55
Table 2.2 : Cytokines used for the research…………………………… 56
Table 2.3: List of antibodies used in study…………………………... 57
Table 2.4 : Chemical and biochemical reagents used for the research……………………………………………………………. 58
Table 2.5 : List of buffers used for assays……………………………… 59
Table 2.6 : Primers used for standard PCR and Sanger Sequencing. 60
Table 2.7 : Oligonucleotide primers and probes used for Real time PCR……………………………………………………………………… 61
Table 2.8 : List of commercially available kits used for the research... 62
Table 2.9 : Standard PCR reagents…………………………………….. 72
Table 2.10 : PCR cycling condition……………………………………… 72
Table 2.11 : SDM reagents………………………………………………. 81
Table 2.12: PCR reactions……………………………………………….. 81
Table 2.13: sFasL and enhancer ligands concentration……………… 83
Table 2.14: Antibodies that were used for apoptosis, cells death and B cell detection………………………………………………………….. 83
Table 3.1: Patient immunological parameters………………………… 89
Table 3.2 : Vaccine strain measles, mumps and rubella and HHV6 were detected by PCR or viral culture in the patient………………….. 90
Table 3.3: Antibody response to measles, mumps and rubella……… 90
Table 3.4 : 40 highest upregulated ISGs in IFNα-stimulated control fibroblasts and extent of induction by alternative IFNs or in IFNγ-treated patient fibroblasts………………………………………………… 97
Table 4.1.1: Clinical features of patient 1 (pre and post transplant) and patient 2………………………………………………………………….. 120
Table 4.1.2 : Immunoglobulin levels of patient 1 and patient 2 pre- and post-transplant……………………………………………………….. 124

Table 4.1.3 : Shared homozygous regions that were detected by homozygosity mapper in patient 1 and patient 2………………………. 127
Table 4.2.1 : Animal models and phenotypes pf TET2-deficient mice. 142
Table 4.2.2 : Similar phenotypes in both patients and TET2-deficient mice……………………………………………………………………. 159
Table 4.3.1 : Pro- and anti-apoptotic genes that were identified to be differentially expressed in patient B cells compared to controls…….... 177

List of Abbreviations

5caC	5-carbyxlcytosine
5fC	5-formylcytosine
ALPS	Autoimmune lymphoproliferative syndrome
AML	Acute myeloid leukemia
ANA	Antinucelar antibody
APCs	Antigen presenting cells
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
ASFV	African swine flu virus
BAFF	B cells activating factor
BCG	Bacillus Calmette-Guérin
Bcl2	B cells lymphoma 2
BCR	B cell receptor
BMA	Bone marrow aspiration
BMT	Bone marrow transplant
CD40L	CD40 ligand
cDC	Conventional dendritic cells
cDNA	Complementary DNA
CLP	Common lymphoid progenitor
CMC	Chronic mucocutaneous candidiasis
CMML	Chronic myelomonocytic leukemia
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CSR	Class switching recombination
CTL	Cytolytic T cells
CVID	Common variable immune deficiency
DAPI	4',6-diamidino-2-phenylindole
DAT	Direct antiglobulin test
DISC	Death inducing signalling complex
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	deoxyribonucleic acid

DNMT	DNA methyltransferase
DNT	Double negative T cells
dNTP	Deoxynucleotide
DSBH	Double stranded beta helix
dsRNA	Double stranded ribonucliec acid
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FADD	Fas associated death domain
FasL	Fas Ligand
FOXP3	Forkhead box P3
GAF	Gamma-Interferon Activation Factor
GATA3	GATA binding protein 3
gDNA	Genomic DNA
GFP	Green Fluorescent protein
GMP	Granulocyte macrophage progenitor
HATs	Histone acetyltransferase
HBSS	Hanks Buffered Saline Solution
HCF1	Host cell factor 1
HCV	Hepatitis C virus
HDACs	Histone deacetylase
HEK293T	Human embryonic kidney 293 T cells
hESC	Human embryonic stem cells
HHV6	Human Herpes Virus 6
HL	Hodgkin lymphoma
HLH	Hameophagocytic Lymphohistiocytosis
hMEDIP	Hydroxymethylated DNA immunoprecipitation
HPLC	High performance liquid chromatography
HPV-16	Human papilloma virus-16
HRP	Horseradish peroxidase
HSCs	Haematopoeitic stem cells
HSCT	Haematopoeitic Stem Cells Transplant
HSE	Herpes simplex encephalitis

HSV	Herpes Simplex Virus
IAP	Inhibitor of apoptosis
IDH1/2	Isocitrate dehydrogenase 1/2
IFN	Interferon
IFNAR1	Interferon alpha receptor 1
IFNAR2	Interferon alpha receptor 2
IFNGR	Interferon gamma receptor
IFNβ	Interferon beta
IFNγ	Interferon gamma
IFNλ	Interferon lambda
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukin-10
IL-10Rβ	Interleukin-10 receptor beta
IL-2	Interleukin-2
IL17	Interleukin-17
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
iPSCs	Induced pluripotent stem cells
IRAK-4	interleukin-1 receptor-associated kinase 4
IRF3	Interferon regulatory factor 3
ISG56	Interferon stimulated genes-56
ISGF3	Interferon stimulated gene factor 3
ISRE	IFN-stimulated response elements
JAK	Janus kinase
KO	Knock out
LB	Luria Bertani
LCMV	Lymphocytic Choriomeningitis Virus
LPS	Lipopolysaccharide
LRBA	LPS-responsive beige-like anchor
MAPK	Mitogen activated protein kinase
MAS	Macrophages activation synrome
MDS	Myelodysplastic syndrome

MeDIP	Methylated DNA immunoprecipitation
MHC	Major histocompatibility complex
MMR	Measles mumps rubella
MPD	Myeloproliferative disease
MPN	Myeloproliferative neoplasm
mRNA	Messenger ribonucleic acid
MZ	Marginal zone
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing
NHL	Non Hodgkin lymphoma
NK cells	Natural killer cells
NOG	N-oxalylglycine
nTregs	Natural T regulatory cells
OGT	O-Linked N-Acetylglucosamine (GlcNAc) Transferase
PAMPs	pathogen associated molecular patterns
pAPCs	Professional antigen presenting cells
PBMC	Peripheral blood mononuclear cells
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principle component analysis
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PHA	Phytohaemagglutinin
PICU	Paediatric intensive care unit
PIDs	Primary Immunodeficiencies
PIV5	ParaInfluenza virus 5
PRRs	Pathogen recognition receptors
pSTAT	Phospho STAT1
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real time polymerase chain reaction
RAG1	Recombination activating gene 1
RF	Rheumatic factor
RIN	RNA integrity number

RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
RSN	Robust spline normalisation
RSV	Respiratory syncitial virus
RT	Room temperature
RUNX	Runt-related transcription factor
SCID	Severe combined immune deficiency
SDS	Sodium dodecyl sulfate
sFasL	Soluble Fas Ligand
SFV	Semliki forest virus
SHM	Somatic hypermutation
SNP	Single nucleotide polymoprhism
SOB	Super Optimal Broth
SP	Sinlge positive
STAT	Signal transducer activators transcription
SV	Simian virus
TAE	Tris-acetate-EDTA
TBST	Tris Buffered-Tween Saline Solution
TCR	T cell receptor
TET	Ten Eleven Translocation
Tfh	T follicular helper cells
TGF-β	Transforming growth factor beta
Th1	T helper 1
Th17	T helper 17
TLR3	Toll like receptors 3
TNF	Tumour necrosis factor
TNFSR	Tumor necrosis factor soluble receptor
TNFα	Tumourn ecrosis factor alpha
Tr1	Type I regulatory cells
Tr3	Type 3 regulatory cells
TRAF	TNF receptor associated factor
Treg	T regulatory cells
TYK2	Tyrosine kinase 2
VAHS	virus-associated HLH syndrome

VST	Variance stabilising transformation
VSV	Vesicular stomatitis virus
WES	Whole exome sequencing
WGS	Whole genome sequencing
XLP	X-linked lymphoproliferative disease

Chapter 1: Introduction

1.1 Primary Immunodeficiency Diseases (PIDs)

1.1.1 Overview

Primary Immunodeficiency Diseases (PIDs) are a heterogeneous group of genetic disorders characterised by malfunctioning of the immune system (McCusker and Warrington, 2011). The immune system comprises of innate and adaptive immunity in which innate is the first line of defence against infections whereas adaptive immunity occurs later after innate immune responses take place. Defects within either of these two main components of the immune system may result in immunodeficiency. PIDs fully meet the definition of "experiments of nature" (Garrod, 1924; Ochs *et al.*, 1999) in that they provide in vivo assessment of the functional consequences when specific genes are defective. For this reason, many clinical and molecular studies have focused on the forward genetic potential of PIDs (Ochs *et al.*, 1999; Fischer, 2007).

The number of genetically defined PIDs has grown from only a handful in 1990 (Ochs *et al.*, 1999), to more than 300 distinct disorders by 2015 (Picard *et al.*, 2015). With the existence of increasingly advanced diagnostic technologies and fruitful international collaborations between PID centres worldwide, it is expected that more PIDs will be understood at genetic level. It should be noted that secondary immunodeficiencies can occur as a result of malnutrition, infections or immunosuppressive drug treatments (McCusker and Warrington, 2011) and may lead to similarly impaired development and function of the immune system (Notarangelo, 2010). Most of the genetic changes described in PIDs are loss of function mutations, however, there are several that lead to gain of function (Fischer, 2007).

It is estimated that the prevalence of overall PIDs is 1:10 000 to 1:100 000 (Joshi *et al.*, 2009; Bousfiha *et al.*, 2013; Nijman *et al.*, 2014) of live births and thus that it is a rare disease, although individual PIDs disorders vary in incidence (Chapel *et al.*, 2014). The clinical presentation of PIDs can be as early as within few days of life to adulthood, and while symptoms may be mild in certain forms, other PIDs are severe or fatal in infancy. Early diagnosis has a

demonstrably favourable impact on outcome for many PIDs. Hence, the Jeffrey Modell Foundation has developed a list of 10 warning signs in order to assist in the early recognition of PID (Table 1.1). The following criteria should raise suspicion: recurrent sinus or ear infections or pneumonias within a one year period; failure to thrive; poor response to prolonged use of antibiotics; persistent thrush or skin abscess; or family history of PID(McCusker and Warrington, 2011).

1. ≥ 8 new ear infections with in 1 year.
2. ≥ 2 serious sinus infections within 1 year.
3. ≥ 2 months on antibiotics with little effect.
4. ≥ 2 pneumonias with in 1 year.
5. Failure of an infant to gain weight or grow normally.
6. Recurrent, deep skin or organ abscesses.
7. Persistent thrush in mouth or elsewhere on skin, after age 1.
8. Need for intravenous antibiotics to clear infections.
9. ≥ 2 deep-seated infections.
10. A family history of PID.

Table 1.1. 10 warning signs of immune deficiency in paediatrics suggested by 'The Jeffrey Modell Foundation'. This table is reproduced from Jeffrey Modell Foundation: Primary Immunodeficiency Resource Centre. Available at http://www.info4pi.org/library/educational-materials/10-warning-signs.

Most PIDs present with increased susceptibility to infection (McCusker and Warrington, 2011). The pattern of susceptibility can be restricted to a certain pathogen (e.g. candida species or herpes simplex encephalitis) or extend to various pathogen types (e.g. common variable immune deficiency (CVID)) (McCusker and Warrington, 2011). Nevertheless, with increasing numbers of identified PIDs, more PIDs have also been associated with other clinical presentations such as autoimmunity and malignancy (Ochs *et al.*, 1999). For example, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome and autoimmune lymphoproliferative syndrome (ALPS) are all monogenic disorders that present with dysregulation of the immune system.

As the numbers of molecular defects known to be associated with PID are increasing tremendously every year, the classification of PIDs are revised and updated frequently by the International Union of Immunological Societies expert committee. In the latest update (2015), PIDs were classified into nine major categories on the basis of the affected immunological compartment including combined immunodeficiencies, antibody deficiencies, diseases of immune dysregulation and defects of innate immunity (Picard *et al.*, 2015). It is quite difficult to adhere strictly to the classification as most PIDs are complex diseases in which certain conditions can be classified under several categories (Picard *et al.*, 2015). In addition, different mutations of the same gene may cause different disease phenotypes, complicating both diagnosis and disease classification. As it is expected that more new PIDs will be discovered, the classification will also undergo major revision and update.

Since my work centres around novel PIDs with viral susceptibility and immune dysregulation, subsequent sections will introduce normal immune responses involving innate antiviral immunity, adaptive immunity and immune homeostasis as well as a selection of PIDs where these mechanisms are defective. Finally, at the end of this chapter, I will discuss approaches that have been used in the identification of disease causing variants in PIDs and the importance of identifying the molecular defect for the patients and family members.

1.2 PIDs involving innate antiviral immunity

1.2.1 Overview

Innate immunity refers to non-specific protective mechanisms that act as the first line of defence against pathogens. Components of innate immunity include physical and chemical barriers, such as epithelial surfaces of the skin, respiratory tract and gastrointestinal tract, which prevent the entry of microbes, and the pH acidity in the stomach, which prevents the growth of pathogens (Abbas *et al.*, 2007). Nucleated cells possess a variety of intrinsic, anti-infective mechanisms that are activated by exposure to microbes and can be powerfully amplified by cytokines such as interferons. The immune system contains specialised innate effectors such as dendritic cells, neutrophils, macrophages, Natural Killer (NK) cells and circulating plasma proteins including members of the complement system.

Viral infections in children and young adults are mainly asymptomatic or result in mild clinical presentations. These patients are able to mount an effective immune response that either eliminates the virus from the body or restricts it to a latent or low level form without further problems. The innate antiviral immune response plays an important role in controlling viral infections at the early stage of the infections. However, it is insufficient to clear viral pathogens. Therefore it requires the adaptive immune response to be generated, in order to fully eradicate the viral infection.

Individuals with primary immunodeficiency disorders have a genetic defect causing an inability to mount effective immune responses. Recently, susceptibility to viral infections due to genetic defects of innate antiviral immunity has been described. Some of these mutations predispose to specific viral infections, while others predispose to viruses and other pathogens (Dropulic and Cohen, 2011). In order to understand how these mutations result in increased viral susceptibility, it is necessary to know how innate antiviral immunity functions in healthy individuals.

1.2.2 Innate Antiviral immunity

1.2.2.1 Virus replication

Viruses are intracellular pathogens that infect and replicate within host cells and exploit the host's cellular machinery for their survival. Viruses exist outside the cell as virion particles, each of which contains a genome consisting of either a double stranded or single stranded DNA or RNA wrapped in a protein coat called a capsid (Strauss and Strauss, 2008). Viruses infect the cells through attachment to a surface receptor which leads to the penetration of the genome into the cytoplasm. The genome is then transcribed, translated, and replicated (Strauss and Strauss, 2008). Most viruses bud from the cell, thereby producing progeny virions which then can infect new cells. Some viruses however spread from cell to cell without forming new virions. The host cell may be killed as a direct result of virus infection, but certain viruses can persist in live cells especially in a transcriptionally inactive or "latent" form.

Healthy individuals have the ability to control viral infections through effective innate and adaptive antiviral immune responses. Critical mediators in the innate immune response are interferons (IFNs), which are cytokines that induce the

transcription of hundreds of genes (the IFN-stimulated genes, ISGs) involved in blocking or impairing viral replication in the host cells. In addition, IFNs also have a profound immunomodulatory effect and stimulate the adaptive immune response (Goodbourn *et al.*, 2000).

1.2.2.2 Interferons (IFNs)

Interferons are widely expressed cytokines that have an antiviral effect, regulate cell growth and activate the immune system (Goodbourn *et al.*, 2000). The interferons are classified into three distinct types, type I, II and III. Type I IFNs are produced in direct response to virus infection and include IFNα (which can be subdivided into 13 subtypes IFNα1, -α2,- α4, -α5,- α6, -α7, -α8, -α10, -α13, -α14, -α16, -α17 and -α21), IFNβ and others of unknown significance in humans such as IFN-δ, IFN-ε, IFN- ƙ, IFN-т and IFN-ω (Platanias, 2005). Most nucleated cells can both produce IFNα/β and respond to it through the ubiquitously expressed type I IFN receptor, which is composed of two subunits, IFNα-receptor 1 (IFNAR1) and IFNα-receptor 2 (IFNAR2) (McNab *et al.*, 2015). Mouse models have played a pivotal role in understanding type I IFN biology. Mice lacking either IFNAR1, IFNAR2 or components of the downstream JAK/STAT signalling pathway were shown to be extremely susceptible to a large number of viruses such as vesicular stomatitis virus (VSV), Semliki Forest virus (SFV), vaccinia virus, lymphocytic choriomeningitis virus (LCMV) (Muller *et al.*, 1994; Van Den Broek *et al.*, 1995; Meraz *et al.*, 1996; Park *et al.*, 2000), dengue virus (Züst *et al.*, 2014), herpes simplex virus 2 (Ank *et al.*, 2008) and Theiler’s virus (Fiette *et al.*, 1995).

IFNγ is the only member of type II IFNs. Rather than being induced directly by viral infection, IFNγ is predominantly synthesised in response to the recognition of infected cells by T cells and natural killer (NK) cells and can act on broad ranges of cells that express the IFNγ receptor (Goodbourn *et al.*, 2000; Platanias, 2005). Similarly to type I IFN, type II IFN also has antiviral properties and its antiviral role was demonstrated by mouse models that lack the IFNγ receptor. These mice were shown to be unable to mount efficient responses to viruses including Theiler’s virus, LCMV and vaccinia virus (Huang *et al.*, 1993; Muller *et al.*, 1994; Fiette *et al.*, 1995; Van Den Broek *et al.*, 1995). Importantly, the mouse studies revealed that for the control of certain viruses, both type I

and type II IFNs are required, each playing a non-redundant role in the antiviral defence.

Type III is another class of IFNs, comprising IFN-λ1, -λ2, -λ3 and newly identified –λ4 (O'Brien *et al.*, 2014). Type III IFNs signal through their receptor complex consisting of IL-10Rβ and IL-28Rα chains (Ank *et al.*, 2008). Type III IFNs are directly induced by virus infection and although their receptors are different, they signal via the same JAK/STAT pathway as type I IFN (Onoguchi *et al.*, 2007; Ank *et al.*, 2008). In addition, type III IFN also triggers similar antiviral effects as type I IFN by inducing the expression of the same set of genes (Sommereyns *et al.*, 2008). However, expression of the IFNλ receptor is mainly on epithelial surfaces (Sommereyns *et al.*, 2008; Witte *et al.*, 2010), in contrast to IFNα/β receptors which are expressed in most cell types. Due to this, it is suggested that innate protection against viral invasion at the epithelia, for example skin and mucosal surfaces, is mainly governed by type III IFN (Ank *et al.*, 2008; Sommereyns *et al.*, 2008; Durbin *et al.*, 2013). In contrast to type I IFNs, the pathways or mechanisms that induce the production of type III IFNs are still unclear.

1.2.2.3 Induction of Type I IFN

Type I IFNs, IFNα and IFNβ, are produced early in response to viral infections (Biron, 1998). Most cells can produce IFNα/β but plasmacytoid dendritic cells (pDC) do so particularly readily and are a major source of IFNα in humans (Colonna *et al.*, 2002). When cells are infected, it results in the stimulation of receptors known as pattern recognition receptors (PRRs) which are located in the cytosol or endosomal compartment (Muller *et al.*, 1994). These receptors are able to detect pathogen-associated molecular patterns (PAMPs), e.g. viral nucleic acid. In the cytoplasm, the main receptors are the RNA helicases retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) which are responsible for detecting the double stranded RNA (dsRNA) of viruses. Both RIG-I and MDA-5 are ubiquitously expressed in most tissues (Goodbourn *et al.*, 2000). Upon stimulation, both RIG-I and MDA-5 recruit and activate a mitochondrion-associated adaptor variously called CARD adaptor inducing IFNβ (Cardiff)/virus-induced signalling adaptor (VISA)/mitochondrial antiviral signalling protein (MAVS)/IFNβ promoter stimulator protein 1(IPS-1) (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*,

2005; Xu *et al.*, 2005). This recruitment then leads to the activation of the IRF3 and NF-κB pathways. Prior to induction, both IRF3 and NF-κB are in the cytoplasm. Once the signal is received, the interaction of TANK-binding kinase 1 (TBK-1) and IKKε leads to phosphorylation of IRF3 and its migration into the nucleus. Similarly, NF-κB is held by the inhibitor of NF-κB (IκB) in the cytoplasm until stimulation, when the IκB is phosphorylated and degraded, causing the release of NF-κB which then translocates into the nucleus. In the nucleus, phosphorylated IRF3 and NF-κB bind to the IFNB promoter with other co-factors such as CBP/p300 and RNA polymerase II (Randall and Goodbourn, 2008), leading to the stimulation of transcription of IFNα/β.

Besides the cytosolic receptors, Toll-like receptors such as TLR3, TLR 7,TLR8 and TLR9 also activate pathways that result in production of IFNα/β. TLR3 recognizes double stranded RNA (dsRNA) (Alexopoulou *et al.*, 2001), whereas TLR7 and TLR8 detect viruses that contain single-stranded RNA (ssRNA) genomes (Diebold, 2008). In addition, TLR9 detects unmethylated CpG motifs in DNA viruses (Krug *et al.*, 2004). Specialised cells such as conventional (cDC) and plasmacytoid dendritic cells (pDC) produce IFNα via these TLR pathways (Colonna *et al.*, 2002). These TLRs are located intracellularly in the endosomal compartment. Upon infection, viral nucleic acid is internalised into endosomes by endocytosis which then activates the TLR signalling pathway (Randall and Goodbourn, 2008). TLRs such as TLR7, 8 and 9 signal through the adaptor molecule myeloid differentiation factor 88 (MyD88) and the recruitment of interleukin-1 receptor-associated kinase 4 (IRAK-4) and IRAK-1 (Randall and Goodbourn, 2008), whereas TLR3 mediates its signal through the Toll-like interleukin-1-resistance (TIR) domain-containing adaptor inducing IFNβ (TRIF) (Yamamoto *et al.*, 2003). This recruitment then leads to the activation of NF-κB and IRF3 pathways, similar to the activation via RIG-I and MDA5.

Based on several sources of evidence, activation of the IRF3 pathway leads to an initial ‘first wave’ of IFNβ production which then triggers the transcription of IRF7 (Marié *et al.*, 1998; Honda *et al.*, 2006; Randall and Goodbourn, 2008). Except in specialised cells such as pDC, IRF7 is normally absent or present in very low amounts (Randall and Goodbourn, 2008). Once upregulated, IRF7 is activated in the same way as IRF3 and provides a positive feedback loop to enhance the transcription of the primary IFN genes and allows transcription of a

second wave of IFN genes including the IFNα genes (Marié *et al.*, 1998; Sato *et al.*, 1998; Randall and Goodbourn, 2008). IRF3 and IRF7 are considered as the main IRF family members important for IFNα/β production. There are others such as IRF1, IRF5 and IRF8 that can also induce production of IFNα/β but their importance still remains unclear (Honda *et al.*, 2006; Randall and Goodbourn, 2008). The induction of IFNα/β via the cytosolic receptors and TLRs pathways are summarised in Figure 1.1.

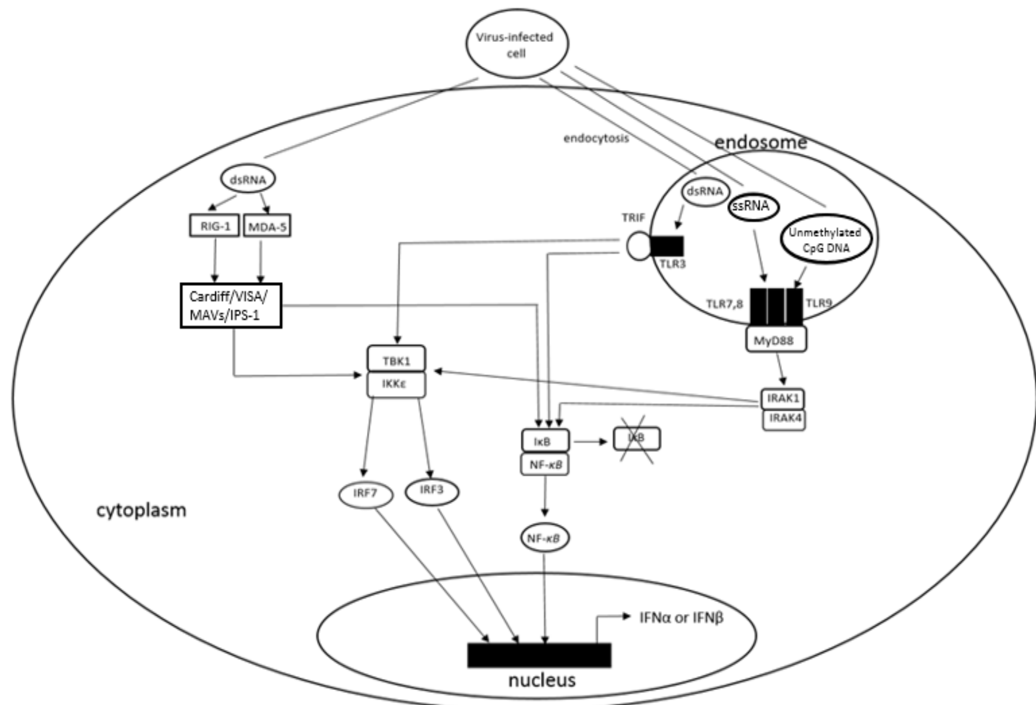


Figure 1.1: Pathways of type I Interferon (IFNα/β) induction. Pattern recognition receptors (PRR) such as RIG-I, MDA-5 and TLRs detect viral nucleic acid leading to induction of IFNα/β transcription, which is mediated by the NF-κB and IRF3 pathways. IRF-7, which is present due to feedback action of IFN, is activated similarly to IRF-3.

1.2.2.4 IFN signalling and induction of IFN-stimulated genes (ISGs)

Type I IFN signalling has been firmly established and characterized in comprehensive reviews (Platanias, 2005; Randall and Goodbourn, 2008). IFN-mediated signalling induces the transcription of IFN-stimulated genes via the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. IFNα/β binds to and signals through a heterodimeric transmembrane receptor composed of IFNAR1 and IFNAR2. The IFNAR1 is associated with tyrosine kinase 2 (TYK2) whereas IFNAR2 is associated with the tyrosine

kinase Janus kinase 1 (JAK1). The binding of IFNs results in phosphorylation of JAK1 and TYK2. These subsequently phosphorylate STAT1 on tyrosine 701 and STAT2 on tyrosine 690 (Randall and Goodbourn, 2008). The phosphorylated STAT1 and STAT2 interact strongly with each other, forming a stable pSTAT1-pSTAT2 heterodimer which then translocates into the nucleus. There, the pSTAT1-pSTAT2 heterodimer interacts with the DNA-binding protein, IRF9, forming a heterotrimeric complex known as IFN-stimulated Gene Factor 3 (ISGF3). The heterotrimeric complex ISGF3 then binds to IFN-stimulated response elements (ISRE), leading to the transcription of the several hundreds of IFN-stimulated genes including dsRNA-dependant protein kinase R (PKR), 2'5'-oligoadenylate synthetase (OAS), Mx and numerous other potent antiviral effectors such as ISG15, 54 and 56 (Goodbourn *et al.*, 2000). The genes mentioned are among those that are studied intensively and were demonstrated to play an important role in the antiviral response (Randall and Goodbourn, 2008). For example PKR is involved in inhibiting the translation of viral mRNAs (Balachandran *et al.*, 2000), whereas Mx proteins were demonstrated to interfere with virus replication (Stranden *et al.*, 1993).

Besides the ISGF3-dependent canonical signalling pathway mentioned above, IFNα/β also signals through STAT1 homodimers, which are the principal mediators of IFNγ-mediated signalling (George *et al.*, 1998). The STAT1 homodimer translocates into the nucleus and binds to the specific IFNγ activating site (GAS) that is present in the promoter of ISGs, thereby promoting their transcription (Platanias, 2005). The IFN signalling pathway is summarised in Figure 1.2.

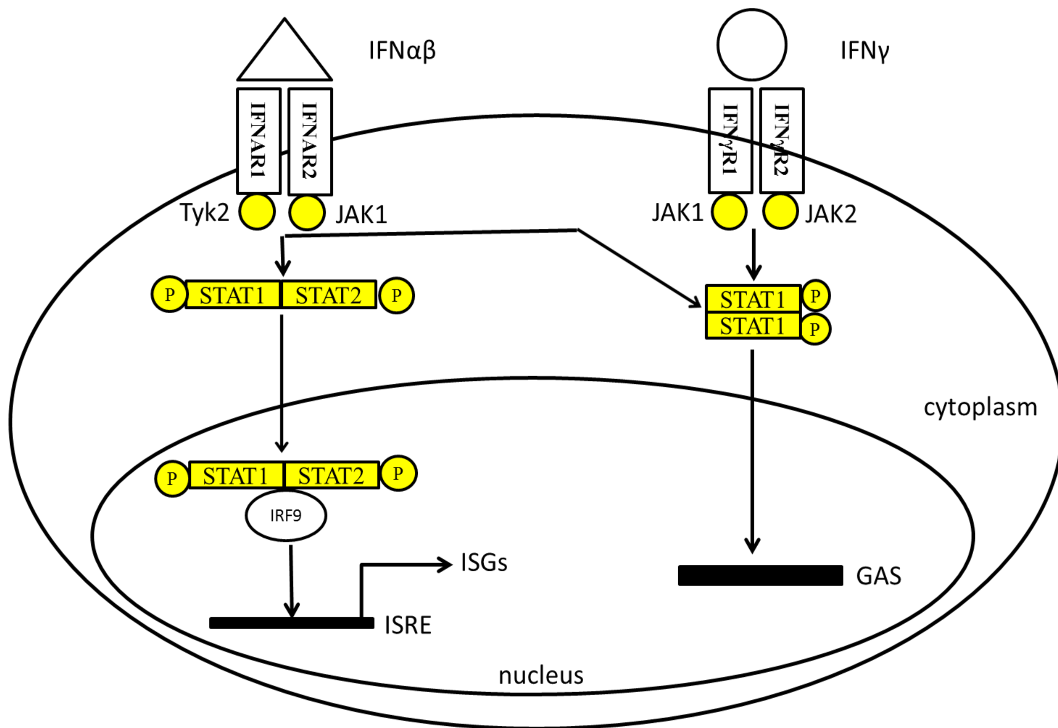


Figure 1.2: Signalling pathways activated by IFNα/β. IFNα/β binds to its receptor and activates the JAK-STAT pathway. The activation leads to phosphorylation of STAT1 and STAT2, which then translocate into the nucleus, together with IRF9, forming a heterotrimeric complex, ISGF3. ISGF3 binds to ISRE, leading to the transcription of ISGs. IFNα/β can also signal through STAT1 homodimers, which are commonly associated with IFNγ-mediated signalling, bind to GAS and initiate gene transcription.

In addition, it should also be noted that there are other members of the STAT family such as STAT3, STAT4 and STAT5. These can also be activated by IFNα/β to induce the transcription of a broad range of genes that encode cytokines, chemokines, antibacterial effectors and metabolic mediators, in addition to those dedicated to viral restriction (Ivashkiv and Donlin, 2014; McNab *et al.*, 2015). The resulting changes in gene expression are important in activating DCs, enhancing NK cell and macrophage function and promoting survival and effector functions of T and B cells (Christensen and Thomsen, 2009), implying that IFNα/β's role in antiviral immunity is not restricted to direct antiviral activities only but is also important for modulating the innate and adaptive immune response.

1.2.2.5 Role of the innate cellular components in antiviral immunity

As discussed in detail in the above section, IFNα/β are critical mediators of innate antiviral immunity by inducing the expression of a broad range of genes. This results in the secretion of cytokines and chemokines which recruit innate cellular components such as natural killer (NK) cells and macrophages to local virus-infected tissues. In turn, in addition to IFNα/β, these cells also produce other cytokines and chemokines that enhance the innate antiviral response.

NK cells are found in the blood, liver, spleen and also in lymph nodes (Christensen and Thomsen, 2009). They can migrate to the site of infection after being activated. NK cells are proposed to play two effector functions in controlling viral infection (Luca and Francis, 2001). Firstly, they can recognise the virus-infected cells at the very early stage of infection by expressing a repertoire of receptors including NKG2D, through which NK cells are able to distinguish between uninfected and infected cells (Eagle and Trowsdale, 2007; Christensen and Thomsen, 2009). NKGD2 ligands such as MHC-class-I-polypeptide-related sequence A (MICA) and MICB are expressed on the infected cells (Eagle and Trowsdale, 2007), which also may downregulate inhibitory ligands such as class I MHC molecules. Following recognition of infected cells, activated NK cells bring various effector mechanisms into play including the directed release of cytolytic granules. Secondly, they augment the local innate inflammatory response by secreting pro-inflammatory cytokines including IFNγ and TNFα, which are required for the activation and expression of other cytolytic effector functions in the control of a large variety of DNA and RNA viruses (McNab *et al.*, 2015).

Another cellular component is macrophages, whose activation represents a key role in the early clearance of many virus infections. Upon activation by viruses, macrophages secrete pro-inflammatory cytokines such as IFNα/β and TNFα that have direct antiviral activity. They also produce other cytokines that have indirect immunomodulatory functions, e.g. Interleukin-12 (IL-12) and IL-18. Both interleukins were demonstrated to have antiviral activities by inhibiting the replication of several number of viruses including HSV (Carr *et al.*, 1997) and vaccinia virus (Tanaka-Kataoka *et al.*, 1999) in vivo, but these antiviral activities are thought to be due to the possibility that these cytokines can induce IFNγ by NK cells (Luca and Francis, 2001; Dinarello and Giamila, 2003), rather than

having a direct antiviral potential themselves. In addition, macrophages also produce chemokines including CCL3/macrophage inflammatory protein (MIP-1α), CCL5/normal T cell-expressed and secreted (RANTES), CCL2/monocyte chemotactic protein-1 (MCP-1) and CXCL10/IFN-γ-inducible protein of 10 kDa (IP-10) (Luca and Francis, 2001; Melchjorsen *et al.*, 2003). These chemokines were shown to be upregulated when infected with either respiratory syncytial virus (RSV) (Haeberle *et al.*, 2001) or Influenza virus (Dawson *et al.*, 2000),. Nitric oxide, which is produced by activated macrophages via the inducible nitric oxide synthase (iNOS)-dependant pathway (Biron, 1998), also has antiviral activity, as it was shown that this substance is able to inhibit the replication of various viruses including vaccinia virus, HSV, poliovirus, rhinovirus and VSV (Reiss and Komatsu, 1998).

Both plasmacytoid (pDC) and conventional dendritic cells (cDC) are also involved in the innate antiviral response. Apart from being major producers of IFNα/β (Colonna *et al.*, 2002), DCs also secrete antiviral cytokines, including TNFα, IL-12 and IL-18 following activation (Luca and Francis, 2001). In resting conditions, immature DCs are poor at stimulating T cells, however following stimulation by e.g. IFNα/β upon viral infection (Le Bon and Tough, 2002), they become mature DCs which are able to present antigens and activate T cells as well as stimulate the differentiation and proliferation of B cells (Luca and Francis, 2001). This important role contributes to the development of the adaptive immune response during viral infections.

From the overall discussion above, it is clear that the induction and signalling of IFNα/β as well as the arrival of the innate effector cells play a direct role in controlling the initial phase of the viral infection. Furthermore, the activation of this innate immune response is critical in promoting the initiation of the adaptive immune response which relies upon the cytolytic T cell (CTL), helper T cell (Th) and B cell functions. The adaptive immune response will be discussed in detail in the adaptive Immunity section.

1.2.2.6 Viral evasion strategies to the IFN response

As the IFN system provides a powerful defence against viral infection, it is not surprising to discover that viruses have evolved numerous mechanisms to evade it (Goodbourn *et al.*, 2000; Haller *et al.*, 2006; Randall and Goodbourn,

2008). Despite their small genomes, many viruses express IFN-antagonists which are multifunctional proteins that interact with multiple viral or host cells components (Haller *et al.*, 2006). These IFN-antagonists use several strategies such as inhibition of the different components of IFN induction, the IFN signalling cascade or even specific IFN-induced antiviral genes.

Several viruses were shown to produce proteins that inhibit NF-κB as well as IRF3, to prevent the induction of IFNα/β (Powell *et al.*, 1996; Ronco *et al.*, 1998); African Swine Fever virus (ASFV) encodes a homologue of IκB that inhibits the activity of NF-κB (Powell *et al.*, 1996), whereas E6 protein of human papillomavirus type 16 (HPV-16) binds IRF3 and inhibits its virus-induced transcriptional activation function (Ronco *et al.*, 1998). In addition, the hepatitis C virus (HCV) protein NS3/4a blocks the TLR3 signalling cascade by cleaving the TLR3 adaptor protein, TRIF, and reducing its abundance (Li *et al.*, 2005). In contrast, RIG-I-mediated signalling is inhibited by NS1 protein of Influenza virus (Guo *et al.*, 2007; Opitz *et al.*, 2007) but the mechanism is unclear. All these inhibitory activities by viruses minimise the IFN production, thereby leading to efficient viral transcription and replication in the host cells.

Other viruses target IFN signalling, either by IFNα/β or IFNγ or even both (Goodbourn *et al.*, 2000). For example, the paramyxovirus human parainfluenza virus 2 (hPIV2) targets only STAT2 (Young *et al.*, 2000), resulting in blocking the IFNα/β signalling, whereas V protein of the paramyxovirus simian virus 5 (SV5) and Sendai Virus (SV) targets STAT1 for proteasome-mediated degradation, therefore blocking both IFNα/β and IFNγ signalling (Young *et al.*, 2000). Interestingly, in contrast, respiratory syncytial virus (RSV, another paramyxovirus) was demonstrated not to block any of the IFN signalling pathways but was able to replicate efficiently in human cells (Young *et al.*, 2000), indicating that it uses a different circumvention strategy. Thus, even within a single virus family (paramyxoviruses), distinct virus-specific mechanisms are used to circumvent the IFN response. Another strategy of the virus in evolving the IFN response is by inhibiting the activity of antiviral genes. For example, the activity of PKR is inhibited by the binding of the non-structural protein NS5A of HSV (Gale Jr *et al.*, 1997) and PK2 protein of baculovirus (Dever *et al.*, 1998).

Intriguingly, there are also viruses that produce IFN-antagonistic proteins that use a combination of these strategies. One example is the NSs protein of Rift valley fever virus which has the ability to block IFN production by inhibiting the IFNβ promoter activity (Billecocq *et al.*, 2004). Surprisingly, the same viral protein was also demonstrated to activate the cellular suppressor of STATs, SOCS-1, thereby blocking IFN signalling (Haller *et al.*, 2006), indicating that the Rift valley fever virus has the ability to evade the IFN response through inhibiting both IFN induction and signalling. Hence, this allows the virus to enhance its replication and spread rapidly from the infected cells to neighbouring cells, as it has suppressed the antiviral state.

By producing IFN-antagonistic proteins which target the components of the antiviral response, viral pathogens are able to multiply extensively and establish a solid infection in the host cells. An important corollary of this is that without these proteins, their ability to evade the immune response becomes limited. Attenuated, mutant viruses that lack the IFN-antagonists are generated in many laboratories in order to have a better understanding of viral pathogenesis and the IFN system. This is one of the approaches that is being used to study a disease mechanism which will be described later in Chapter 3. These viruses also open up the possibility of therapeutic application as live attenuated vaccines in which the viral IFN antagonists have been deleted and thus will provide a robust IFN and immune response against the viral infection.

1.2.2.7 Mutations affecting the IFN induction and signalling pathway

Mutations affecting the IFN pathway have been described in humans and represent a form of Primary Immunodeficiency Disease (PID) (Dropulic and Cohen, 2011). These include lesions that affect the production of IFN and those that affect IFN signalling. These mutations predispose individuals to severe viral infections mainly, but some are also associated with infections by other organisms such as fungi and mycobacteria.

Mutations of the TLR3 signalling pathway have been linked mainly to herpes simplex encephalitis (HSE) in children (Dropulic and Cohen, 2011). Two patients with autosomal recessive homozygous mutations in UNC-93 homolog B1 (UNC93B1) have been reported. The functions of this protein are in TLR7

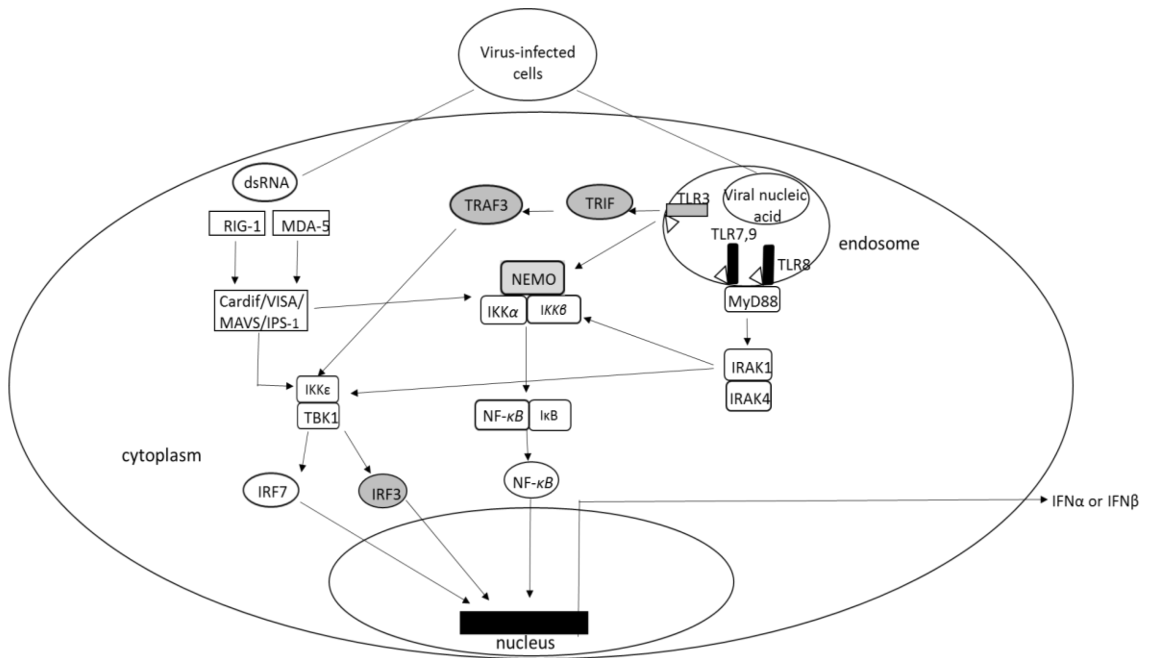
and TLR9 trafficking from the endoplasmic reticulum to endosomes and in TLR3, TLR7, TLR8 and TLR9 signalling (Casrouge *et al.*, 2006; Kim *et al.*, 2008). Another genetic cause of HSE is the heterozygous autosomal dominant mutation in TLR3 in two siblings (Zhang *et al.*, 2007). Interestingly, it was noted that disease associated with this mutation shows incomplete clinical penetrance as other family members who also had the same mutation did not have any clinical manifestations. In addition, autosomal recessive homozygous mutations in TLR3 were also identified that predispose the individual to HSE whilst having normal resistance to other infections (Guo *et al.*, 2011b).

One child with HSE that recovered was reported with an autosomal dominant-negative mutation in tumour necrosis factor receptor-associated factor 3 (TRAF3) (Pérez de Diego *et al.*, 2010), a protein downstream of UNC93B and TLR3 in the cytoplasm. The mutation results in the impaired production of Type I IFN. Furthermore, autosomal recessive or autosomal dominant mutations in TRIF, another protein downstream of the TLR3, have also been observed to cause HSE (Sancho-Shimizu *et al.*, 2011). Since no other severe viral infection was observed in these patients, all these mutations demonstrated that impaired TLR3 signalling via UNC93B-TRAF3-TRIF dependant pathway causes selective susceptibility to Herpes Simplex Virus (HSV-1) infection in the central nervous system.

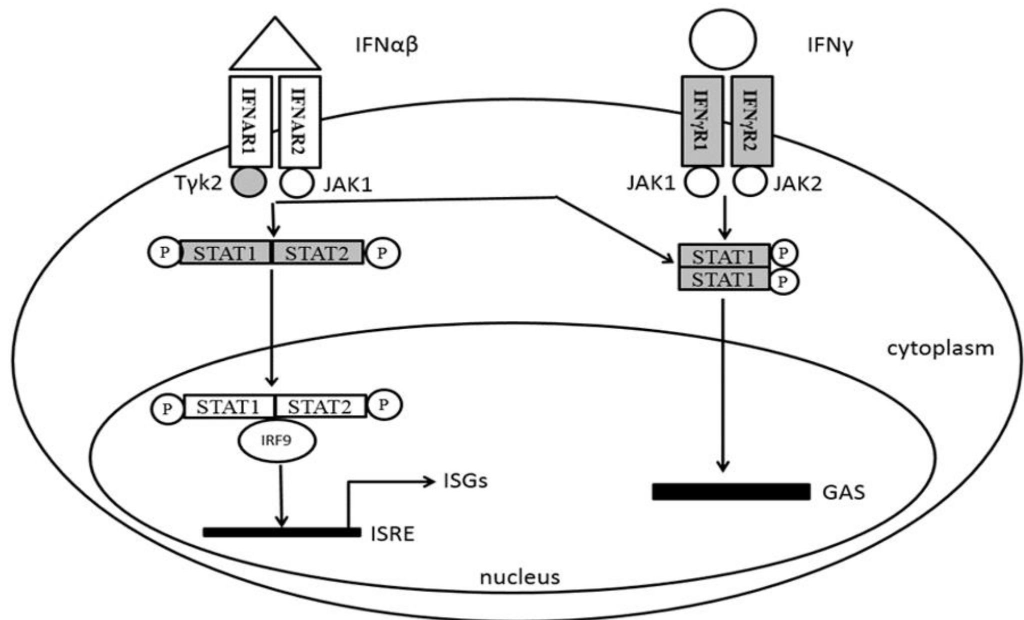
The IRF3-mediated pathway is activated by most of the PRRs that play an important role in the induction of IFNα/β. Very recently, a new and incompletely penetrant autosomal dominant disorder of *IRF3* was identified in a 15 years old adolescent with HSE (Andersen *et al.*, 2015). This is the first identification of a mutation in a member of the IRF family that is involved in the induction of type I IFNs. The NF-κB-mediated pathway is another pathway that induces the production of type I IFNs in the cells. It was identified that hypomorphic mutations in inhibitor of NF-κB kinase γ, known as NEMO (NF-κB essential modulator), cause susceptibility to herpesviruses (e.g. CMV sepsis and colitis, HSV stomatitis, pharyngitis and encephalitis) (Orange *et al.*, 2004). However, this mutation results in combined immunodeficiency with susceptibility to other organisms as well as, such as susceptibility to pyogenic bacteria and atypical mycobacteria (Orange *et al.*, 2004).

Susceptibility to viruses in patients with mutations affecting IFN signalling have also been described. Homozygous complete autosomal recessive (AR) mutations of STAT1, which cause both defective IFNα/β and IFNγ signalling, were identified in two patients. Both patients presented with disseminated Bacille Calmette -Guerin (BCG) disease but died due to recurrent HSE caused by HSV-1 (Dupuis *et al.*, 2003). In addition, one of two patients with partial AR STAT1 deficiency was reported to suffer severe herpesviral infections in addition to salmonellosis (Chapgier *et al.*, 2009). Other mutations that also predispose to increased susceptibility to severe infections with herpesviruses and certain respiratory viruses were homozygous or heterozygous dominant-negative mutations in IFNγ receptors 1 or 2; however, these mutations primarily predispose to disseminated infections with mycobacteria (Dorman *et al.*, 1999).

Binding of IFNα/β to its receptor, composed of IFNAR1 and IFNAR2, leads to the phosphorylation of JAK1 and TYK2. TYK2 deficiency due to a homozygous mutation has been described in a 22 year old boy with a complex phenotype (Minegishi *et al.*, 2006). Initially the patient was diagnosed with autosomal recessive hyper-Immunoglobulin E syndrome as he exhibited recurrent staphylococcus abscesses, respiratory tract infections and markedly increased serum Immunoglobulin (Ig) E concentrations (> 2000IU/ml). However, he also demonstrated unusual susceptibility to various microorganisms such as HSV-1, mycobacteria and salmonella. Evaluation of the patient's immune response revealed defects in multiple cytokine signalling pathways including IFNα/β, IL-6, IL-10, IL-12 and IL-23, implying that TYK2 is essential in multiple cytokine signals involving innate and acquired immunity. Subsequently other TYK2-deficient patients have been described with similar broad susceptibility to infection but without the hyper-IgE element of this phenotype (Kreins *et al.*, 2015). Finally, another defect of the IFN signalling pathway was reported by our group to be a homozygous autosomal recessive mutation in STAT2; here, the index patient developed disseminated vaccine strain measles following routine immunization (Hambleton *et al.*, 2013). Given the severity of viral susceptibility in the Stat2-deficient mouse model (Park *et al.*, 2000), it was surprising to find that the STAT2-deficient patient could cope well with common viral infections and remained generally healthy, with no obvious defects in adaptive immunity.



IFN induction



IFN signalling

Figure 1.3: Known mutations affecting the IFN induction and signalling pathway. Mutated genes are coloured in grey.

1.3 PIDs involving adaptive Immunity

1.3.1 Overview

Adaptive antiviral immunity implies a highly specific and potent immune response to infection. In contrast to innate immunity, it does not act immediately but requires up to several days to generate a primary response. Once established, it can be extremely effective in eliminating infection, although this varies from one virus to another. It is distinct from innate immunity in two important respects: 1) it exhibits fine antigenic specificity against the invading pathogen, and 2) it has the ability to form memory against previously encountered pathogens, responding more rapidly upon second encounter. Two major cell types that participate in adaptive immunity are B lymphocytes and T lymphocytes. B cells mediate humoral immunity by developing into plasma cells that secrete antibodies, whereas T cells play a central role in cell-mediated immunity; extensive cross-talk also occurs between T and B cells.

Adaptive immune responses are required to eliminate not just viruses but also other pathogens such as bacteria, parasites and fungi. Patients with numerical deficiency of T cells suffer severe combined immunodeficiency (SCID) which is marked by susceptibility to multiple viruses, bacteria and fungi, resulting in severe and fatal illness (van der Burg and Gennery, 2011). Less catastrophic defects of T cell development and function produce varying patterns of susceptibility to infection as well as immunodysregulatory phenomena such as autoimmunity and lymphoproliferation. Likewise disorders of antibody production vary in degree and consequence, according to the particular defect. In order to understand how PIDs impair cellular and humoral immune response, subsequent sections will introduce lymphocyte biology involving their development, activation and tolerance as well as highlighting the genetic defects that have been identified in humans within these processes.

1.3.2 T lymphocyte biology and function

1.3.2.1 T lymphocyte development

Both T and B cells are derived from common lymphoid progenitor (CLP) cells which in turn originate from haematopoietic stem cells (HSCs), cells that are found in the bone marrow and have self-renewal potential, to maintain the

number of blood cells to ensure lifelong production. Besides lymphocytes, CLP also gives rise to NK cells which play a more important role in the innate immune response, as has been described in the innate antiviral immunity section earlier. Besides CLPs, HSCs also differentiate into common myeloid progenitors that give rise to macrophages, granulocytes, mast cells, erythrocytes and platelets. For T lymphocyte development, CLPs migrate via the blood to the cortex of the thymus. Here, the precursor T cells lacking expression of T-cell receptor (TCR), CD4 and CD8 are known as double negative (DN) thymocytes. DN thymocytes further differentiate in a series of four stages (DN1, DN2, DN3 and DN4) (Germain, 2002). These cells can develop into either TCRαβ or TCRγδ-expressing cells (E Robey and Fowlkes, 1994; Germain, 2002), but the majority will give rise to TCRαβ-expressing cells.

It is during the DN3 stage that the commitment to be either αβ or γδ-TCR expressing cells is made (Ochs *et al.*, 1999). At this stage, the rearrangement of the VDJ segments of the TCRβ chain occurs, which requires the expression of Recombination-activating Gene 1 (RAG1) and RAG2 (Germain, 2002). If the TCRβ chain successfully pairs with the pre-TCRα chain to form a pre-TCR, the DN3 cells undergo proliferation and proceed to further development into DN4 or late pre-T cells. DN4 cells that expand successfully undergo transition into double positive (DP) cells that express both CD4 and CD8. Replacement of the pre-TCRα chain with the newly rearranged TCRα-chain yields complete TCRαβ expression on the cell surface (Germain, 2002). Cells that fail the TCR gene rearrangement process will die through apoptosis.

As the DP thymocytes move d eeper into the cortex, they encounter self-antigens presented on MHC molecules and positive and negative selection occurs. The fate of the DP thymocytes is determined by the quality of these interactions. DP cells whose TCR binds with low affinity to peptide-MHC complexes undergo positive selection resulting in cell survival and expansion of cells that can recognise MHC complexes at least weakly. If they are not able to bind MHC, they are eliminated by “death by neglect”. On the other hand, DP cells whose TCR recognises self-antigens with high affinity are subject to negative selection. These cells, which might otherwise be harmful, are eliminated mainly by apoptosis although other processes such as anergy (Hammerxing *et al.*, 1991) and receptor editing (McGargill *et al.*, 2000;

Nemazee, 2006) have been described; it is unclear how prominent the latter processes are in establishing central tolerance of T cells (Holman *et al.*, 2003; Hogquist *et al.*, 2005).

During positive selection, another process also occurs which is known as CD4/CD8 lineage commitment. If the TCR on the DP cells recognises peptide-loaded MHC class I molecules, the cells become single positive CD8+ T cells, and if the TCR recognises peptide-loaded MHC class II molecules, the cells become single positive CD4+ T cells. These SP cells proliferate, then leave the thymus and populate the peripheral tissues. Most thymocytes die during the developmental processes and only few of them successfully emerge from the thymus and become mature competent T cells in the periphery (Ochs *et al.*, 1999). The development of T cells is summarised in Figure 1.4

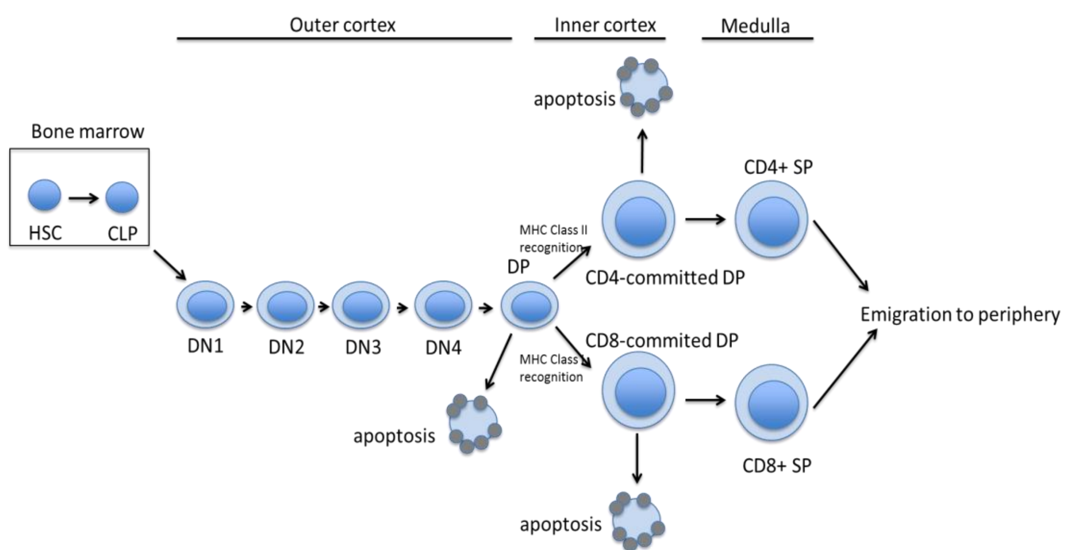


Figure 1.4: Overview of T cell development in the thymus. DN-double negative, TCR-T cell receptor, DP-double positive.

It should be noted that a variety of cytokines and transcription factors are involved in initiating and maintaining lymphopoiesis including Interleukin-7 (IL-7), which is known to be critical for the early development of T cell progenitors (He and Kabelitz, 1994) along with GATA-binding protein 3 (GATA3), RUNT-related transcription factor (RUNX) and several others transcription factors (Naito *et al.*, 2011) which are involved at various stages of T cell development.

Several genetic defects affecting T-cell development have been described to cause PID (Picard *et al.*, 2015). These include mutations in genes encoding components of the IL-7 signalling cascade such as Interleukin-2 (IL-2) receptor common γ chain (*IL2RG*) (Noguchi *et al.*, 1993), IL-7 receptor α chain (*IL7R*) (Puel *et al.*, 1998) and Janus kinase 3 (*JAK3*) (Macchi *et al.*, 1995). Defects of several components of the TCR associated CD3 complex also prevent T cell development by their failure to support positive selection. The above all lead to T-B+ SCID, in which T- but not B-cell development is affected; NK cell development is also impaired in both *IL2RG*- and *JAK3*-SCID but spared in deficiencies of IL7R or CD3. Another type of SCID, T-B- most frequently involves mutations in *RAG1* or *RAG2* which result in defective VDJ recombination and affects both T- and B-cell compartments. Different genotypes of SCID can result in unique symptoms but most SCID patients presented with failure to thrive and severe recurrent infections of multiple pathogens including bacteria, protozoa, fungi and viruses (Kalman *et al.*, 2004). The symptoms usually present in the first few months of life and if not treated early, it leads to the death of the patient.

1.3.2.2 Antigen presenting cells (APCs)

As described in section 1.2.1.1, once T cells completed their development in the thymus, they emerge into the periphery in response to chemokine cues. Naïve T cells circulate from the blood into lymphoid organs and back until they encounter their cognate antigen. Most cells can present antigen but only specialised cells, which are known as professional APCs (pAPCs) are capable of priming naive T cells (Jacques *et al.*, 2000). APCs act by processing the protein antigens to peptides and displaying them on the MHC-complex to be recognised by T cells in the presence of a co-stimulatory signal. There are 3 major pAPCs that play important roles in the antigen presentation process including dendritic cells (DCs), B cells and macrophages (Abbas *et al.*, 2007).

Immature DCs reside in most tissues. Although they are poor at antigen presentation, they are efficient in capturing antigens (Banchereau and Steinman, 1998). When they encounter pathogens, their pattern recognition receptors (PRRs), such as the Toll like receptors (TLRs), recognise these and activate the DCs to mature and migrate to lymphoid tissues such as lymph nodes and spleen (Murphy *et al.*, 2012). DC maturation sees the upregulation of co-

stimulatory molecules (e.g. CD80/86) which contribute importantly to the activation of T cells (Banchereau and Steinman, 1998).

There are two main classes of DCs 1) conventional DCs (cDCs) and 2) plasmacytoid DCs (pDCs). Both classes of DCs are involved in antigen presentation, but cDCs mostly participate in antigen presentation and activation of naïve T cells, whereas pDCs are important in generating a large amount of type I IFN especially important in the response against viral infections (Murphy *et al.*, 2012). The CD141+DC subset of cDCs is specialised in the cross-presentation of exogenously acquired antigens on class I MHC molecules, whereas conventional peptide loading is restricted to endogenously expressed proteins. Another class of dendritic cells, known as inflammatory DCs (infDCs) have been discovered and they are monocyte-derived cells (Geissmann *et al.*, 2003). The infDCs are recruited to the inflamed tissues and also have the ability to participate as antigen presenting cells (Geissmann *et al.*, 2003; Hespel and Moser, 2012)

B cells also act as professional APC. In addition to their role in secreting antibodies, they are also capable of presenting antigens and express co-stimulatory molecules that result in the priming and differentiation of T cells. B cells acquire antigen through binding to their cell-surface immunoglobulin, the BCR, and internalize the antigen by receptor-mediated endocytosis (Abbas *et al.*, 2007). The internalised protein antigen is processed into peptide fragments and displayed as peptide: MHC Class II complexes which are recognised by CD4+ T cells, driving their differentiation. Antigen uptake through the endocytic pathway is considered to be a highly efficient mechanism, especially for antigens that are present as soluble protein, as most toxins are.

Macrophages are phagocytic cells that are ubiquitous in most tissues including lymphoid organs (Murphy *et al.*, 2012). Several types of tissue macrophage have been described which are characterised based on their location and specialised function, for example Kupffer cells in the liver and microglia in the central nervous system (Murphy *et al.*, 2012). Inflammatory macrophages can also be generated by the ingress of blood monocytes to inflamed tissues. Macrophages are involved in engulfing the microbes and particulate antigens which are then enzymatically destroyed by phagosome-lysosome fusion. This function leads to the generation of peptide fragments which can be loaded onto

MHC Class II molecules for surface presentation. Similarly, like the other two major APCs that have been mentioned, macrophages are also capable to upregulate co-stimulatory molecules which promote the activation of T cells.

1.3.2.3 T cells activation

For the full activation, proliferation and differentiation of naive T cells into effector cells, they require two important signals. The first signal is provided by peptide–MHC interaction with the TCR/CD3 complex of naïve T cells and this interaction predominantly occurs in the lymphoid tissue. Naïve CD4 T cells recognise peptide-MHC II, whereas naïve CD8 T cells recognise peptide-MHC I complexes. The second signal is provided by the co-stimulatory molecules CD80/86 (also known as B7 family) that interact with CD28, a co-receptor protein which is expressed on T cells. The expression of these co-stimulatory molecules is upregulated on pAPCs.

TCR/CD3 ligation initiates a signalling cascade which culminates in the transcriptional induction of a variety of genes that are important for the regulation of T cell growth, survival and differentiation. The binding results in the recruitment of Src protein tyrosine kinases, Lck and Fyn, to the TCR/CD3 complex which then phosphorylate immunoreceptor tyrosine-based activation motifs (ITAM) on CD3 molecules. Phosphorylation of ITAM allows the recruitment of ζ-chain associated protein kinase of 70 kDa (ZAP70) which results in its activation. Activated ZAP70 then recruits the linker for the activation of T cells (LAT). LAT then recruits phospholipase C-γ1 (PLC-γ1) which also interacts with IL-2 inducible T cell-kinase (ITK) and this mediates the hydrolyses of membrane lipid PIP_2 into diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3). DAG activates two major pathways involving the Protein Kinase C θ (PKC θ) and RAS. RAS mediates the mitogen-activated protein kinase (MAPK) pathway that results in the activation of extracellular signal-regulated kinase 1 (ERK1) followed by activation of activator protein-1 (AP-1), a transcription factor which forms a component of many transcriptional complexes. PKC θ activation leads to the activation of transcription factor of NF-κB. IP_3 opens Endoplasmic reticulum (ER) Ca^{2+} store leading to Ca^{2+} flux into the cytoplasm. Increased intracellular calcium activates calcineurin that is required for the activation of the transcription factor Nuclear Factor Activated T-cells (NFAT). The TCR signalling cascade is summarised in Figure 1.5.

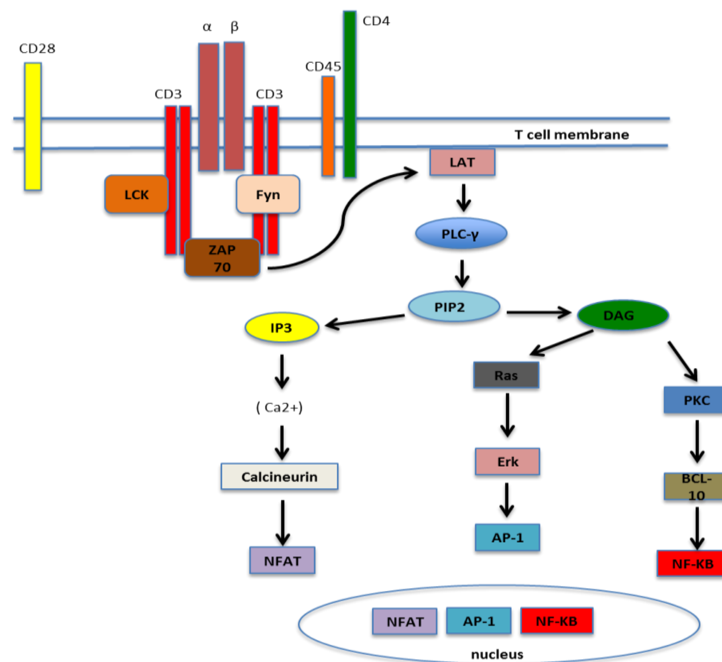


Figure 1.5: TCR signalling cascade. The binding of antigen to TCR activates the signalling cacade. Early signalling events include the activation of Lck, Fyn and Zap70, resulting in activation of LAT. Following this, PLC-γ signalling pathway is initiated, leading to activation of transcription factors AP-1, NFAT and NF-κB.

The importance of TCR signalling for T cell responses in humans have been elucidated through the discovery of several genetic defects in PIDs. For example, AR deficiency of ZAP70 results in the typical phenotypes of SCID despite the presence of residual T cells in the periphery (Elder, 1996). LCK deficiency leads to combined immunodeficiency, considered to be generally less profound than SCID (Picard *et al.*, 2015); patients displayed recurrent infections with bacteria and fungi, severe inflammation in the gut and mucosa (Hauck *et al.*, 2012). In contrast to ZAP70 deficiency, both CD4 and CD8 counts were reduced in LCK deficiency. Similar to some cases of ZAP70 deficiency (Picard *et al.*, 2015), the patient also displayed features of autoimmunity.

1.3.2.4 Effector T cells

Upon receiving activating signals and depending upon the context of antigen presentation, the CD4+ T cells polarise towards the expression of different cytokines and transcription factors characteristic of alternative subsets of CD4+ T helper cells including T helper (Th)1, Th2, Th17 ,T regulatory (Treg) and T Follicular helper (Tfh)cells. Naïve CD8 T cells differentiate into cytotoxic T cells

(CTLs). Each of these effector cells has their own role in mediating T-cell immune responses.

1.3.2.4.1 Th1

The critical cytokines that are involved in differentiating naïve T helper cells to Th1 cells are IL-12 and IFN-γ (Luckheeram *et al.*, 2012). The transcription factor T-bet is the master regulator as it also enhances the production of IFN-γ for Th1 differentiation and upregulates the expression of IL12Rβ (Luckheeram *et al.*, 2012). In addition, the transcription factor signal transducer and activator of transcription 1 (STAT1) is activated by IFN-γ which in turn stimulates the expression of T-bet. This provides a positive amplification loop that drives the T helper cells towards Th1 differentiation (Luckheeram *et al.*, 2012). The principal effector function of Th1 cells is in eliminating intracellular pathogens. They mainly secrete IFN-γ, TNFα, and IL-2. Both IFNγ and TNFα have antiviral properties, i.e. they are able to inhibit viral replication, thus they are important in eliminating viral infections. In addition, IFNγ is important for the activation of phagocytic activity and killing by macrophages as well as stimulating production of immunoglobulins from B cells that opsonise the microbes for phagocytosis. Another cytokine, IL-2 helps in promoting CD8+ memory after antigen priming, hence it participates in a robust secondary immune response.

1.3.2.4.2 Th2

The differentiation of Th2 cells is regulated by IL-4 and IL-2. STAT6 is the transcription factor that is involved in upregulating the expression of GATA-binding protein (GATA3), both of which are considered major transcription factors for Th2 differentiation (Luckheeram *et al.*, 2012). The function of Th2 as effector cells is to support eosinophil/mast cell-mediated immune reactions including those towards helminths and other extracellular parasites (Murphy *et al.*, 2012). Furthermore, Th2 cells are involved in the induction and persistence of asthma as well as other allergic diseases. The Th2 subset secretes IL-4, IL-5 and IL-10 which are involved in stimulating B cells to produce antibodies. In addition IL-5 also activates eosinophils to release their granule contents that are capable of destroying the helminths.

1.3.2.4.3 Th17

The major cytokines that are involved in Th17 differentiation are IL-6, IL-21, IL-23, and TGF-β as well as the transcription factors of RORγT and STAT3 (Murphy *et al.*, 2012). Th17 cells are responsible for mediating the immune response against extracellular bacteria and fungi (Murphy *et al.*, 2012) and they release mainly cytokine IL-17 (IL-17A, IL-17F), which gives rise to their name, and other cytokines including IL-21 and IL-22.

IL-17 promotes the secretion of IL-1, IL-6, IL-8, CXC ligand 1 and TNF from stromal, epithelial and endothelial cells. These proinflammatory cytokines are involved in recruiting neutrophils to the site of infection, thus providing an acute immune response at the infection site (McKenzie *et al.*, 2006). Sustained IL-17 production supports ongoing recruitment of neutrophils, contributing to long term maintenance of antimicrobial responses such as granuloma formation and other immune responses during chronic bacterial infection (as demonstrated in *a Klebsiella* lung infection model (McKenzie *et al.*, 2006)). In addition to its role in recruiting neutrophils, IL17 is also important in resisting *Candida albicans* infection. This role has been elucidated in humans with a disease known as Chronic Mucocutaneous Candidiasis(CMC) (McDonald, 2012), a unique form of PID. Mutations in the cytokine or its receptors *IL17RA*, *IL17RC* and *IL17F* result in recurrent infections at the skin, nails and mucosal membrane with *Candida albican* and, to a lesser extent, with Staphylococcus aureus, indicating that Th17 responses are essential for mucocutaneous antifungal immunity (Puel *et al.*, 2011). A similar vulnerability results in the context of impaired Th17 development (STAT3 loss-of-function) or autoantibody neutralisation of IL17 (APECED, resulting from AR mutations in AIRE).

1.3.2.4.4 Treg cells

The crucial function of Tregs is in maintaining the peripheral tolerance of autoreactive T cells. They are also known as ‘suppressor T cells’ as they limit inappropriate or excessive responses of conventional T cells (Workman *et al.*, 2009). There are two types of Tregs; natural Tregs (nTregs) and induced Tregs (iTregs) (Workman *et al.*, 2009).

nTregs develop in the thymus during positive and negative selection stages and they comprise 5-10% of peripheral CD4+ T cells (Workman *et al.*, 2009). nTregs

express the transcription factor Forkhead family transcription factor (FOXP3) which is a master regulator of Treg development and function (Sakaguchi *et al.*, 2008). The importance of FOXP3 in nTregs was first identified in the mouse strain scurfy which bore a defect of the *Foxp3* gene (Brunkow *et al.*, 2001). The scurfy mouse displayed hyperactivation of CD4+ T cells and increased pro-inflammatory cytokine production (Brunkow *et al.*, 2001; Sakaguchi *et al.*, 2008). Consistent with the finding in mice, a conserved role of FOXP3 in regulating Treg function had also been validated in humans in which mutations in *FOXP3* results in a genetic disease of immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (Le Bras *et al.*, 2006). In addition to FOXP3, nTregs also express high levels of IL-2 receptor α chain(CD25), CTLA4 and glucocorticoid-induced TNFR-related protein (GITR) (Wan and Flavell, 2009) which is also important for nTreg function. The critical cytokine in nTreg development and maintenance is IL-2. IL-2 was shown in vitro to be involved in sustaining the expression of FOXP3 and CD25 in nTregs and to enhance their suppressive function (Fontenot *et al.*, 2005). The importance of CD25 to humans is highlighted by the fact that AR mutations of CD25 predispose to an IPEX-like syndrome which is clinically indistinguishable from that caused by FOXP3 mutations (Caudy *et al.*, 2007)

Another type of Tregs is the adaptive or also known as induced Tregs (iTregs) which, unlike nTregs, develop in peripheral lymphoid organs after antigen priming. There are two main subsets that are generated from iTregs; type 1 regulatory T cells (Tr1) which are induced by IL-10 and T helper 3 (Th3) which are induced by TGFβ (Workman *et al.*, 2009). Similar to nTregs , both Tr1 and Tr3 express CD25, GITR and only Th3 express FOXP3 (Workman *et al.*, 2009). They mediate their suppressive function through the same cytokines that are responsible for their induction, IL-10 and TGFβ, which are also produced at high levels by nTregs. IL-10 and TGFβ are considered inhibitory cytokines and represent the major mechanism of suppression utilized by Tregs.

Besides producing inhibitory cytokines, it is proposed that other modes of maintaining peripheral tolerance and suppressive mechanism by Tregs are through cytolysis and modulation of APC function (Vignali *et al.*, 2008). Tregs kill autoreactive T cells through granzyme-dependent apoptosis (Vignali *et al.*, 2008; Workman *et al.*, 2009). In addition, Tregs also suppress target T cells by

downregulating APC function through CTLA-4 dependant mechanism, for example it may downmodulate CD80/86 expression on APC or stimulate dendritic cells to form the enzyme indoleamine 2, 3-dioxygenase, which this enzyme is involved in catabolising the essential amino acid tryptophan to kynurenines that are toxic to T cells (Workman *et al.*, 2009).

1.3.2.4.5 Tfh cells

Tfh cells express high levels of CXCR5, the receptor for Chemokine (C-X-C motif) ligand-13 (CXCL13) which is secreted by follicular stromal cells in the B cell zone. Tfh cells are found enriched in the edge of the B cell zones and follicular regions and germinal centres (GCs) (Wan and Flavell, 2009; Ma *et al.*, 2012). Tfh express effector molecules such as ICOS, CD40 Ligand (CD40L), OX40, PD-1 and BTLA-4 which are critical for their development and function and through these molecules Tfh provide help to B cells by promoting the activation, differentiation and survival of B cells (Ma *et al.*, 2012). For example, CD40L interaction with CD40 expressed on B cells results in activation and promotes their proliferation whereas ICOS induces the production of B helper cytokines such as IL-10 and IL-21 (Ma *et al.*, 2012).

1.3.2.4.6 Cytotoxic T cells (CTLs)

CTLs are the only cells that are differentiated from CD8+ T cells. CD8+ T cells undergo expansion and differentiate into CTLs with the help from cytokines such as IL-12, IL-15 and IL-17. The main function of CTLs is in eliminating infected target cells which is mediated through the perforin-granzyme and death ligand pathways (Abbas *et al.*, 2007), both of which eventually lead to programmed cell death or apoptosis of the infected target cells. In addition, CTLs are also major producers of cytokines including IFNγ, TNFα and to a lesser extent IL2 (Luca and Francis, 2001).

1.3.2.4.7 Memory T cells

Once the T cells have become activated and differentiated into effector cells, these cells circulate and peak for a week. At the end, most of these will die, however a small proportion of primed CD4+ and CD8+ T cells will persist as memory T cells (MacLeod *et al.*, 2010). Memory cells differ from naïve T cells in their superior ability to respond very quickly to subsequent exposure to the same antigen; this is very useful in preventing future infections and disease

from the same pathogens (MacLeod *et al.*, 2010). There are two types of memory cells, central memory T cells (TCM) and effector memory T cells (TEM) which differ in terms of their homing capacity and effector function (Sallusto *et al.*, 2004).

TCM express CCR7 and CD62L which are important for cell extravasation through high endothelial venules and migration to the T-cell area of secondary lymphoid organs (Sallusto *et al.*, 2004). They are highly sensitive to antigenic stimulation and are less dependent on co-stimulation than naïve T cells (Sallusto *et al.*, 2004). TCM mediate the reactive memory; following TCR triggering, TCM produce mainly IL-2 and proliferate, after which they efficiently differentiate into effector cells (Sallusto *et al.*, 2004)

TEM are memory cells that have lost the expression of CCR7 but express CD62L and exhibit chemokine receptors and adhesion molecules that are required for homing to inflamed tissues (Sallusto *et al.*, 2004). In contrast to TCM cells, they mediate protective memory and provide rapid effector function by producing cytokines or displaying cytotoxic function within hours of antigenic stimulation (Sallusto *et al.*, 2004).

1.3.2.5 Peripheral tolerance of T cells

Central tolerance of T cells occurs during T-cell development in the thymus. Although central tolerance mechanisms are potent and efficient, not all self-reactive T cells are eliminated and a small number escape and exit from the thymus. Therefore, peripheral tolerance mechanisms exist (Xing and Hogquist, 2012) including anergy, apoptotic deletion and suppression of T cells, to control these self-reactive T cells (Xing and Hogquist, 2012). If the peripheral tolerance breaks down, this results in autoimmune tissue damage and disease.

T cell activation requires recognition of antigen by TCR (signal 1) and ligation of the co-stimulatory molecule CD28 by CD80 and CD86 (signal 2). T cell activation in the absence of signal 2 results in a state of long term hyporesponsiveness or "anergy", characterised by repressed TCR signalling and failure to express IL2. Anergy can also result when TCR stimulation is accompanied by negative second signals delivered via co-receptors such as Cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD-1). CTLA-4 is a structural homologue of CD28 and competes with CD28 for

CD80/CD86 binding sites, stripping these ligands from the APC. Hence it attenuates the costimulatory signal and inhibits TCR signalling. Interaction of PD-1 with its ligands, PD-L1 and PD-L2, inhibits TCR signalling, leading to inactivation of T cells and anergy (Xing and Hogquist, 2012). The roles of CTLA-4 and PD-1 are highlighted in mice lacking CTLA-4 or PD-1, which develop autoimmune diseases. Heterozygous mutations in CTLA-4 have also been identified in humans in patients with immune dysregulation and autoimmune phenomena (Schubert *et al.*, 2014), similar phenotypes as the mouse model.

Tolerogenic DCs, the subset of DCs that is involved in inducing tolerance, are thought to be generated from incomplete maturation of DCs (Xing and Hogquist, 2012). They present antigen without the co-stimulatory second signal, resulting in anergy in cognate T cells. Depletion of DCs in mice breaks self-tolerance of CD4+ T cells and results in fatal autoimmunity (Ohnmacht *et al.*, 2009; Saei and Hadjati, 2013), further strengthening the crucial role of tolerogenic DCs in peripheral immune tolerance.

As mentioned earlier, self-reactive lymphocytes in the thymus are eliminated by apoptosis. Similarly, the deletion of self-reactive T cells in the periphery is achieved through apoptotic cell death. There are two major pathways that lead to apoptosis, the death receptor (extrinsic) and mitochondrial (intrinsic) pathways. Both pathways will be discussed in detail in chapter 4.3.

1.3.3 B lymphocyte biology and function

1.3.3.1 Early B cell development in the bone marrow

Similar to T cells, B lymphocytes derive from CLPs which are formed from HSCs in the bone marrow. In contrast to T cells, B-cell development takes place in the bone marrow. Each stage of B cell maturation is marked by a specific pattern of gene expression and immunoglobulin heavy (H) chain and light (L) chain gene rearrangements[10] by RAG-dependent V(D)J recombination as for TCR rearrangement (Tob *et al.*, 2013). CLPs first differentiate into pro B cells. At this stage, the RAG1/RAG2 dependent-arrangement of D-gene and J-gene segments of H chain from germline commences (Pieper *et al.*, 2013). This is followed by the second rearrangement joining an upstream V region to the rearranged DJ segment. The next cell stage is the pre B cells where the

functional H-chains (VDJ-Cμ) pair with V-pre B and λ-like, leading to the formation of the pre-BCR which is not detected on the surface but expressed within the cells (Pieper *et al.*, 2013). The formation of the pre-BCR induces signals for shutting down RAG expression, preventing rearrangement of the second H-chain allele, and allowing the cells to proliferate. Subsequently, RAG genes are re-expressed to initiate V-J rearrangements of L chains. Successfully rearranged κ or λ chain replaces the V-preB/λ and pairs with H chain (Pieper *et al.*, 2013). This then results in the formation of cells expressing BCR of class IgM on the cell surface. At this stage the cells are now known as immature B cells. As mentioned earlier in the T cell development section, null mutations of RAG1 and RAG2 lead to complete failure of T and B cell development and result in T-B- SCID. Hypomorphic mutations of RAG1 or RAG2 were also found to cause Omenn syndrome (Villa *et al.*, 2008). Affected patients displayed severe susceptibility to infection similar to SCID, such as viral or fungal pneumonitis, chronic diarrhoea, and failure to thrive but they also present additional features such as enlarged lymphoid tissue, severe erythroderma, increased IgE levels, and eosinophilia. These features reflect immunopathology caused by excessive expansion of oligoclonal and dysregulated T cells in the presence of very low levels of VDJ recombination. In addition, a low or absent number of B cells is a characteristic of Omenn syndrome due to RAG1/RAG2 mutation. Milder missense mutations within RAG1/2 can lead to more subtle immunodeficiency states still.

In the development of B cells, their autoreactivity is screened at several check points due to the enormous diversity of antibody specificities (Pieper *et al.*, 2013). The first checkpoint occurs when the pre-BCR is expressed, which is between the differentiation of pro into pre B cells (Pieper *et al.*, 2013). The next checkpoint is when the immature B cells express BCR, where the BCR is tested for reactivity against autoantigens. The fate of B cells that recognise self-antigen is determined by BCR signal strength. A strong BCR signal by binding with high affinity to autoantigen will lead to negative selection of the respective B cell. It is estimated that 75% of the human early immature B cells express BCRs that are specific for self-antigens (Wardemann *et al.*, 2003). Some of these self-reactive immature B cells are deleted via apoptosis or induced to change their BCR specificity by receptor editing (Tob *et al.*, 2013). Receptor

editing involves further antibody gene rearrangements, resulting in an altered specificity of the BCR and allowing the cells to escape negative selection.

The immature B cells that escape negative selection travel to the spleen, to complete their maturation before migrating to other peripheral lymphoid organs. On arrival in the spleen, the immature B cells pass through two transitional stages designated transitional 1 (T1) and T2. From transition of T1 to T2 phase, the B cells express IgD in addition to IgM on the cell surface. Subsequently, the T2 B cells finalize their early development by differentiating into follicular B cells, recirculating cells that proliferate in germinal centres (GCs), or into marginal zone B cells (MZs) that populate the marginal zones. The mechanism(s) that determine(s) differentiation towards the follicular or MZ phenotype are not really understood, however, it is suggested that it may be due to the BCR specificity (Cariappa and Pillai, 2002).

It should be noted that while immature B cells enter the spleen and become transitional B cells, they receive survival signals generated by the binding of B-cell activating factor (BAFF) to BAFF-receptor (BAFF-R). BAFF-R is first expressed on immature B cells in bone marrow and increases when the transitional cells differentiate into MZ and GC B cells. This survival signal is important for the maturation and maintainance of B cells in the periphery. The role of BAFF-R in B cell survival in humans was demonstrated in patients with common variable immunodeficiency (CVID) in which these patients had low numbers of B cells with impaired development and homeostasis of follicular, IgM memory/marginal zone, and class-switched memory B cells (Warnatz *et al.*, 2009).

1.3.3.2 BCR signalling

The development and survival of B cells in providing effective humoral response is dependent on signalling pathways which are similar downstream of both pre BCR and BCR (Pieper *et al.*, 2013). Ligation of the BCR leads to conformational changes, allowing the binding of spleen tyrosine kinase (SYK) and initiation of the signalling cascade. SYK phosphorylates Igα/Ig and adaptor protein, B-cell linker protein (BLNK) which serves as a scaffold for Bruton Tyrosine Kinase (BTK). Following this, the phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K) and phospholipase C (PLCγ2) pathways are activated. The PLCγ2 signalling is

mediated through Protein kinase C (PKC) which then interacts with caspase recruitment domain-containing protein 11 (CARD11), mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) and B-cell lymphoma 10 (BCL10) to activate the transcription of NF-κB. PLCγ2 also activates the transcription factor, serum response factor (SRF), through mitogen-activated protein kinase (MAPK). In addition, PLCγ2 also activates transcription of NFAT through IP_3. As for the PI3K pathway, it is mediated by AKT which induces the protein synthesis and cellular fitness, prolonging cell survival (Pieper *et al.*, 2013). The preBCR/BCR signalling cascade is summarised in Figure 1.6.

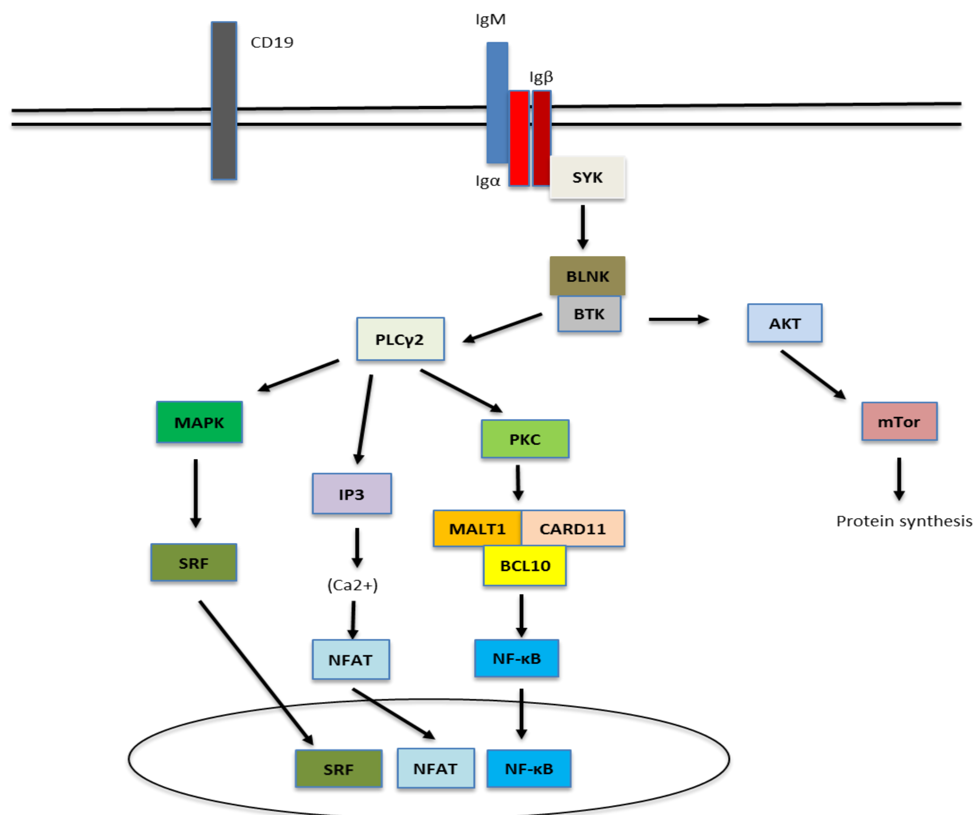


Figure 1.6: BCR signalling cascade. Ligation of BCR leads to the binding of SYK, initiating the signalling cascade. Syk phosphorylates Igα/Igβ and BLNK, which act as a scaffold for BTK. This results in the activation of PI3K and PLCγ2 signalling pathway, followed by the activation of transcription factors NFAT, SRF and NF-κB.

BTK is involved in proximal BCR signalling. In humans, the role of BTK was demonstrated by the identification of mutations in the *BTK* gene located on the X chromosome, causing a disease known as X-linked agammaglobulinemia (XLA, also known as Bruton's disease) (Tsukada *et al.*, 1993). This mutation

results in a lack of peripheral blood B cells that leads to severely reduced antibody titres of all Ig isotypes. In addition, analysis of the patients' bone marrow samples revealed abnormalities of the pre-B cell numbers and proliferation, indicating that BTK is involved in the early progenitor cells. Similar phenotypes are also observed in patients with BLNK deficiency (Minegishi *et al.*, 1999), suggesting that both genes are important for the development of early B cell progenitors . Another mutation that has been identified in humans in the downstream signalling cascade is homozygous deletion of CARD11, resulting in an accumulation of transitional B cells due to blocking of B cell differentiation (Snow *et al.*, 2012).

1.3.3.3 Development of follicular B cells in germinal centres of the spleen

Activation of follicular B cells in GCs results in the development long lived plasma cells that secrete antibodies, and switched memory B cells (Pieper *et al.*, 2013). For their differentiation and proliferation, follicular B cells require antigen-induced BCR signalling in addition to signals from cytokine receptors and co-stimulatory receptors (Rickert, 2013). Furthermore this occurs in a T-cell dependent manner, requiring help from Tfh cells through the interaction of CD40L expression on Tfh cells and CD40 expression on B cells. Within GCs, the follicular B cells undergo somatic hypermutation (SHM) of the variable regions of the rearranged immunoglobulin genes, leading to a process of affinity maturation of the antibody response. Mutations that result in surface immunoglobulins with low affinity to antigens prevent the B cells from becoming activated efficiently, therefore these B cells are eliminated by apoptosis. Whereas B cells with mutated immunoglobulins that have improved ability to bind to antigens survive. In parallel, the cells also undergo immunoglobulin class-switch recombination (CSR) which replaces the IgM constant region with those of IgG, IgA or IgE (Pieper *et al.*, 2013). The SHM and CSR machineries require two essential enzymes that are expressed on the follicular B cells, activation-induced cytidine deaminase (AID) (Rickert, 2013) and uracil-N-glycosylase (UNG) (Pieper *et al.*, 2013).

Genetic defects of AID (Revy *et al.*, 2000) and UNG (Kavli *et al.*, 2005) have been identified in humans and result in Hyper IgM syndrome, a form of humoral immunodeficiency characterised by low or absent IgG and IgA despite relatively preserved levels of IgM in patients' serum. Both AID and UNG deficiency

present with increased susceptibility to bacterial infections, lymphoid hyperplasia, and a failure of both CSR and SHM (Etzioni and Ochs, 2004). Mutations of CD40L have also been identified in humans, causing X-linked Hyper IgM syndrome. This accounts for 65-70% of all cases of Hyper IgM syndrome, mostly affecting males in early infancy (Etzioni and Ochs, 2004). The typical presentation is with interstitial pneumonia caused by Pneumocystis jirovecii (PCP) and/or bacterial sinopulmonary infections, other features being autoimmunity, cryptosporidiosis and resulting hepatobiliary disease (DiSanto *et al.*, 1993). Another gene in which autosomal recessive mutations can cause Hyper IgM syndrome is *CD40,* which is normally expressed on B cells as well as a variety of APCs and epithelia. Similar to CD40L, patients with CD40 deficiency display susceptibility to opportunistic infections including cryptosporidiosis (Etzioni and Ochs, 2004) as well as bacterial infections. The phenotypes of CD40L and CD40 deficiency thus demonstrate that both partners in this interacting pair are essential, not only for normal humoral responses but also for effective cellular immunity.

Finally, after SHMs and CSR processes, long lived plasma cells are generated which then leave the germinal centres and travel to peripheral tissues such as bone marrow, spleen red pulp and mucosal-associated lymphoid tissues (MALT) where they live for a long period and are a source of long-lasting high affinity antibodies(Tangye, 2011). In addition, long lived memory B cells are also generated after these processes (Tangye, 2011).

1.3.3.4 Development of marginal zone B cells

Another type of B cells are the marginal zone B cells which, after leaving the bone marrow, migrate to the marginal zone (MZ) of the spleen. MZ B cells provide an early and rapid immune response against blood-borne pathogens, mainly in a T-cell independent manner. Within a few days of encountering antigens, the MZ B cells rapidly differentiate and proliferate into short lived plasma cells that secrete antibodies, mainly consisting of IgM with limited switching to some IgG subtypes (Abbas *et al.*, 2007). The full development of B cells in bone marrow, spleen and lymph nodes up to the production of plasma cells and memory cells is summarised in Figure 1.7.

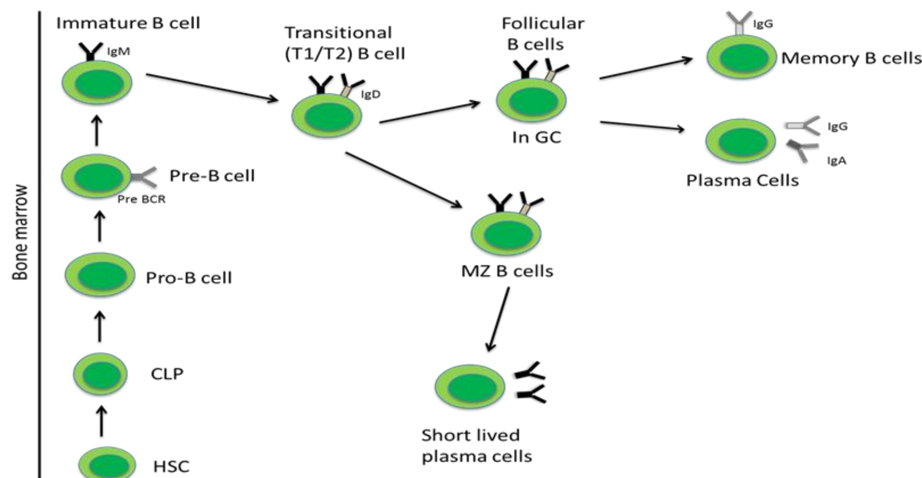


Figure 1.7: Overview of B-cell development. The early stage of B cell development is in bone marrow (HSC, CLP, Pro B cells, Pre B cells,immature B cells). Immature B cells leave bone marrow and travel to spleen and enter as transitional B cells (T1 then T2 transitional cells). They complete the early development either as follicular B cells or marginal zone (MZ) B cells. In GC, upon antigen encounter, follicular B cells are activated in a T cell-dependant manner. After undergoing SHM and CSR, long lived plasma cells are generated and travel to peripheral tissues providing long lasting antibodies. Memory B cells are also generated from these process. As for MZ B cells, once they encounter antigen, they will develop into short lived plasma cells secreting mainly IgM antibodies.

1.3.3.5 Memory B cells

Memory B cells are defined as long-lived quiescent B cells expressing somatically mutated Ig V genes that are capable of eliciting rapid and robust responses compared to naïve B cells (Tangye and Tarlinton, 2009). It has been postulated that memory B cells are generated from GCs B cells in a T-cell dependent manner, however, Berkowska et al. demonstrated that there are several distinct memory B cell subsets based on their antigen-experienced phenotype and differential expression of CD27 and IgH isotypes (Berkowska *et al.*, 2011). These memory cells subsets are differentiated from three different origins and occur in T-cell dependent and T-cell independent manner.

CD27+IgA+ and CD27+IgG+ memory B cell subsets are considered as true memory B cells (Good *et al.*, 2009) due to high expression of activation and extensive replication history compared to naïve B cells (Berkowska *et al.*, 2011). They develop in GCs in a T-cell-dependant manner. Two subsets have been

identified that develop in a T-cell independent manner: natural effector cells, which originate from MZ of spleen and CD27-IgA+ cells from the gastrointestinal system. The fact that these cells were found in patients with CD40L deficiency suggests that they develop in a T-cell independent manner (Berkowska *et al.*, 2011). In contrast to GC memory B cells, these two subsets are characterised by limited replication history and reduced degrees of SHM.

1.3.3.6 Peripheral tolerance of B cells

Similar to T cells, some of the autoreactive B cells manage to evade central tolerance mechanisms and enter the periphery. Peripheral tolerance mechanisms of B cells include the induction of anergy. Anergy in B cells is a state of unresponsiveness in which B cells that are capable of a normal initial response to antigen fail to receive secondary signals (Cambier *et al.*, 2007). This occurs when self-reactive B cells are exposed to self-antigens in the absence of T cell help (Cambier *et al.*, 2007). Therefore, anergy silences potentially harmful self-reactive cells in the periphery and blocks the cells from forming plasma and memory cells expressing high affinity autoantigens. Another mechanism is through apoptotic deletion. B cells that manage to escape any of these mechanism result in autoreactive cells, causing autoimmune diseases.

1.4 PIDs involving immune dysregulation

1.4.1 Overview

So far, I have described the normal innate antiviral immunity and adaptive immune response. It is clear that both systems play an important role in preventing the host from susceptibility to infection. Any defects affecting either one or both arms of the immune response results in immunodeficiency, which has been elucidated by genetic defects identified in PIDs.

In addition to immunodeficiency, many PIDs with immune dysregulation have also been identified recently, thus implying that it is important that the immune response must be counterbalanced by regulatory mechanisms to contract the size and activity of the antigen-specific population. During normal immune responses, as mentioned before, the production of co-stimulators and cytokines allows the clonal expansion and differentiation of lymphocytes into effector cells.

In addition, anti-apoptotic genes of the Bcl2 family are upregulated, providing survival stimuli for the lymphocytes. In normal homeostasis of the immune system, after successful elimination of the pathogenic foreign antigens, these survival stimuli also decline and the lymphocytes will return to their basal resting state. The majority of the activated cells will die through apoptosis either via the activation-induced cell death (AICD) pathway which involves the upregulation of the expression of death receptors such as Fas on the lymphocytes surface and the binding of Fas to its ligand, or by passive apoptosis via the mitochondrial pathway, i.e. induction of the expression of the proapoptotic protein Bim (both mechanisms will be described in detail in Chapter 4.3). Defects in immune homeostasis may lead to a lymphoproliferative disorder that could predispose to the formation of lymphoma, a type of lymphocyte cancer. Another important aspect that has been discussed in earlier sections is the lymphocyte tolerance. A failure of immune tolerance results in autoreactive immune cells attacking their own host cells and leads to autoimmunity.

According to the latest update of the International Union of Immunological Societies' (IUIS') PID classification, genetic defects that are associated with immune dysregulation, autoimmunity and lymphoproliferation are classified under diseases of immune dysregulation (Picard *et al.*, 2015). Five diseases have been categorized under this classification including autoimmune lymphoproliferative syndrome (ALPS), haemophagocytic lymphocytosis (HLH), T regulatory genetic defects, immune dysregulation with colitis and type 1 interferonopathies (Picard *et al.*, 2015). For the purpose of my work, ALPS and HLH will be discussed in detail.

1.4.2 Autoimmune lymphoproliferative syndrome (ALPS)

The importance of the death receptor pathway, mediated by the interaction of Fas receptor with its ligand FasL, in maintaining lymphocyte homeostasis was first discovered in two related mouse strains with lymphoproliferation (denoted lpr) and generalised lymphoproliferative disease (denoted gld) (Sneller *et al.*, 1997). The lpr mice were found to have a complete defect of Fas expression whereas the gld mice had a recessive defect of Fas ligand; both defects abrogated the function of the respective protein. As well as lymphoproliferation, these mice showed autoantibody formation and autoimmune nephritis. Later, a human disease with similar features of coexistent lymphoproliferative disease

and autoimmunity was found to be caused by heterozygous Fas mutations (Rieux-Laucat *et al.*, 1995). The disease of these patients was called autoimmune lymphoproliferative syndrome (ALPS). ALPS is defined as a genetic disorder “resulting in a failure of apoptotic mechanisms to maintain lymphocyte homeostasis, permitting accumulation of lymphoid mass and persistence of autoreactive cells that often manifest in childhood with chronic nonmalignant lymphadenopathy, hepatosplenomegaly, and recurring multi lineage cytopenias “ (Rao and Oliveira, 2011).

1.4.2.1 Clinical Features and diagnosis

The typical onset of ALPS is usually in early childhood; however, it can also occur later in life, from 18 to 35 years of age (Oliveira, 2013). The main clinical features of ALPS are the presence of chronic, non-malignant lymphadenopathy and/or splenomegaly, in addition to which the majority of patients develop autoimmunity, commonly autoimmune cytopenias such as haemolytic anaemia and/or thrombocytopenia (Fleisher *et al.*, 2010). Other autoimmune disorders such as glomerulonephritis, polyneuropathy and autoimmune hepatitis have also been documented (Fleisher *et al.*, 2010). A life-threatening complication of ALPS is lymphoma, a type of cancer that arises from lymphocytes, of either Hodgkin’s (HL) or non-Hodgkin’s (NHL) type. The risk of developing HL is 150 time greater and NHL is increased 61-fold compared with the general population(Price *et al.*, 2014). Laboratory features that are useful in diagnosing ALPS are elevated numbers of T cells that express TCRα/β but neither CD4 nor CD8 co-receptors (CD4-CD8-, called double negative T (DNT) cells), and high serum or plasma levels of IL-10, IL-18, soluble Fas ligand and vitamin B12 (Rao and Oliveira, 2011). The demonstration of defective Fas-mediated lymphocyte apoptosis in vitro is highly supportive (Oliveira *et al.*, 2010). Oliveira and colleagues proposed a set of diagnostic criteria for ALPS, shown in Table 1.2.

Required
1. Chronic(>6 months),non malignant,non-infectious lymphadenopathy or splenomegaly or both
2. Elevated DNT cells (≥1.5% of total lymphocytes or 2.5% of CD3+ lymphocytes in the setting of normal or elevated lymphocyte counts
Accessory
Primary
1. Defective lymphocyte apoptosis (in 2 separate assays)
2. Somatic or germline pathogenic mutation in FAS, FASLG, or Caspase 10
Secondary
1. Elevated plasma sFASL levels (>200pg/ml) OR elevated plasma IL-10 levels (>20pg/ml) OR elevated serum or plasma vitamin B12 levels (> 1500ng/L) OR elevated plasma IL-18 levels > 500pg/ml
2. Autoimmune cytopenias (haemolytic anemia, thrombocytopenia, or neutropenia) AND elevated immunoglobulin G levels (polyclonal hypergammaglobulinemia)
3. Family history of a non-malignant/non-infectious lymphoproliferation with or without autoimmunity

Table 1.2. Diagnostic criteria for ALPS. A definitive diagnosis is made when the patient demonstrates both required criteria and one primary accessory criterion. A probable diagnosis of ALPS is considered when the patient presents with both required criteria and one secondary accessory criterion. The table is adapted from (Oliveira *et al.*, 2010).

For a definitive diagnosis of ALPS, the patients should show both chronic non-malignant lymphadenopathy or splenomegaly and elevated DNT cells, plus one of the primary accessory criteria.

1.4.2.2 Genetics and classification

So far, several causative mutations within the FAS-mediated pathway have been identified in patients with ALPS (Oliveira and Fleisher, 2004; Fleisher *et al.*, 2010; Oliveira *et al.*, 2010; Rao and Oliveira, 2011). The majority of ALPS patients bear autosomal dominant germline heterozygous mutations of *FAS*. However, it was demonstrated that as many as 60% of family members who are carriers of the same genetic defects have mild or absent phenotypic expression of the disease, indicating that other factors such as haploinsufficiency and gene modifier may be involved in determining the phenotypic expression (Price *et al.*, 2014). Germline *FAS* mutations affecting the intracellular portion of the Fas protein are associated with severe clinical manifestations and higher penetrance (Jackson *et al.*, 1999). These patients have been reported to have

increased risk of developing HL and NHL (Price *et al.*, 2014). Somatic Fas mutations have also been described in a small number of patients with sporadic ALPS (Fleisher *et al.*, 2010).

Apart from Fas, heterozygous or homozygous mutations in Fas ligand (*FASL*) have also been identified, with homozygous mutations causing a severe ALPS phenotype (Del-Rey *et al.*, 2006). Other mutations that affect the Fas-mediated apoptosis pathway, such as germline mutations of caspase 10, have also been described (Wang *et al.*, 1999). All these mutations contribute to the classification of ALPS. Initially, the classification of ALPS was based on numbers, however, due to chaotic nomenclature, the classification has been changed to simpler nomenclature (Oliveira *et al.*, 2010). The full previous and revised classification of ALPS is shown in Table 1.2.

Previous nomenclature	Revised nomenclature	Gene	Definition
ALPS type 0	ALPS-FAS	FAS	Patients fullfill ALPS diagnostic criteria and have germline mutations in FAS
ALPS type Ia	ALPS-FAS	FAS	Patients fullfill ALPS diagnostic criteria and have germline heterozygous mutations in FAS
ALPS type Im	ALPS-sFAS	FAS	Patients fullfill ALPS diagnostic criteria and have somatic mutations in FAS
ALPS type Ib	ALPS-FASLG	FASLG	Patients fullfill ALPS diagnostic criteria and have germline mutations in FAS Ligand
ALPS type IIa	ALPS-CASP10	Caspase 10	Patients fullfill ALPS diagnostic criteria and have germline mutations in caspase 10
ALPS type III	ALPS-U	Unknown	Patients meet ALPS diagnostic criteria; however,genetic defect is undetermined
ALPS-related apoptosis disorder			
ALPS type IIb	Caspase-8 deficiency state	Caspase 8	Patients present with lymphadenopathy and/or splenomegaly,elevated DNT,recurrent infections,and germline mutations in caspase 8
ALPS type IV	RALD	NRAS/KRAS	Patients present with autoimmunity, lymphadenopathy and/or splenomegaly, elevated DNT, somatic mutations in NRAS or KRAS

Table 1.3: Revised classification ALPS and ALPS-related disorders. FASLG – Fas Ligand, RALD- RAS-associated autoimmune leukoproliferative disease. This table is adapted from (Oliveira *et al.*, 2010).

In addition, there are also apoptotic disorders that cause distinct phenotypes, now classified as ALPS-related disorders. Patients with mutations in the gene encoding caspase 8 demonstrate typical features of ALPS such as lymphadenopathy, splenomegaly, elevated DNTs and defective Fas-induced

apoptosis. However, they also show additional features such as defective T-, B- and NK-cell activation with consequent recurrent bacterial and viral infections (Chun *et al.*, 2002). Somatic mutations of NRAS and KRAS are also classified under ALPS-related disorders; they share the clinical features of ALPS but without defective Fas-mediated apoptosis as NRAS and KRAS regulate the intrinsic-mitochondrial apoptosis pathway. Patients with RAS-associated autoimmune leukoproliferative disorder show chronic persistent monocytosis and significant leucocytosis (Oliveira *et al.*, 2007; Niemela *et al.*, 2011). Recently, evaluation of a patient who presented with chronic lymphadenopathy, splenomegaly, autoantibodies, elevated immunoglobulin levels and natural killer cell dysfunction associated with chronic, low-grade Epstein-Barr virus infection revealed a novel homozygous, loss-of-function mutation in the gene encoding Protein kinase C delta (PKCδ) (Kuehn *et al.*, 2013). This mutation results in B cell hyperproliferation and defective apoptosis with consequent lymphocyte accumulation, a phenotype that is mirrored in the PKCδ knock out mouse model (Kuehn *et al.*, 2013). Furthermore, homozygous loss-of-function mutations in LPS-responsive and beige-like anchor (LRBA) have been discovered very recently by whole exome sequencing analysis in three patients from unrelated families presenting with ALPS-like features with raised serum Fas ligand levels and defective Fas-mediated apoptosis (Revel-Vilk *et al.*, 2015). In addition, another mutation that has been identified through whole exome sequencing is FADD deficiency in which the patient exhibited typical ALPS features with severe susceptibility to bacterial and viral diseases as well as recurrent hepatopathy and encephalopathy, and cardiac malformations (Bolze *et al.*, 2010). Interestingly, these patients did not display any autoimmune features. Both of these newly identified gene mutations contribute to the expanding list of genes underlying ALPS-like diseases.

In addition to the ALPS classifications described above, there is ALPS-U, in which the patients present with ALPS features, but the genetic defect is unknown. It is estimated that about one third of patients fall into the ALPS-U group. It is likely that more advanced technologies such as whole exome sequencing will help in identifying the molecular defect in these patients. It is important to know the genetic defect for establishing a diagnosis in order to enable proper care and follow up of patients with this disease.

1.4.2.3 Treatment for ALPS

Almost half of ALPS patients in a large US series underwent splenectomy in order to manage their chronic and refractory cytopenias (Rao and Oliveira, 2011), however, in the recent study by Price et al. demonstrated that patients underwent splenectomy, likelihood to have cytopenia relapse, estimated about 30% by 4 years and exceeded 70% by 20 years (Price *et al.*, 2014). In addition, young patient who underwent splenectomy had increased risk of developing sepsis (Price *et al.*, 2014). Cytopenias may also be successfully treated with immunosuppressive drugs such as corticosteroids, as well as intravenous Immunoglobulin G, rituximab and the mTOR inhibitor rapamycin (sirolimus) (Teachey *et al.*, 2009). Besides treating the cytopenias, rapamycin is also useful in reducing the lymphoproliferation as well as significantly reducing the numbers of DNT cells. However, due to its toxicity, patients on rapamycin need to be monitored for a long time. As most ALPS patients respond to these treatments, haematopoietic stem cell transplantation (HSCT) is rarely considered and so far the role of HSCT in ALPS patients is not really clear (Rao and Oliveira, 2011). However, HSCT has been successful in treating selected ALPS patients with lymphoma or a very severe phenotype of FAS mutation and refractory cytopenias (Sleight *et al.*, 1998). Failures of HSCT have also been observed, which were due to progression of malignancy, ineffective engraftment resulting from non-myeloablative conditioning and opportunistic infections (Rao and Oliveira, 2011). Finally, the morbidity and mortality of ALPS depends on the severity of autoimmune disease, hypersplenism (or susceptibility to fatal sepsis secondary to splenectomy) and development of lymphoma. As mentioned earlier, intracellular FAS mutations usually result in severe phenotypes with increased risk to develop lymphoma, therefore these patients need diligent lifelong follow up. Since ALPS patients develop multiple problems, they sometimes require collaborative follow up from multiple subspecialists that are familiar with ALPS for long term monitoring.

1.4.3 Haemophagocytic Lymphohistiocytosis (HLH)

The term HLH describes a pattern of immune dysregulation marked by overwhelming immune/inflammatory responses. It arises due to a reactive process resulting from persistent and excessive activation of antigen-presenting cells (macrophages, histiocytes) and T cells (Filipovich, 2009) and

overproduction of cytokines such as IFN-γ, IL-6 and IL-10 (Faitelson and Grunebaum, 2014). There are two forms of HLH, primary and secondary. Primary HLH results from inborn errors of the immune system and includes familial HLH (fHLH) and X-linked lymphoproliferative diseases (XLP). Typically, disease onset in fHLH is during infancy or early childhood and may be triggered by infection such as Epstein Barr virus (EBV), cytomegalovirus or parvovirus (Henter *et al.*, 1993), while EBV is the classic trigger in boys with XLP.

HLH may also occur outside the context of PID in response to overwhelming activation of the immune system, for example severe inflammatory reactions to viral infection, including EBV, HIV and Avian influenza (Henter *et al.*, 2007). This form is called virus associated haemophagocytic syndrome (VAHS) and has been demonstrated to occur with increased frequency in immunocompromised patients (Henter *et al.*, 2007). In addition, other precipitating factors for HLH are autoimmune diseases (e.g. rheumatoid arthritis) and malignancies (e.g. leukaemia, lymphoma). The term “macrophage activation syndrome” (MAS) is used interchangeably with HLH especially in the rheumatological context (Henter *et al.*, 2007) where this is a feared complication.

1.4.3.1 Genetics of primary HLH

Several autosomal recessive genetic defects have been linked to fHLH and interfere with the mechanisms responsible for cytotoxicity. The first gene linked to fHLH was PRF1, encoding perforin, a soluble, pore-forming cytolytic protein that is secreted by cytotoxic T cells and NK cells upon conjugation between effector and target cells. It is known to be sequestered along with granzyme serine proteases in secretory cytotoxic vesicles. Perforin can perforate the membrane of the target cell and polymerize to form a cell death-inducing pore which allows the entry of granzymes that trigger apoptosis. The second gene in which autosomal recessive mutations cause HLH is UNC13D. This encodes Munc 13-4 which is important for the fusion of cytolytic granules with the plasma membrane and its deficiency results in defective degranulation (Filipovich, 2009). Syntaxin 11 deficiency similarly leads to defective degranulation (Filipovich, 2009). Other cytotoxic defects that have been associated with life threatening episodes of HLH are Chediak-Higashi syndrome (defect in the lysosomal trafficking regulator (*LYST/ CHS1*) gene) (Faitelson and Grunebaum, 2014), Griscelli type 2 (mutation in the *RAB27A* gene that is involved in

vesicular fusion, trafficking and docking at the plasma membrane) (Faitelson and Grunebaum, 2014) and Hermansky Pudlak type 2 (mutations in the *AP3B1* gene that encodes the adaptor protein AP-3 complex, involved in trafficking of proteins to lysosomes) (Faitelson and Grunebaum, 2014). The latter disorders lead to severe impairment of secretory lysosomes of melanocytes, platelets and neutrophils as well as of T and NK cells, with corresponding associated features such as oculocutaneous albinism and bleeding diathesis as well as susceptibility to HLH.

HLH arises as a complication of EBV infection in 35% of boys with X-linked lymphoproliferative syndrome (XLP1), other outcomes of EBV including fulminant infectious mononucleosis, aplastic anaemia and lymphoma (Booth *et al.*, 2011). XLP1 is caused by hemizygous mutations in *SH2D1A* encoding SAP (SLAM-associated protein) which results in deficiency of invariant Natural killer T (iNKT) cells and impaired T and NK cytotoxic responses towards EBV-infected B cells (Filipovich, 2009). XLP1 is also characterised by decreased activation-induced apoptosis of lymphocytes, which contributes to associated lymphoproliferation (Filipovich, 2009). Dysgammaglobulinaemia is prominent and may precede the acquisition of EBV. XLP2 is due to a hemizygous mutation in X-linked inhibitor of apoptosis (*XIAP*) and has been described in male patients with EBV-associated HLH (Rigaud *et al.*, 2006). In contrast to XLP1, lymphomatous change does not appear to occur in XIAP deficiency but patients often experience splenomegaly and colitis.

1.4.3.2 Clinical features and diagnostic criteria

In the absence of a positive family history, it is difficult to distinguish primary from secondary HLH on clinical grounds alone as their presentations overlap. Most patients with HLH present with prolonged fever and hepatosplenomegaly (Henter *et al.*, 2007). Other findings that are associated with HLH are neurological symptoms including seizures and/or ataxia, hypo- or hypertonia, cranial nerve palsies, meningismus, signs of increased intracranial pressure and altered consciousness (Filipovich, 2009). Less common findings in HLH are rashes, lymphadenopathy and diarrhoea. Laboratory findings reveal hyperferritinaemia, cytopenias (especially anaemia and thrombocytopenia), liver dysfunction, hypofibrinogenemia, hypertriglyceridemia, hypoalbuminemia and hyponatremia(Filipovich, 2009). Furthermore, other abnormal laboratory

findings in HLH are low NK cell cytotoxic activity and elevated soluble IL-2 receptor (sIL-2r) levels in serum and cerebrospinal fluid (Henter *et al.*, 2007). Histological examination of bone marrow or other tissues may demonstrate frank haemophagocytosis (ingestion of erythrocytes by macrophages) but this is neither sensitive nor specific for HLH.

Diagnostic guidelines were formed in order to assist the diagnosis of HLH. The guidelines had been revised twice and the latest was in 2004. The main five guideline criteria that was generated in 1991 is still maintained in 2004 , including :1) fever 2) splenomegaly 3) cytopenias affecting at least two or three lineages in the peripheral blood 4) hypertriglyceridemia and hyperfibrinoginemia and 5) hemophagocytosis in bone marrow, spleen or lymph nodes (Henter *et al.*, 2007). Three other criteria that are added to the 2004 guidelines are : 6) low or absent NK cells activity 7) hyperferritinemia and 8) high levels of sIL-2r (Henter *et al.*, 2007). For the diagnosis of HLH, altogether five of the eight criteria must be fulfilled , however exception for patients with molecular diagnosis of HLH, these diagnostic criteria not need to be fulfilled (Henter *et al.*, 2007). The diagnostic criteria are summarised in Table 1.4.

The diagnosis HLH can be established if one of either 1 or 2 below is fulfilled
(1) A molecular diagnosis consistent with HLH
(2) Diagnostic criteria for HLH fulfilled (five out of the eight criteria below)
(A) Initial diagnostic criteria (*to be evaluated in all patients with HLH*)
Fever
Splenomegaly
Cytopenias (affecting ≥2 of 3 lineages in the peripheral blood):
Hemoglobin <90 g/L (in infants <4 weeks: hemoglobin <100 g/L)
Platelets <100 × 10^9/L
Neutrophils <1.0 × 10^9/L
Hypertriglyceridemia and/or hypofibrinogenemia:
Fasting triglycerides ≥3.0 mmol/L (i.e., ≥265 mg/dl)
Fibrinogen ≤1.5 g/L
Hemophagocytosis in bone marrow or spleen or lymph nodes
No evidence of malignancy
(B) New diagnostic criteria
Low or absent NK-cell activity (according to local laboratory reference)
Ferritin ≥500 µg/L
Soluble CD25 (i.e., soluble IL-2 receptor) ≥2,400 U/ml

Comments:

(1) If hemophagocytic activity is not proven at the time of presentation, further search for hemophagocytic activity is encouraged. If the bone marrow specimen is not conclusive, material may be obtained from other organs. Serial marrow aspirates over time may also be helpful.
(2) The following findings may provide strong supportive evidence for the diagnosis: (a) spinal fluid pleocytosis (mononuclear cells) and/or elevated spinal fluid protein, (b) histological picture in the liver resembling chronic persistent hepatitis (biopsy).
(3) Other abnormal clinical and laboratory findings consistent with the diagnosis are: cerebromeningeal symptoms, lymph node enlargement, jaundice, edema, skin rash. Hepatic enzyme abnormalities, hypoproteinemia, hyponatremia, VLDL ↑, HDL ↓.

Table 1.4: 2004 Diagnostic Guideline for HLH. VLDL- very low density lipoprotein, HDL- high density lipoprotein. This table is reproduced from (Henter *et al.*, 2007).

1.4.3.3 Treatments for HLH

The prognosis of untreated active HLH is extremely poor and it can rapidly progress to a fatal disease. Thus, treatment must be started urgently when there is a high clinical suspicion of HLH. Treatment consists of combinations of chemotherapy and immunosuppressive drugs targeting the abnormal and hyperactivated T cells (Filipovich, 2009). The treatment protocol for HLH has been revised twice, firstly in 1994 and secondly in 2004 in which the protocol is designed for the patients with HLH, with or without evidence of familial or genetic disease, regardless of suspected or documented viral infections (Henter *et al.*, 2007). The treatment protocol includes initial therapy that covers the first 8 weeks of treatment involving etoposide, dexamethasone and cyclosporine A as well as other supportive medications such as prophylactic antibiotics. This is followed by continuation therapy with the same therapy and, if an acceptable

donor is available, HSCT should be performed as soon as possible. HSCT is the curative treatment for HLH. Through follow up, it was demonstrated that 5 years after HSCT, up to 66% of HLH patients are still alive, providing a good prognosis after receiving HSCT treatment (Trottestam *et al.*, 2011).

1.5 Approaches to the identification of defective genes of PIDs

1.5.1 Overview

As mentioned earlier, in the latest update (2015), PIDs were classified according to the basis of the affected immunological compartment (Picard *et al.*, 2015). In each classification, they were further classified based on their diseases and phenotypes and finally the genetic defects that cause the disease. Although many genetic defects have been identified, nonetheless , still many patients with clinical and laboratory evidence of PID lack a molecular genetic diagnosis (Conley and Casanova, 2014). These patients present a challenge in providing an appropriate prognosis and therapy. There have been several approaches that have been used to identify the disease causing variants in some of these patients. The three major approaches that are currently favoured are: 1) investigating candidate genes within pathways that are known to be important and shown to be dysfunctional, 2) using unbiased genomic approaches such as next generation sequencing (NGS) and/or linkage analysis and 3) comparative genetics, i.e. similarity of the clinical phenotypes to mouse models (Platt *et al.*, 2014). These approaches are discussed below.

1.5.2 Investigating known signalling pathways

The phenotypic investigation of known signalling pathways can be helpful in candidate gene identification for targeted Sanger sequencing (Platt *et al.*, 2014). For instance, previously our own group has successfully identified a novel genetic defect associated with increased viral susceptibility via this approach (Hambleton *et al.*, 2013). With the knowledge that type I IFN signalling is crucial for antiviral immunity, we investigated this pathway in a patient with disseminated vaccine strain measles following routine immunization and by Sanger sequencing found a homozygous STAT2 mutation to be the cause of the disease. This approach was also useful in identifying the Interleukin-17 receptor (IL-17R) as a novel defect causing impaired Th17 responses in certain

patients with chronic mucocutaneous candidiasis (CMC) (Puel *et al.*, 2011). Likewise, molecular defects affecting tumor necrosis factor receptor–associated factor 3 (TRAF3) were identified by investigating type I IFN induction pathways (Pérez de Diego *et al.*, 2010), adding to mutations in TLR3 and UNC93B that had already been described in this pathway as causing Herpes simplex Encephalitis (HSE) (Dropulic and Cohen, 2011).
It is easier to use this approach if the disease manifests with specific phenotypes as one can concentrate on one rather than multiple signalling pathways. However, such a hypothesis-driven approach may be limited by prior knowledge and preconceptions. Therefore, to overcome this limitation, other methods such as using the advanced genetic approaches can be used to broaden the scope of gene discovery.

1.5.3 Unbiased genetic approaches

Next Generation sequencing (NGS) is currently widely used and has been demonstrably successful in identifying many novel disease genes in PID In contrast to Sanger sequencing, NGS enables fast and cost-effective sequencing of the whole genome (WGS) or whole exome (WES) within a few days (Moshous *et al.*, 2013; Nijman *et al.*, 2014). WES captures the coding regions that, while constituting only 1% of the genome, harbour approximately 85% of deleterious mutations (Botstein and Risch, 2003). Currently, WES is the preferred method due to its lower cost and complexity. However, WGS provides better coverage of non coding as well as coding exons, intronic sequences, promoters and regulatory regions (Conley and Casanova, 2014). There are several NGS platforms in widespread use, including Solexa/Illumina (the most common method), 454 Sequencing (Roche) and SOLID (Sequencing by Oligonucleotide Ligation and Detection, Life Technologies), which differ in their sequencing chemistries and technical details (Heather and Chain, 2015). Newer platforms continue to enter the market and differ dramatically in read length, error rate and cost.

Briefly, to perform WES involves shearing the patient's genomic DNA into small fragments, followed by ligation of adaptors to the ends of the sheared DNA. Next, the coding regions are selected by allowing a labelled "bait', which consist of exons with flanking splice sites, to hybridise to the patient DNA (Conley and Casanova, 2014). Finally, the labelled bait and the patient's coding region are

purified, amplified and primers hybridising to adaptors are used for massively parallel sequencing (Conley and Casanova, 2014) where the sequencing of each patient coding region must be sequenced at least 10 up to 100 times. This is to avoid mistaking a sequencing error for a true DNA alteration. To analyse the sequencing data, a bioinformatic analysis or pipeline is required and to finally identify the pathogenic variants, it involves several filtering processes (will be discussed in chapter 4.1). Although, this technique provide a comprehensive sequencing data but it is challenging to analyse the data as it requires multistep of bioinformatics analysis or pipeline as well as involving several filtering processes to finally identify the pathogenic variants.

Nevertheless, this technique has already been shown to be useful in identifying causative genetic defects in PIDs showing both recessive and dominant models of inheritance (Casanova *et al.*, 2014; Conley and Casanova, 2014). The first successes were in patients from consanguineous families or belonging to isolated populations, in which context homozygous disease-causing variants are likely (Conley and Casanova, 2014). For example, one young woman was found to have inherited a homozygous premature stop codon of exon 6 of *PI3KR1* which resulted in agammaglobulinaemia and failure of B cell development (Conley *et al.*, 2012). WES has also been used successfully to investigate groups of unrelated patients who share a distinctive and rare phenotype, and are likely to bear mutations in the same gene. One example is mutations in *GATA2,* affecting 4 patients with similar phenotypes of dendritic cell, monocyte, B and natural killer lymphoid deficiency (Dickinson *et al.*, 2011). The familial occurrence of this phenotype in adjacent generations of outbred families strongly suggested an autosomal dominant model of inheritance. Heterozygous mutations were found at different sites of the same gene in each patient but all mutations were predicted be deleterious, affecting the C-terminal zinc finger domain of GATA2 (Dickinson *et al.*, 2011). Some *GATA2* mutations were de novo and hence sporadic.

In addition to WES, another available genetic approach is classical linkage analysis which uses SNPs flanking a pathogenic mutation to define the disease locus shared by the affected subjects (Platt *et al.*, 2014). To identify the causative variant, the genes within the linkage region are then sequenced. There have been studies that have demonstrated the success of this technique

in identifying novel disease-causing variants. One example is in 5 patients within 4 consanguineous families who presented with hypogammaglobulinemia, decreased B cell numbers and autoimmunity (Lopez-Herrera *et al.*, 2012). Initially 81 genes were identified through linkage analysis and Sanger sequencing was performed for selected genes. A homozygous mutation in *LRBA* was shown to be causative. This technique is most powerful in large families with multiple affected members but often identifies large linkage regions within which to spot and analyse the disease gene by Sanger Sequencing.

Another related and useful genomic approach is homozygosity mapping, a special case of linkage analysis (Platt *et al.*, 2014). This approach is helpful in the setting of rare disorders in consanguineous families where homozygous autosomal recessive mutations are expected. It identifies the genomic regions that most likely harbours the pathogenic mutation within a locus containing clusters of homozygous SNPs specific to the affected subjects (Platt et al., 2014). It is commonly combined with WES to assist in filtering candidate variants in PID. An example is the identification of a homozygous mutation in tripeptidyl peptidase (*TPP2*) in four patients from two families (two members of each family were affected) with combined immunodeficiency, severe autoimmunity and developmental delay (Lu *et al.*, 2014). Another example is in 4 members of an extended consanguineous family who presented with biological features of ALPS and susceptibility to viral and bacterial infections. Both WES and homozygosity mapping were performed and their intersection revealed a homozygous missense mutation of Fas-associated death domain (FADD) to be disease-causing (Bolze *et al.*, 2010). These examples demonstrate that homozygosity mapping and WES provide a powerful combination in identifying the molecular defects in PIDs.

After candidate pathogenic variants are identified using these genetic approaches, extensive functional assays are often required in order to confirm that the identified variants are disease-causing. However, in certain cases, such functional assays fail to provide a mechanistic link to the clinical phenotype. In part this may reflect technical limitations of the experimental system such as the use of different cell types and lines in vitro. In addition, additional factors, such as the modifying genetic factors, environmental factors or infectious exposures

may influence the expression of the disease phenotype in different settings (Conley and Casanova, 2014).

1.5.4 Similarity of the clinical phenotypes to mouse models

It has been demonstrated by many studies that mouse models are useful in enabling the identification of disease causing variants when the phenotypes of the knock-out mouse models are similar to the patients' (Bolze *et al.*, 2010). This approach has been used in three patients with Interferon Regulatory Factor 8(IRF8) deficiency where they displayed disseminated mycobacterial disease and absence of dendritic cells including both myeloid and plasmacytoid dendritic cells in the blood in which these phenotypes were similar to phenotypes observed in IRF8 knock out mice (Hambleton *et al.*, 2011).

In addition, mouse models can also be used to help prioritize candidate genes that were derived from NGS. For example, in a family with congenital asplenia , 32 candidate genes had been identified in 3 affected siblings by WES (Koss *et al.*, 2012). Among the candidate genes, NKX2-5,stood out as it had already been shown to be essential for mouse spleen development (Koss *et al.*, 2012). To confirm, in vitro studies demonstrated that the missense mutation of NKX2-5 results in abolition of its function (Koss *et al.*, 2012) .

Whilst comparative genetics can be a powerful approach, there is a major limitation since not all mouse models recapitulate human disease. A good example is provided by a mouse deficient in TANK-binding kinase 1 (Tbk1), the susceptibility phenotype of which suggested that this protein is important for multiple antiviral and antibacterial pathways (O'Neill, 2008). However, in contrast, so far human TBK1 deficiency only results in susceptibility to herpes simplex virus (HSV), causing herpes simplex encephalitis (Herman *et al.*, 2012). Thus, the mouse model of Tbk1 deficiency did not predict the limited scope of human disease. Although only one example, it can still show that in certain cases the phenotypes observed in patients may not be similar to the mouse models.

In recent years, many mouse models were generated in order to help providing an understanding of the human immune system, but mostly the mouse generated from the studies had null mutation, complete loss of function. However since the phenotypes of null and hypomorphic mutations can be

dramatically different, knock out mice are imperfect models of the many PIDs due to hypomorphic mutations. In addition, due to mouse and humans being two different species, it is unavoidable to expect that there may be differences in the phenotypes and the scope of diseases between them.

1.5.5 The importance of molecular diagnosis for the patient and family members

The identification of a molecular defect provides a huge impact for the patients and family members. Firstly, by knowing the molecular diagnosis, this provides a better understanding on how the gene defects lead to the pathogenesis of the diseases. Secondly, the mutation analysis help to provide precise diagnosis and accurate prognosis for the patient. Since patients with PIDs are associated with increased morbidity and mortality, the molecular diagnostics enable the appropriate therapy and treatment to be given to the patients which include in the development of long term preventive strategies to limit complications and irreversible organ damage. In addition, a lifesaving and permanent curative interventions such as HSCT can be performed.

In recent years, Haematopoietic Stem Cell Transplantation (HSCTs) is considered to be the treatment of choice for many forms of PID as it offers cure and HSCT-associated morbidity and mortality is constantly improving (Cant *et al.*, 2013). Although it is not the main criterion in assessing whether the patient is suitable for HSCT or not, knowing the genetic defect may influence and help to predict the outcome of the HSCT (Ochs *et al.*, 1999). For most SCID patients, the definitive treatment is HSCT, however, different gene mutations have different prognosis. For example, patients with Artemis deficiency have a worse prognosis of HSCT compared to RAG deficiency due to the associated cellular radiosensitivity. Besides SCID, patients with life-limiting combined immunodeficiencies such as that caused by mutations in the dedicator of cytokinesis 8 (*DOCK8*) have been successfully transplanted and this results in HSCT as the choice of treatment for this molecular diagnosis (Barlogis *et al.*, 2011).

The definitive treatment that requires knowledge of the molecular defect is gene therapy. This is a technique whereby a functional copy of the defective gene is introduced into the patient’s stem cells by means of viral vectors (Cant *et al.*,

2013). Although in some cases such as in Adenosine Deaminase deficiency that gives rise to severe combined immunodeficiency (SCID) gene therapy was demonstrated to be successful (Cavazzana-Calvo *et al.*, 2012), due to the evident serious potential complications such as activating oncogenes (Cant *et al.*, 2013), this treatment has yet to enter the mainstream.

Finally, the molecular diagnosis is not only for the patient's benefits but also for other family members as proper genetic counselling can be given to the family members. Knowing the genetic diagnosis can help the family members to plan and expect the risk of having other children that will be affected with the same disease.

1.6 Aims of the study

For my study, I have two different Primary Immunodeficiencies where:

1) Patient 1 demonstrated increased viral susceptibility after receiving measles, mumps, rubella (MMR) immunization.
2) Two patients (siblings) who had viral susceptibility and immune dysregulation that results in autoimmune lymphoproliferative syndrome (ALPS).

These patients were diagnosed with PID based on clinical and laboratory evidence, but their molecular diagnosis was unknown. Since it is important to provide the molecular diagnosis, the aim of my study was to identify the genetic defects causing these two novel PIDs using the approaches that have been described earlier. In addition, I anticipated that the identification of the genetic defects would enable me to elucidate the pathogenesis of their disease, which would provide new and better knowledge of the human immune system.

Chapter 2: Materials and Methods

2.1 Study subjects

Specimens were obtained with signed informed consent from patients and healthy volunteers. For samples from children age <16 years old, the informed consent was obtained from parents of the children.

For the novel defect in the IFN signalling study, patient and control fibroblasts and parents' whole blood were provided by the Clinical consultant in charge of the patient, Dr Ronan Leahy, Our Lady's Children Hospital, Crumlin, Dublin.

For the novel defect of autoimmune lymphoproliferative syndrome study, fibroblasts and frozen DNA from patients, family members and controls were prepared and collected from Human Genetics Laboratory, Centre for Life, Newcastle University. Control DNA was extracted from whole blood from healthy volunteers recruited from the Musculoskeletal Research Group, Newcastle University.

2.2 Materials

Reagents/medium	Company	Catalogue number
Phosphate buffered Saline (PBS	Lonza	BE10-543F
Hanks Buffered Saline Solution (HBSS)	Lonza	BE17-512F
Dulbecco's Modified Eagle's Medium	Gibco	41965-039
RPMI 1640 medium	Sigma	R0883
Fetal Calf Serum (FCS)	Lonza	14401F1
Lymphoprep	Axis-Shield Diagnostic	NYC1114547
Trypsin-EDTA	Sigma	59418C
200 mM L-glutamine Solution	Sigma	G6392
10x Penicillin-Streptomycin Solution (104 units per ml penicillin, 10 mg/ml streptomycin)	Sigma	P0781

Sodium Pyruvate	Sigma	S8636
2-mercaptoethanol	Gibco	31350-010
Non essential amino acid	Sigma	M7145
FuGENE HD Transfection Reagent	Promega	E2311
CD19 Microbeads, human	Miltenyi Biotec	130-050-301
Whole blood CD19 Microbeads human	Miltenyi Biotec	130-090-880
Phytohaemaglutinin (PHA)	Sigma	L1668

Table 2.1: Cell culture reagents and medium used for the research.

Cytokine	Company
Interferon-α (Intron A, recombinant)	Schering-Plough
Interferon-γ (Immunikin)	Boehringer Ingelheim
Interferon-β (Avonex)	Biogen Idec
Interleukin-2 (Proleukin)	Novartis

Table 2.2: Cytokines used for the research

Antibodies	Species	Company	Catalogue number	Dilution	Assay
Tet2	Mouse	Active Motif	61389	1:1000	WB
IFNAR2 C-terminal	Sheep	R & D systems	AF7014	1:500	WB
ISG56/IFIT	Goat	Santa Cruz	sc-82946	1:10000	WB
MxA(Mx1/2/3)	Rabbit	Santa Cruz	sc-34128	1:500	WB
STAT2	Rabbit	Millipore	07-140	1:500	WB
pSTAT2 (Tyr690)	Rabbit	Cell Signalling	4441	1:500	WB
STAT1	Rabbit	Cell Signalling	9172	1:500	WB
pSTAT1(Tyr701)	Rabbit	Cell Signalling	7649	1:500	WB
STAT3	Rabbit	Cell Signalling	4904	1:500	WB
pSTAT3(Tyr705)	Rabbit	Cell Signalling	9131	1:500	WB
GAPDH	Rabbit	Cell Signalling	5174	1:2000	WB
CD20-PE,human	Mouse	Miltenyi Biotec	130-098-084	1:100	Flow cytometry
Pacific blue α-mouse CD19	Mouse	Biolegend	115526	1:100	Flow

					cytometry
5-hydroxymethylcytosine (5 hmC)	Rabbit	Active Motif	39769	1:1000	IF
Anti-Flag M2	Mouse	Sigma	F3165	1:500	IF
Donkey α-sheep IgG HRP conjugated	Sheep	R & D system	HAF106	1:500	WB
Horse α-mouse IgG HRP	Mouse	Cell Signalling	7076	1:5000	WB
α-rabbit IgG HRP	Rabbit	Cell Signalling	7074S	1:2000	WB
Alexa Fluor 488 Goat anti-rabbit IgG	Rabbit	Invitrogen	A11070	1:500	IF
Alexa Fluor 546 Goat anti-mouse IgG	Mouse	Invitrogen	A21237	1:500	IF

Table 2.3: List of antibodies used in study

Chemical/reagent	Company	Catalogue number
Sodium Dodecyl Sulfate(SDS)	Sigma	71727
Sodium Deoxycholate	Sigma	D6750
Protease inhibitor cocktail	Roche	04693116001
DL-Dithiothreitol(DTT)	Sigma	646563
NuPAGE LDS Sample Buffer	Life Technologies	NP0007
Tris-HCL	Sigma	T5941
Sodium chloride	Sigma	S9888
Triton-X-100	Sigma	T8787
Trypan blue solution, 0.4%	Thermo Fischer Scientific	15250-061
Sodium Fluoride	Sigma	201154
Sodium Orthovanadate	Sigma	S6508
Pierce Protein Assay Reagent	Thermo Scientific	22660
Bovine Serum Albumin	Sigma	A2058
4-12% Tris-Glycine polyacrylamide gel	Life Technologies	EC60352BOX
Nu-PAGE Tris Glycine SDS Running Buffer	Life Technologies	LC2675
Immobilon™ Western	Millipore	WBKLS0500

Chemiluminescent HRP Substrate		
Paraformaldehyde	Sigma	P6148
Vectashield® Mounting Media with DAPI	VectorLabs	H-1200
Super Optimal Broth (SOB) medium	Invitrogen	15544-034
NEB 5-alpha Competent E. coli (Hig Efficiency)	New England Biolab	C2987H
Staurosporine	Calbiochem	569397
TaqMan® Gene Expression Master Mix	Life Technologies	4369016
Tris Base	Sigma	T1503
PageRuler Prestained Protein Ladde	Thermo Scientific	26616
Acetic Acid	Sigma	537020
Ethylenediaminetetraacetic acid (EDTA)	Sigma	E9884
Tween-20	Sigma	P1379
Glycine	Sigma	410225
LB Broth Powder	Sigma	L3022
NP-40 (Tergitol)	Sigma	S5439

Table 2.4: Chemical and biochemical reagents used for the research

Buffer	Components	Assay
MACS buffer	0.5% (w/v) Fetal Calf Serum (FCS), 2mM EDTA, Phosphate Buffered Saline (PBS)	B cells isolation using Miltenyi Biotec
FACs buffer	1% (w/v) Bovine Serum Albumin , Phosphate Buffered Saline (PBS)	B cells purity assessment by flow cytometry
50xTAE (Tris-acetate-EDTA) buffer	242 g Tris Base, 57.1 mL Acetic Acid 100 mL 0.5 M EDTA	Agarose gel electrophoresis
Tris Buffered-Tween Saline Solution (TBS/T	100mM Tris Base, 0.15M Sodium Chloride 0.1% (v/v) Tween-20	Western Blotting
Stripping buffer	15 g glycine, 1 g SDS, 10 ml Tween20, pH 2.2, dH_2O 1L	
Tris- HCL Buffer	100mM Tris HCL, distilled H_2O	Immunofluorescence
Blocking buffer	1% (w/v) Bovine Serum Albumin(BSA),	Immunofluorescence

	0.05% (v/v) Tween-20, PBS	
LB Broth medium	5.15g mixed LB Broth powder(Tryptone, Sodium Chloride, Yeast extract), 250ml distilled water	Plasmid cloning
RIPA buffer	50mM Tris HCL (pH 7.5), 150mM Sodium Chloride, 0.5%(w/v) Sodium Deoxycholate 0.1% (w/v) Sodium Dodecyl Cholate (SDS) 1% (v/v) NP-40, 1x complete protease inhibitor	Extraction of nuclear protein lysate
Whole cell lysis	20 mM Tris-HCL (pH 7.4), 150mM Sodium Chloride, 1% (v/v) Triton X-100, 5 mM EDTA, 1x complete protease inhibitor, phosphatase inhibitor(Sodium Fluoride, Sodium Orthovanadate)	Whole cell lysate extraction

Table 2.5 List of buffers used for assays.

Gene	Sequence (5'-3')
IFNAR2 cDNA	For - GGCGAGAGCTGCAAAGTTTA Rev – GCTTGCTCATCACTGTGCTC For – TTCTGGCTGGCCATAGACAT Rev – ACATCAACCTCAGGCAGGTC For – CCATGGATATGGTGGAGGTC Rev – TTGGAGAACACTTGCAGACG For – GCCTCGTGTTTGGTATTTCA Rev – GATGGAAATTTCACCATCACA For - CCCGAAATAAAAGGAAACATGA Rev - CCATATCCATGGCTTCCAAC For – CTGACCTGCCTGAGGTTGAT Rev - GGAAAGGTTGGCTGTGTCC
IFNAR2 gDNA (Exon 5)	For - AAGGCGCCCAAAAATAGACT Rev - GGGACGAAGTGGAGAAACAG
IFNAR1 cDNA	For - GGGCGGTGTGACTTAGGAC Rev - TGGTGGAGGTAGTTCATTTTCA For - AAGCTCAGATTGGTCCTCCA Rev – GTGCTCTGGCTTTCACACAA

	For - GCGTACAAGCATCTGATGGA Rev – CAGGGAAACGTCCTCTCTGT For – GATGACAACTTTATCCTGAGGTG Rev - CACCTGAAGAGTTTTTCCAGA For - TGAAAATGAACTACCTCCACCA Rev – TTCCAGACTGTTTTGGAGCA For – TTGGGAAAACACTTCAAATGC Rev - TGTTCCTCAGAAGTTGAAAGCA
Tyk2 cDNA	For – ACTGAGGCCCAGAATTGCTA Rev - AGGGCCAGGGTCTGTAGG For – CTTCGGCACAGAGCGTGT Rev – CCCTCATACACGTTGGTCCT For - CCCAGGACACTCAACCTCAG Rev – CTCCCTCTGCAGCCACTG For – CCACAATCTTGCTGACGTCTT Rev – CTCCCTCTGCAGCCACTG For - CGGCTGTGTACCGTTGTG Rev – AGGGCCAGGGTCTGTAGG For - ACTGAGGCCCAGAATTGCTA Rev - TTGCCCTGCTCAAAGAGGTA For - CTTCGGCACAGAGCGTGT Rev - GGTGCTCCAGTGAATGAGGT For - ACTCCAGCCACTACCTGTGC Rev – CCCTCATACACGTTGGTCCT For - GTAATGTGTGTGGCCGGAAC Rev – AAGCTGACCTTGCCGAAGT For – CCCAGGACACTCAACCTCAG Rev – CCAGGGGATCCTCTCCAC For - CCACAATCTTGCTGACGTCTT Rev - GGTAGTACTCGTGGCCTTCG For – CAGCACTACATCCACCGAGA Rev - CTCCCTCTGCAGCCACTG
TET2 gDNA	For – CTTTCGCATTCACACACACTTT

	Rev - TGTGCAGGGGAACTC
TET2 (SDM)	For - TTCTGTGCTCATGCCCGCAGAGACTTGCACAAC Rev - GTTGTGCAAGTCTCTGCGGGCATGAGCACAGAA
TET2 cDNA	For - TGAACACAGAGCACCAGAGTG Rev - AAAAGTCAGCCCCATCACGT

Table 2.6: Primers used for standard PCR and Sanger Sequencing.

Gene	Sequence (5’-3’)	Universal Probe Library Probe number
TET1	For – TCTGTTGTTGTGCCTCTGGA Rev - GCCTTTAAAACTTTGGGCTTC	57
TET2	For – AAAGATGAAGGTCCTTTTTATACCC Rev - ATAGCTTTACCCTTCTGTCCAAAC	68
TET3	For – CACTCCGGAGAAGATCAAGC Rev - GGACAATCCACCCTTCAGAG	1
TIA1	For – CCCTGTGCTTGTACATCTTCC Rev - TGTCTTTGTACATGCAATCTAGTTCTT	8
Smad3	For – GAAGAAGGGCGAGCAGAA Rev - TTTCTTGACCAGGCTCTTGAC	51
DUSP1	For – TGGGTACATCAAGTCCATCTGA Rev - GCAAAAAGAAACCGGATCAC	29
CDKN1A	For – TCCAGACACAGACATATCCACA Rev - TGCTCTGGTGGCTGTACTGA	76
TRAF5	For – TGCTATGCAGAGACCCATACC Rev - ATTTCTTTTGTGGACTCTTCATCC	67

Table 2.7: Oligonucleotide primers and probes used for Real time PCR.

Kit	Catalogue number	Company	Method
QiAmp DNA Mini	51304	Qiagen	DNA extraction
Quest 5-hmC™DNA ELISA	D5425	Zymo research	ELISA (quantification of global 5hmC)
ReliaPrep™ RNA Cell Miniprep System	Z6011	Promega	RNA extraction
SuperScript® III Reverse	18080-044	Invitrogen	cDNA production

Transcriptase			
MyTaq™ HS DNA Polymerase	BIO-21111	Bioline	PCR DNA amplification
PureYield Plasmid Midiprep System	A2492	Promega	Plasmid DNA purification
QuikChange II XL Site-Directed Mutagenesis	200521	Agilent technologies	Site-Directed Mutagenesis (SDM)
QIAprep Spin Miniprep	27104	Qiagen	Mutated plasmid DNA purification
FasL, soluble (human) (recombinant) set	ALX-850-014-KI02	Enzo Life Sciences	Fas-mediated apoptosis
FITC Annexin V Apoptosis Detection	556547	BD Pharmingen	Fas-mediated apoptosis

Table 2.8 : List of commercially available kits used for the research.

2.3 Methods

2.3.1 Preparation and culture of human cells

2.3.1.1 Fibroblast culture

Dermal fibroblasts obtained from patients and controls were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin and L-glutamine. Fibroblasts were cultured in T75 flasks and incubated in a 5% CO2 incubator at 37^0 C until they were 80% confluent. Cells were split, maximum up to passage 14, to maintain them in a sub- confluent state. The cells were removed from T75 flasks by trypsinisation: cells were washed once with PBS before 1x trypsin EDTA was added to detach the cells from the flask over 2 to 3 minutes at room temperature. Serum-containing DMEM was added, cells and medium harvested and centrifuged at 600g for 5 minutes at RT. The cells were seeded again in T75 flasks, containing fresh medium until ready to be used for further experiments.

2.3.1.2 HEK293T cell culture

HEK293T is an adherent cell line, originally derived from human embryonic kidney cells. I obtained an aliquot from the Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University. The cells were grown and subcultured as described for dermal fibroblasts, except that as this is an immortalised cell line it could be grown continuously.

2.3.1.3 PBMC isolation

Whole blood samples were collected in heparinized tubes. Blood was mixed 1:1 with Hanks Buffered Saline Solution(HBSS) (containing 1% FCS). The mixed blood was layered on top of Lymphoprep 2:1 and centrifuged at 825g for 30 minutes at RT. The PBMC were collected from the interface, and washed with cold HBSS by centrifugation at 600g for 5 minutes. The PBMC pellet was washed with HBSS once and centrifuge at low speed for 5 minutes. The pellet was then resuspended in fresh HBSS medium kept on ice for further experiments and an aliquot was taken and counted for determination of cell numbers.

2.3.1.4 B cell isolation and purity assessment by flow cytometry

B cells were isolated using two methods:

2.3.1.4.1 Positive selection from whole blood for microarray analysis

For microarray analysis, B cells were isolated from fresh whole blood using positive selection and either an autoMacs Pro Separator (patient samples, carried out by Dr Dawn Barge, Clinical Immunology Laboratory, Royal Victoria Infirmary, Newcastle) or an autoMacs Classic Separator (healthy controls). Whole blood (4ml) was collected in EDTA tubes and labelled with 200ul of whole blood CD19 Microbeads (Miltenyi Biotec). The blood and beads were mixed well and incubated for 15 minutes at 4°C. The cells were washed with 10ml of autoMacs running buffer and centrifuged at 445g for 10 minutes at RT. The supernatant was discarded except for a residual volume (approximately 1-2ml deep) which was left to avoid cell loss. The pelleted cells were resuspended in autoMacs running buffer to a total volume of 2ml. B cells were then isolated using positive selection by autoMacs Separator. Isolated B cells of both healthy

controls and patient were kept on ice for RNA extraction and an aliquot was taken for cell counting and assessed for purity by flow cytometry.

2.3.1.4.2 Positive selection of B cells from PBMC for Real Time PCR

Another method that was used to isolate healthy control B cells was by positive selection from PBMCs. This method was used to isolate more B cells from a higher volume of blood (60ml). This was performed to investigate the TET1, TET2 and TET3 mRNA level in healthy control B cells by qRTPCR. PBMCs were centrifuged at 600g for 5 minutes at RT. The cell pellet was resuspended in 80ul of MACs buffer and CD19+ cells were labelled with 20ul of CD19 Microbeads per 10^7 total cells. The cells were then incubated for 15 minutes at 4^0C. The cells were washed with 2ml of MACs buffer per 10^7 cells and centrifuged at 300g for 10 minutes. The supernatant was discarded completely and the pelleted cells were resuspended in 500ul of buffer. The suspended cells were applied to a prepared LS column attached to a magnet. The column was washed three times and was removed from the magnet. The magnetically labelled CD19+ cells were retained within the column and eluted as the positively selected cell fraction. An aliquot of cells was counted for determination of cell number and assessed for purity by flow cytometry. The remaining B cells were kept on ice for RNA extraction.

2.3.1.4.3 Purity assessment

Following B cell isolation, cells were assessed for purity. 500ul of isolated B cell suspension was aliquoted into a FACs tube. The cells were centrifuged at 400g for 5 minutes. The supernatant was discarded and pelleted cells were washed initially with 1ml PBS, centrifuged then washed again with 1ml FACs buffer and centrifuged. Both centrifugations were at 400g for 5 minutes. The pelleted cells were resuspended in 100ul of FACs buffer containing 10ul of PE-CD20 antibody (Miltenyi Biotec) and incubated on ice for 30 minutes. Then the cells were washed with 2 ml of FACs buffer and centrifuged at 400g for 5 minutes. The cells were washed again with FACS buffer and finally resuspended in 300ul FACs buffer and assessed on the BD FACs Canto II flow cytometer (BD Bosciences). The data was then analysed using FlowJo 7.6 (Treestar, USA) software.

2.3.1.5 Cell counting

Trypan Blue-exclusion method was used to distinguish between live and dead cells. Due to membrane rupture, dead cells absorb the blue dye which stains the cytoplasm and were thus visualised under the microscope in blue, whereas viable cells were visualised as clear.

For cell counting, a 20ul aliquot of the cells was diluted at 1:5 with 30ul of growth media and 50ul of 0.4% Trypan blue. 10ul of the cell dilution was applied to the surface of a haemocytometer at the edge of the coverslip. The number of cells within a 5 x 5 grid was counted under inverted microscope at 40x magnification. The concentration of cells was calculated as :

Total number of cells/ml = Number of cells within 5 x 5 grid x 5 (dilution factor) x 10^4

2.3.1.6 Cell transfection

HEK293T cells that were 80% confluent in T75 flasks were removed by trypsinisation (method is similar to splitting the cells). The cells were then seeded on chamber slides at 18000 cells/well in 100 ul of DMEM/10% FCS (antibiotic free) in the morning. At the end of the day, cells were transfected with DNA of the WT TET2 or mutant $TET2^{p.H1382R}$ plasmids. A Fugene HD transfection reagent/DNA mixture was prepared by incubating 2ug DNA of either construct with 6ul of Fugene HD (at 3:1 Fugene HD:DNA ratio), a non-liposomal transfection reagent, and 90ul of serum and antibiotic free DMEM medium for 15 minutes at room temperature. Then 6ul of each mixture was added per well to the HEK 293T cells that were seeded earlier in the morning. The cells were incubated in 37^0C CO_2 incubator for 48 hours prior to staining for immunofluorescence microscopy.

In addition, to assess the transfection efficiency by Western blot, 400,000 cells per well were seeded in a 6-well plate in 1ml of serum and antibiotic free media. The amount and ratio of DNA:Fugene HD were the same as before and 90ul of the mixture was added per well to the cells. The cells were left in the 37^0C CO_2 incubator for 48 hours. The transfection efficiency was assessed by Western blot.

2.3.1.7 Cell stimulation

For the novel defect of IFN signalling study, both patient and control fibroblasts that were 80% confluent were removed from T75 flasks as described and seeded at 60,000 cells per well in a 24-well plate. The cells were left in 37^0C CO_2 incubator overnight. For PBMCs, 2 $x10^6$ cells per well were seeded per well in a 24-well plate. Both fibroblasts and PBMCs were left unstimulated or stimulated with :

a) IFNα at 100IU/ml for overnight stimulation. The cells were lysed the next day and subjected to Western blotting. This cell stimulation experiment was performed to assess the upregulation of Interferon stimulated genes (ISGs).
b) IFNα and IFNγ at 1000 IU/ml for 15 minutes, 30 minutes and 60 minutes. The cells were lysed after the indicated times, and subjected to Western Blotting (see below). This experiment was performed to assess the expression and phosphorylation of STAT1, STAT2 and STAT3.
c) IFNα , IFNγ and IFNβ at 1000IU/ml for 10 hours. RNA was extracted from the cells and subjected to microarray analysis.

2.3.2 DNA methods

2.3.2.1. DNA extraction and quantification

Total DNA was extracted from patient and control PBMCs or fibroblasts. Cells were collected and pelleted in a sterile microcentrifuge tube. DNA extraction was performed using QiAmp DNA Mini kit (Qiagen, UK) according to the manufacturer's protocol. Cells were lysed enzymatically by Qiagen protease. DNA was isolated with a fast spin column procedure in which the DNA bound specifically to the QiAmp silica gel membrane while the contaminants passed through. DNA in the spin column was washed in two efficient steps with wash buffer. Finally, DNA was eluted in TE buffer provided with the kit. Isolated DNA was quantified using a Nanodrop 2,000 Spectrophotometer and stored frozen at -20^0C for further experiments.

2.3.2.2 Whole exome sequencing and homozygosity mapping

Patient genomic DNA was submitted for whole exome sequencing using Sure Select Human All Exon 50Mb kit (Agilent Technologies) coupled with massively parallel sequencing by Illumina GA II Sequencing system. The DNA sequences were mapped to the hg19 human genome by NovoAlign (http://novocraft.com/main). Bioinformatic analysis was performed by Dr Helen Griffin (Institute of Genetic Medicine, Newcastle University) and Dr Yaobo Xu (Institute of Genetic Medicine, Newcastle University) using an in-house pipeline.In parallel, homozygosity mapping was also performed using the Affymetrix Genome-Wide Human SNP 5.0 microarray. Homozygous regions were identified using HomozygosityMapper (http://homozygositymapper.org) and further analysed using microsatellite markers. Homozygosity mapping was performed by Dr Neil Morgan, Birmingham University.

2.3.2.3 5-hydroxymethylcytosine (5hmC) enzyme-linked Immunosorbent Assay (ELISA)

To quantify the global 5hmC level in patient and control B-cell DNA for the study of novel defect of the lymphocyte apoptosis disorder, a Quest 5hmC^{TM}DNA ELISA kit (Zymo Research,UK) was used. This kit uses a sandwich-based ELISA format in which firstly, the anti-5hmC polyclonal antibody was coated to the bottom of the well. Then, DNA was added and single stranded 5hmC-containing DNA binds to the anti-5hmC antibody. Captured DNA is then recognised by an anti-DNA-HRP antibody. Finally, the addition of HRP developer produces a greenish colour, due to the enzymatic reaction of the anti-DNA-HRP antibody to the chemical substance in the HRP developer. A summary of this method is shown in Figure 2.1.

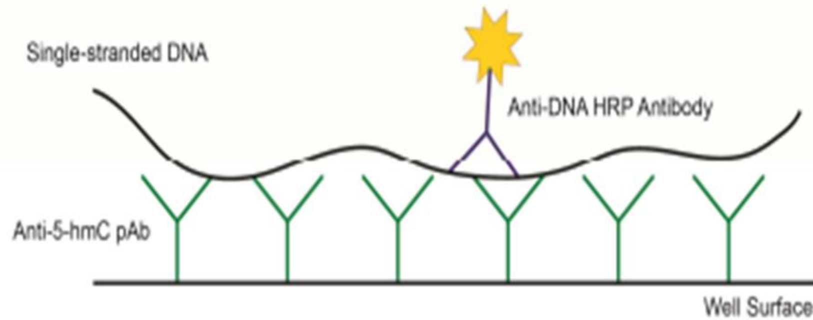


Figure 2.1 : A sandwich-based ELISA format. Anti-5hmC polyclonal antibody is coated onto the bottom of the well. The single stranded 5hmC-containing DNA binds to anti-5hmC and this is recognised by an anti-DNA-HRP antibody. HRP developer is added which produces a greenish colour.

Method : This techniques involved five steps :

1. Coating

Anti-5hmC polyclonal antibody (4ng/ul) in coating buffer was coated onto the 96-well ELISA plate and was incubated in an incubator at 37^{0}C for 1 hour.

2. Blocking

The wells were washed with 1x ELISA buffer three times. 1x ELISA buffer was added into the wells and incubated at 37^{0}C for 30 minutes.

3. DNA addition/binding

Patient and control DNA was denatured at 98^{0}C for 5 minutes in a thermal cycler. Then, the samples were incubated on ice for 10 minutes, after which 250ng of each sample was diluted in 1x ELISA buffer to a concentration of 100ng. The samples were added into the wells and incubated at 37^{0}C for 1 hour.

4. Addition of anti-DNA HRP antibody

The wells were washed with 1x ELISA buffer three times. Anti-DNA HRP antibody in 1x ELISA buffer (1:100) was added into the wells and incubated at 37^{0}C for 30 minutes.

5. Colour development

The wells were washed again with 1x ELISA buffer three times. 1X developer was added into the wells. The absorbance of each well was measured with an ELISA plate reader at 450nm. Readings were taken 30 minutes after the developer was added.

2.3.3 RNA methods

2.3.3.1 RNA extraction and quantification

RNA was extracted from fibroblasts and B cells using the ReliaPrep RNA Cell Miniprep System (Promega, US), as per the manufacturer's instructions. This method uses a spin-column purification protocol. Briefly, it requires five essential steps :

1) disruption and lysis of the cells
2) denaturation of nucleoprotein complexes, allowing the RNA to be released into the solution and isolated free of the protein
3) After clearing the protein and cellular debris by centrifugation, RNA is precipitated with ethanol and loaded onto the spin column, where it binds to the silica membrane
4) contaminating DNA is removed by adding DNase which is an enzyme that digests DNA
5) Finally, after several washing steps, the purified RNA is eluted in nuclease free water.

Purified RNA was quantified using the Nanodrop 2000 Spectrophotometer, aliquoted at 100ng/ul and kept at -80^0C for further experiments

2.3.3.2 Complementary DNA (cDNA) production

cDNA for use in Real Time PCR and Sanger sequencing was synthesised from RNA using Superscript III Reverse Transcriptase (Invitrogen, UK) reagents. 100ng of RNA sample was mixed with 100ng Random Hexamer primers, 1ul dNTP Mix and sterile distilled H_20 up to 13ul in a microcentrifuge tube. The mixture was heated on a thermal cycler at 65^0C for 5 minutes and then incubated on ice for 1 minute. Then, 4ul 5x First strand buffer, 1ul 0.1M DTT , 1ul SuperScript II RT and 1ul sterile distilled H_20 were added to the mixture. The tube was incubated at 25^0C for 5 minutes, followed by 50^0C for 60 minutes on thermal cycler. Finally the reaction was inactivated by heating at 70^0C for 15 minutes.

2.3.3.3.Microarray analysis

2.3.3.3.1 Illumina HT-12 labelling: Nugen

For the study of the novel defect of autoimmune lymphoproliferative disease, a microarray analysis was performed on RNA extracted from patient and control B cells. Quality control, RNA labelling, hybridization and data extraction for microarrays were performed by ServiceXS BV (Leiden, Netherlands). The RNA quality and integrity was determined using Lab on-Chip analysis on the Agilent 2100 Bioanalyzer (Agilent Technologies, US). Biotinylated ss-cDNA was prepared from 50ng of total RNA with the WTA system kit using the NuGEN Ovation PicoSL. This was a specialised protocol as the total amount of RNA used was low. Then 750ng of biotinylated ss-cDNA samples was hybridized onto the Illumina HT-12 v4 Expression Bead Chips (Illumina Inc, San Diego, USA). Image analysis and extraction of raw expression data was performed with Illumina GenomeStudio v2011.1.
Once the Illumina Human HT12v4 Expression BeadChip data was received from ServiceXS BV (Leiden, Netherlands), further bioinformatics analysis was performed in conjunction with Andrew Skelton (Bioinformatics Unit, Newcastle University). The data was background corrected in Illumina Beadstudio, subsequent analysis proceeded using the Lumi and Limma packages in R (Bioconductor). Normalization of background corrected data was applied through Variance Stabilising Transformations (VST) and Robust Spline Normalisation (RST) in Lumi. A technical effect was identified using principal component analysis and linked to scanning batches, this effect was corrected for using the ComBat function from the SVA package. Differential expression was detected using linear models and empirical Bayes statistics in Limma. A list of genes for each comparison was generated using a Benjamini Hochberg false discovery rate correct p-value of 0.01 and a fold change of 2 as cut-offs.

2.3.3.3.2 Illumina HT-12 labelling: Total Prep

For the study of the novel defect of IFN signalling, RNA extracted from patient and control fibroblasts were subjected to microarray analysis which was performed by ServiceXS BV (Leiden, Netherlands). The RNA quality and integrity was determined using Lab-on-chip analysis on the Agilent 2100

Bioanalyzer (Agilent Technologies, USA). Biotinylated cRNA was prepared using Illumina Total prep RNA amplification kit with an input of 200ng total RNA. Per sample, 750ng of biotinylated cRNA was hybridized onto the Illumina HT-12 v4 Expression Bead Chip (Illumina Inc, San Diego, USA). Image analysis and extraction of raw expression data was performed with Illumina GenomeStudio v2011.1.

Bioinformatic analysis of the Illumina Human HT12v4 Expression BeadChip data was performed in conjunction with Andrew Skelton (Bioinformatics Unit, Newcastle University). Data from two Illumina HT-12 v4 Expression Bead Chips were background corrected in Illumina Beadstudio with further analysis carried out using the Lumi and Limma Bioconductor packages in R. Variance Stabilising Transformations (VST) and Robust Spline Normalisation (RSN) in Lumi were applied for normalization of the background corrected data. Differential expression was detected using linear models and empirical Bayes statistics, utilizing a multi-level experiment approach through Limma. A gene list for each comparison was generated using a Benjamini Hochberg false discovery rate correct p-value of 0.01 and a fold change of 2 as cut-offs.

2.3.4 PCR based methods

2.3.4.1 PCR DNA amplification for Sanger Sequencing

Amplification of genomic DNA for Sanger sequencing was performed by the standard Polymerase Chain Reaction (PCR) method. PCR relies on thermal cycling, including cycles of repeating heating and cooling of the reaction mixture for DNA melting and enzymatic replication of DNA. First, heating to 95°C results in denaturation of the DNA template, yielding single stranded DNA molecules. This is followed by heating to around 56°C (depending on the melting temperatures of the primers), allowing the annealing of two primers, forward and reverse primers, which contain sequences complementary to the target region, to the single stranded DNA template. Next is the extension step at 72°C, where the enzyme DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand. At this step, the amount of DNA is doubled, resulting in exponential amplification of the specific DNA fragment. These steps will then be repeated usually for 20-40 cycles.

PCR : PCR was performed using MyTaq HS DNA polymerase (Bioline) reagents. All reagents were added to a 0.2ml centrifuge tube and mixed prior to cycling, as per table 2.9

Reagents	One reaction (50ul)
5x MyTaq reaction buffer (containing dNTPs, $MgCl_2$ and enhancers	10ul
DNA	100ng
Forward Primer (10uM)	1ul
Reverse Primer (10uM)	1ul
MyTaq HS DNA Polymerase	0.25ul
Water (ddH_2O)	Up to 50ul

Table 2.9 : Standard PCR reagents

Reactions were cycled on a thermocycler using the following condition (Table 2.10)

Step	Temperature	Time	Cycles
Initial denaturation	95°C	3 minutes	1
Denaturation	95°C	15 seconds	35
Annealing	57°C	15 seconds	
Extension	72°C	1 minute	
Final Extension	72°C	5 minutes	1

Table 2.10 : PCR cycling condition

Forward and reverse primers were designed using NCBI ePCR software (www.ncbi.nlm.nih.gov/projects/epcr/reverse.cgi) with DNA target sequences including the exon/intron boundaries. Specificity of the primers was confirmed using NCBI blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Sequences of the DNA were obtained from human Ensembl website (www.ensembl.org/Homo_sapiens). Primers were ordered from Sigma Aldrich. A list of primers is shown in the Section 2.2(Table 2.6).

Agarose gel electrophoresis : To check whether the PCR generated the anticipated DNA fragment, agarose gel electrophoresis was employed to separate the DNA fragments based on their size. 2% agarose (w/v) gels were prepared by dissolving agarose in 1x TAE buffer by heating in the microwave. Ethidium bromide was then added to the cooled agarose-TAE solution and the solution was poured into a gel form and allowed to set. Once the gel was formed, it was placed in the electrophoresis tank and submerged in 1x TAE buffer. 20ul of the PCR products were mixed with loading buffer and loaded into the gel. A DNA ladder, a molecular weight marker which contains DNA fragments of known sizes, was also loaded into the gel, alongside with the PCR products. An electric voltage of 100V was applied for 45 -60 minutes. The PCR products were visualised using ChemiGenius II BioImager (Syngene, Cambridge, UK).

Sanger sequencing : Once the PCR products were shown to be present and at the correct size, the remainder of the PCR products were sent to Geneius Laboratories, UK, for Sanger Sequencing using the same primers that were used for amplification of the DNA fragments.

2.3.4.2 PCR cDNA amplification for Sanger Sequencing

Primer design, amplification of cDNA for Sanger sequencing for the novel defect of IFN signalling study by PCR, visualisation of PCR products by gel electrophoresis and Sanger sequencing was done as described in Section 2.3.4.1(above). A list of primers is shown in the Section 2.2(Table 2.6).

2.3.4.3 Real time PCR

Real time PCR was used to test for the expression of TET genes in control B cells and to validate the microarray transcriptional analysis of the patient and control B cells in the study of novel defect of autoimmune lymphoproliferative syndrome. This method measures mRNA expression relative to a housekeeping gene and relies on a fluorescent probe that binds to a short target sequence between the PCR primers. The oligonucleotide primers and matching Universal Probe Library probe were designed using Universal Probe Library Assay Design Centre software (http://lifesciences.roche.com). The oligonucleotide primers (Section 2.2 (Table 2.7)) were purchased from Sigma Aldrich, UK. Each of the universal probes incorporates a fluorophore at the 5' end and a quencher molecule the 3' end. When Taq polymerase encounters the probe bound to its target DNA, Taq displaces and degrades the probe, resulting in dissociation of the fluorophore from the quencher. The resulting fluorescence is therefore directly proportional to the amount of product produced in the PCR reaction.

Method : Real time PCR was done with cDNA that was synthesised as described in Section 2.3.3.2. 4.5ul of cDNA (diluted 1:5 in H2O for all genes except 18S, which was diluted 1:100) was added into a Fast optical 96-well reaction plate. Then, 5ul/well of Taqman Fast Universal PCR master mix was added along with 0.2ul/well of each forward and reverse primers (final concentration of 10uM each) and 0.1ul/well of probe (final concentration of 150nM) making a total volume of 10ul. This was subjected to PCR cycling (500C for 2 minutes, 950C for 10 minutes and 40 cycles of 950C for 15 seconds and 600C for 1 minute) on ABI 7900HT Fast Real Time PCR system.

Data analysis: The expression of each gene was normalised to the expression of 18s rRNA. Data was expressed in the 2-ΔCt format, where ΔCt = Ct (target) – Ct (18s). Ct (cycle threshold) value is described as the number of cycles required for the fluorescent signal to cross the threshold and Ct levels are inversely proportional to the amount of target nucleic acid in the sample.

Statistic : Statistical analysis and graphing of data was performed with Prism 4.0 software (GraphPad Software, USA).

2.3.5 Western blot

Western blotting (also known as Immunoblotting) is a widely used technique for detection of specific proteins in a sample. This technique involves three elements to complete the task: 1) through gel electrophoresis, proteins contained within a sample are separated according to their molecular weight; 2) the proteins are transferred onto a solid support such as polyvinylidene fluoride (PVDF) membrane; and 3) the targeted proteins are recognised by specific primary and secondary antibodies followed by a detection step.

For the novel defect of IFN signalling study, Western blot was performed for the assessment of Interferon stimulated gene (ISG) upregulation and STAT2 phosphorylation.

For the novel defect of autoimmune lymphoproliferative study, this method was used to investigate TET2 protein expression in patients compared with controls.

2.3.5.1 Preparation of lysates

2.3.5.1.1 Extraction of nuclear protein

Radioimmunoprecipitation (RIPA) buffer was used to extract TET2 as this protein is nuclear. RIPA buffer contains Sodium dodecyl sulphate (SDS, Sigma) and Sodium Deoxycholate (Sigma), both active constituents that are useful in disrupting the nuclear membrane for the preparation of nuclear extracts. Cells in T75 flask (80% confluence, roughly at 2 million cells) were removed by trypsinisation, as described before. Cells were washed once with cold PBS and pelleted by centrifugation at 600g for 5 minutes at 4^{0}C. Cell pellets were lysed in RIPA buffer (50mM Tris HCL (pH 7.5), 150mM Sodium Chloride, 0.5%(w/v) Sodium Deoxycholate, 0.1% (w/v) Sodium Dodecyl Cholate (SDS), 1% (v/v) NP-40, 1x complete protease inhibitor) and left on ice for 45 minutes. The cells were then pelleted at 8000g for 3 minutes at 4^{0}C. Lysates were removed carefully from the cell pellet and transferred into a new Eppendorf tube. 100mM dithiothreitol (DTT,Sigma) and 1x NUPAGE loading buffer (Life technologies) were added to the lysates.

2.3.5.1.2 Extraction of whole cells lysate

For the novel defect of IFN signalling study, lysis buffer that contains less strong denaturing detergents such as Triton X-100 was used to lyse the cells. Fibroblasts or PBMCs that were seeded and stimulated with IFNs in a 24-well plate were washed once with cold PBS. Cells were lysed on ice with lysis buffer (20 mM Tris-HCL (pH 7.4), 150mM Sodium Chloride, 1% (v/v) Triton X-100, 5 mM EDTA, 1x complete protease inhibitor, phosphatase inhibitor(Sodium Fluoride, Sodium Orthovanadate)) containing 100mM dithiothreitol (DTT,Sigma) and 1x NUPAGE loading Buffer (Life technologies).

2.3.5.2 Protein Quantification

To measure the concentration of proteins in cell extracts, a Pierce Bicinchoninic Acid (BCA) Assay (Pierce, Thermo Scientific) was used. Standards within the assay's working range were prepared by diluting Bovine Serum Albumin (BSA) in PBS at concentrations of 0mg/ml, 0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml and 1.0mg/ml. Standards and extracted samples were prepared in duplicate. 10ul of each of standards and extracted samples were mixed with 150ul of Pierce Protein Assay reagent (Pierce, Thermo Scientific) in a 96-well plate and were prepared in duplicate. After 5 minutes of incubation at room temperature, absorbances were measured at 660nm using a Tecan sunrise microplate absorbance reader.

A standard curve was generated by plotting the absorbance measurement of each BSA standard against its concentration. The standard curve was used to determine the protein concentration of the extracted samples.

2.3.5.3 Immunoblotting

After the lysates were heated at 90^{0}C on hot block for 10 minutes, equal amount of lysates were loaded and subjected to 4-12% Tris-Glycine Polyacrylamide gel electrophoresis (Novonex, Life Technologies) in 1x Sodium Dodecyl Sulphate (SDS) NuPAGE MOPS Running Buffer (Life Technologies) at a constant voltage of 160V between 1 ½ and 2 hours. 5ul of pre-stained Ladder (Thermo Scientific, Life Technologies) was used as the size marker. After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Thermo Scientific Pierce, Life Technologies) in NuPAGE Tris -

Glycine Buffer (Life Technologies). The membrane was then blocked with 5% (w/v) Bovine Serum Albumin or 5% milk in Tris Buffered Saline- 0.1% Tween(TBS/T) for 1 hour at room temperature, followed by incubation with anti-human primary antibodies for overnight at 4^{0}C. The blots were then washed three times with TBS/T and incubated with appropriate Horseradish Peroxidase (HRP)-conjugated secondary antibodies in 5% (w/v) milk in TBS/T for 1 hour at room temperature. After washing the blots three times, the blots were developed with Immobilon^{TM} Western Chemiluminescent HRP substrate (Millipore) according to the manufacturer's instruction. The Chemiluminescent images were captured on a G:BOX Chemi (Syngene, India) using GeneSnap Software (Syngene). A list of primary antibodies and secondary antibodies used in WB is available in Section 2.2 (Table 2. 3)

2.3.6 Immunofluorescence Microscopy

Immunofluorescence is a microscopy based technique that is used to assess the localization and endogenous expression levels of the protein of interest. This technique utilizes fluorescent-labelled antibodies to detect specific target antigens (Odell and Cook, 2013). There are two techniques in Immunofluorescence including 1) direct Immunofluorescence which uses a single antibody that is conjugated to a fluorescent dye, and 2) indirect Immunofluorescence utilizing a two-step technique in which in the first step a primary antibody recognizes and binds to the target biomolecules, followed by the detection of the primary antibody by a fluorescent-labelled secondary antibody. For the assessment of 5hmC level in the recombinant system in the novel defect of autoimmune lymphoproliferative study, the indirect immunofluorescence technique was used.

Method : HEK293 T cells were transfected as described in Section 2.3.1.6. After 48 hours of transfection, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and permeabilised with 0.2% of Triton-X in PBS for 15 minutes at room temperature (RT). Subsequently, to reveal the epitope, the cells were incubated with 2M HCl at RT for 30 minutes and neutralized with 100mM Tris-HCl buffer (pH 8.0) for 10 minutes. The cells were washed with PBS for 2 times.

For blocking, the cells were treated with 1%BSA, 0.05% Tween-20 in PBS for 1 hour at RT. Then, a rabbit monoclonal anti-5-hmC antibody (1:1000 dilution, Active Motif, UK) and a mouse monoclonal anti-Flag antibody (1:500 dilution, Sigma Aldrich, UK), were added into the blocking buffer overnight at 4^0C. The cells were washed 3 times with 1% Tween-20-PBS solution, after which an Alexa Fluor 488 goat anti-rabbit IgG antibody (1:500 dilution, Invitrogen, UK), and an Alexa Fluor 546 goat anti-mouse IgG antibody (1:500 dilution, Invitrogen, USA) were added in blocking buffer for 1 hour at RT in the dark. The cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with VectaShield reagent. Images were obtained with Zeiss AscioImager 2 microscope using Asciovision 4.8 software.

2.3.7 TET2 wild type plasmid cloning

For the study of novel defect of the autoimmune lymphoproliferative syndrome, a recombinant system was used in which a TET2 WT and mutant construct were overexpressed in cell lines such as HEK293 T cells to investigate the effect of TET2 mutation on its functional activity. To perform this, TET2 WT plasmid was generously given by Dr Skirmantas Kraucionis and his PhD student, Melania Zauri from Oxford University, UK. The next step was to replicate the WT TET2 plasmid for further experiments.

2.3.7.1 Preparation of Luria-Bertani (LB) Broth/ Medium

LB Broth was prepared by dissolving 5.15g of Mixed LB Broth Powder, containing Tryptone, NaCl and Yeast Extract (Sigma Aldrich, USA) in 250 ml of dH_2O. The LB Broth was autoclaved, allowed to cool and stored at room temperature prior to use.

LB plates were prepared by dissolving 5g of Bacto Agar in 250ml LB Broth. After autoclaving, the LB agar was left at room temperature to allow it to cool and 100ug/ml of ampicillin was added. The LB agar was then poured into petri dishes (working around the flame of a penunu bunsen). The LB agar was allowed to set and dry. Plates were stored in up-side position at 4^0C until further used.

2.3.7.2 Transformation into competent cells

Transformation is the process of getting the plasmid DNA into the competent bacteria cells. Competent cells are cells that have the ability to take up free, extracellular genetic material. A common strain of competent bacteria used is *E.Coli*. In order to select transformed cells, plasmids have a selection marker such as an antibiotic resistance gene so that only cells containing the plasmid will be able to grow in medium containing the respective antibiotic. Furthermore, plasmids have an origin of replication allowing for independent replication within the bacterial cell.

Method : The WT TET2 plasmid was received on filter paper and dissolved in sterile dH_2O. 1ul of dissolved plasmid DNA was added to 50ul of NEB 5-alpha competent *E.Coli* cells (New England, BioLabs, USA). The mixture was incubated on ice for 30 minutes. The mixture was heat shocked at 42^0C water bath for 30 seconds, followed by incubation on ice for 2 minutes. 250ul of Super Optimal Broth with Catabolic repression (SOC) medium was added and the mixture was placed in a 37^0C shaker for 60 minutes at 250 rpm. While waiting, the LB agar plate was warmed in a 37^0C incubator. Following this, the mixture was centrifuged at 13000 rpm and the cells were pelleted. 250ul of the supernatant was discarded and the pelleted cells were resuspended in the 50ul remaining supernatant. The cells were plated on LB-ampicillin agar plates and incubated in upside down position in 37^0C incubator overnight.

2.3.7.3 Purification of Plasmid DNA

Next day, several colonies were selected from the LB-ampicillin agar plate and each colony was grown in 5ml of LB Broth, containing 100ug/ml of ampicillin. The colonies were incubated in 37°C shaker for 5hours. Following this, the colonies were then transferred into 100ml of LB Broth with 100ug/ml of ampicillin and grown in 37^0C shaker overnight. Purification of plasmid DNA was performed using the PureYield™ Plasmid Midiprep system (Promega, USA) according to the manufacturer's protocol. The purified DNA was quantified using Nanodrop 2000 Spectrophotometer and kept in -20^0C.

2.3.8 Site Directed Mutagenesis

To investigate the effect of TET2 mutation on its functional activity in the novel defect of autoimmune lymphoproliferative syndrome study, mutant TET2$^{p.H1382R}$ plasmid was generated from TET2 WT plasmid, described in Section 2.3.6, by QuikChange II XL Site-Directed Mutagenesis (SDM) kit. This method is used to introduce a specific point mutation or other intentional changes to a DNA sequence. This method is a PCR-based technique that requires synthetic primers which contain the desired mutation and are complementary to the template DNA around the mutation site to allow it to hybridize to the DNA of the gene of interest during PCR reaction. The single stranded primers are extended during temperature cycling by Pfu Ultra HF DNA Polymerase, which copies the rest of the DNA. Thus, this generates a mutated plasmid containing staggered nicks. Following temperature cycling, Dpn 1 endonuclease is added to digest the parental DNA. The mutated plasmid is then transformed into ultracompetent cells and cloned.

Method: The detailed procedure of QuikChange II XL Site-Directed Mutagenesis (SDM) is as follows :

1. Mutant strand synthesis
Firstly, sense and antisense primers were designed using Primer3 Plus software (http://primer3plus.com) which incorporated the desired mutation into the sequence.
Sense primer : 5’ – ttctgtgctcatgcccgcagagacttgcacaac - 3’
Antisense primer : 5’ – gttgtgcaagtctctgcgggcatgagcacagaa – 3’

To synthesise the mutant, TET2 WT plasmid (DNA template) was mixed with QuikChange II XL Site-Directed Mutagenesis (SDM) reagents listed in Table 2.11. PCR reactions were performed in a thermal cycler as described in Table 2.12.

Reagents	One reaction (51ul)
10x Buffer	5 ul
Sense primer	X ul (125ng)
Antisense primer	X ul (125ng)
dNTP mix	1 ul
DNA template	X ul (200ng)
QuikSolution	3 ul
ddH2O	To a final volume of 50ul
Pfu Ultra HF DNA Polymerase	1 ul

Table 2.11 : SDM reagents.

Segment	Cycles	Temperature	Time
1	1	95^{0}C	1 minute
2	18	95^{0}C	50 seconds
		60^{0}C	50 seconds
		68^{0}C	13 minutes 19 seconds (1 minute/kb of plasm length)
3	1	68^{0}C	7 minutes

Table 2.12: PCR reactions

2. Dpn 1 Digestion of template

Following the PCR reaction, the reaction tubes were placed on ice for 2 minutes, to cool the reactions to 37^{0}C. 1ul of Dpn 1 restriction enzyme was added to the reaction tubes and incubated at 37^{0}C for 1 hour.

3. Transformation into Ultracompetent cells

45 ul XL10-Gold ultracompetent cells were thawed on ice and mixed with 2ul of β-ME mix provided with the kit. The mixture was incubated on ice for 10 minutes. 4ul of Dpn 1-treated DNA sample was added the ultracompetent cells and incubated on ice. The mixture was heat shocked in 42^{0}C water bath for 30 seconds, followed by incubation on ice for 2 minutes. Then, 250 ul of Super Optimal Broth with Catabolic repression (SOC) medium was added to the mixture and incubated in a 37^{0}C shaker at 225 rpm for 1 hour. Finally the cells were plated and cloned on LB-ampicillin agar plate in 37^{0}C incubator overnight.

4. Purification of mutated plasmid DNA

Between 5-10 colonies were picked from the LB-ampicillin agar plate and each colony was grown in 5ml LB – ampicillin broth overnight in 37^{0}C shaker. The mutated plasmid DNA was extracted using the QIAprep Spin MiniPrep Kit (Qiagen, UK) according to manufacturer's protocol. The extracted DNA was quantified using Nanodrop 2000 Spectrophotometer.

5. DNA sequencing

DNA sequencing was performed by Geneius Laboratories, Uk, to confirm that the selected DNA contains the desired mutation. The sequencing was performed using the following primers :

Forward primer : 5' – TGAACACAGAGCACCAGAGTG – 3'

Reverse primer : 5' – ACGTGATGGGGCTGACTTTT – 3'

2.3.9 Fas-mediated apoptosis assay for mouse B cells

For the study of novel defect of autoimmune lymphoproliferative syndrome, a Fas-mediated apoptosis assay was performed using mouse B cells, to investigate the effect of TET2 mutation on Fas-mediated apoptosis pathway.

2.3.9.1 Cells culture for mouse cells

Mouse spleen was dissected from the body using aseptic technique. This was performed by Dr Consuelo Anzilotti, Oxford University. The splenocytes were removed from the spleen by mashing the spleen using the plunger end of the syringe through a 100um cell strainer into a 60mm petri dish. The cells were removed into a centrifuge tube and centrifuged at 600g for 5 minutes at 4^{0}C. The cells were washed once and counted (method described in Section 2.3.1.5). Splenocytes were seeded at 1x 10^6cells/ml in 48-well plates in RPMI media supplemented with 10% FCS, L-Glutamine, 1x Penicillin/Streptomycin, 50uM β-ME, Na Pyruvate and 1x non-essential amino acids.

2.3.9.2 Cells stimulation with soluble Fas Ligand (sFasL)

The cells were then simulated with a soluble Fas Ligand set (Enzo Lifesciences, US) including enhancer ligands, to enhance the apoptosis activity. The cells were stimulated with different concentration of sFasL as follow (Table 2.13).

Cells were also stimulated with staurosporine (10uM,Sigma Aldrich, UK) as positive control.

sFasL concentration	Enhancer concentration
100ng/ml	0.5ug/ml
50ng/ml	0.5ug/ml
25ng/ml	0.5ug/ml
0ng/ml	0ug/ml

Table 2.13: sFasL and enhancer ligands concentration

The cells were incubated overnight in a 37^0C incubator.

2.3.9.3 Annexin V/ PI staining

For cell death and apoptosis detection, the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, UK) was used according to manufacturer's protocol. Briefly, the cells were collected and pelleted by centrifugation at 600g for 5 minutes at 4^0C. The cells were washed twice with cold PBS. The cells were stained as described (Table 2.14) in 100 ul of 1x Annexin V Binding buffer for 15 minutes in the dark at room temperature.

Antibodies	Volume	Detection
FITC Annexin V	5ul	Apoptotic cells
Propidium Iodide (PI)	5ul	Dead cells
CD19 (Biolegend)	0.5ul	B cells

Table 2.14: Antibodies that were used for apoptosis, cells death and B cell detection.

Then, 400ul of 1x Annexin V Binding buffer was added to the tubes. Data acquisition was performed using BD FACs Canto II flow cytometer (BD Biosciences) and the output data was analysed by FlowJo 7.0 (Treestar, USA).

2.3.9.4 Statistics

Statistical analysis and graphic data was performed as described in Section 2.3.4.3. Two way Anova analysis was used to compare the apoptosis differences between wild type and knock out mouse B cells. P value less than 0.05 (< 0.05) was considered as significant.

Chapter 3: A novel defect in the Type 1 IFN signalling pathway

3.1 Introduction

Primary Immunodeficiencies that predispose individuals to severe viral infections have been identified and present either as isolated susceptibility to specific viruses or susceptibility to multiple viruses in addition to disease with other microbes (Dropulic and Cohen, 2011). IFNs are the first line of defence against viruses. IFNs achieve their various biological responses by inducing the expression of hundreds of IFN-stimulated genes (ISGs) (Platanias, 2005). Type I IFN is known to be potent in interfering with viral infection (Perry *et al.*, 2005). Type I IFN which consists of IFNα/β is induced by the detection of viral pathogen associated molecular patterns (PAMPs) by the pattern recognition receptors in infected cells. IFNα/β binds to its heterodimeric transmembrane receptor which is composed of two subunits, IFNAR1 and IFNAR2. This is followed by phosphorylation of JAK1 and TYK2, which in turn activate STAT1 and STAT2 through phosphorylation (Platanias, 2005). Phosphorylated STAT1 and STAT2 translocate to the nucleus and form together with IRF9 the heterotrimeric complex ISGF3, which binds to IFN-stimulated response elements (ISRE) and initiates transcription of the ISGs (Platanias, 2005). In addition, IFNAR signaling also leads to limited formation of phosphorylated STAT1 homodimers (also known as GAF) (Platanias, 2005). Type II IFN, IFNγ, is the prototypical inducer of GAF; binding of IFNγ to IFNGR results in the activation of JAK1 and JAK2, causing STAT1 phosphorylation, and the resulting GAF translocates to the nucleus and binds to GAS elements in the regulatory region of IFN-γ-induced genes (Platanias, 2005). It should be noted that the engagement of IFNα/β to its receptor also causes phosphorylation of another STAT, STAT3, which indirectly suppresses pro-inflammatory gene expression, another known role of Type 1 IFNs (Ivashkiv and Donlin, 2014).

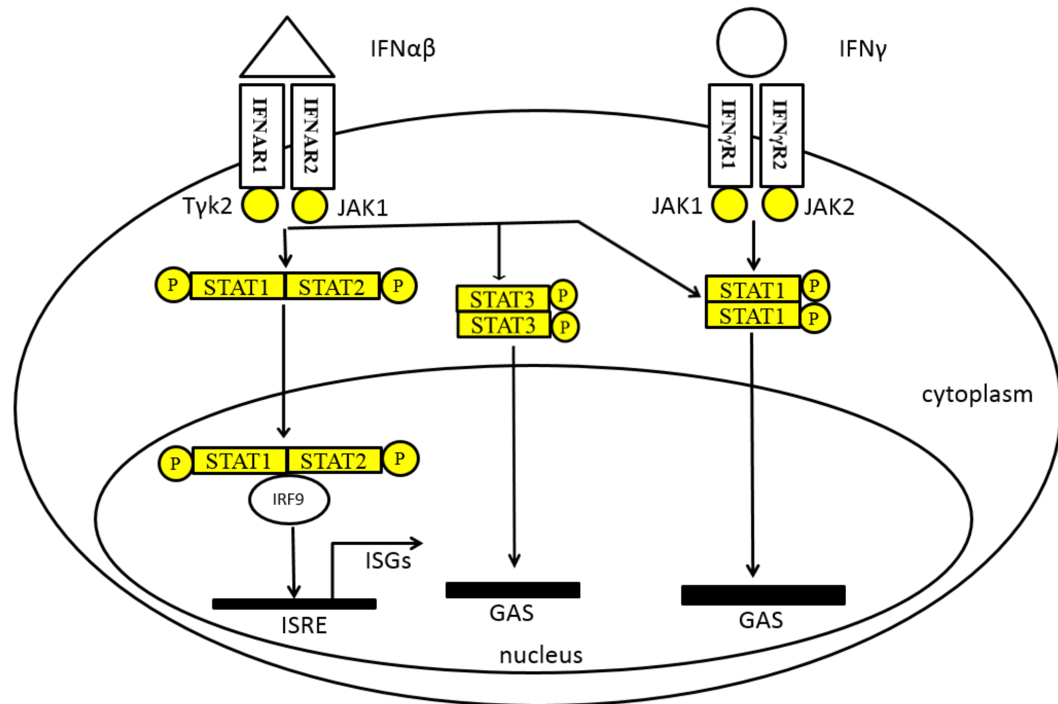


Figure 3.1 : Type I and Type II IFN signalling pathway. Type I IFN, IFNα/β, binds to its receptor, IFNAR1 and IFNAR2, leading to phosphorylation of Tyk2 and JAK1, which results in STAT1/STAT2 phosphorylation. Phosphorylated STAT1 and STAT2, together with IRF9, form ISGF3, in the nucleus and binds to ISRE, leading to transcription of ISGs. STAT1-STAT1 homodimers translocate into the nucleus and bind to GAS. STAT3 homodimers translocate into the nucleus and bind to GAS, which suppresses pro-inflammatory gene expression. Type II IFN, IFNγ, binds to IFNGR, leading to JAK1/2 phosphorylation, resulting in phosphorylation of STAT1 and formation of STAT1 homodimers. STAT1 homodimers then binds to GAS elements in the nucleus.

Many mouse knock out models were generated in order to study the role of Type I IFN signalling in antiviral immunity. IFNAR1 (Muller *et al.*, 1994), IFNAR2 (Muller *et al.*, 1994), STAT1 (Durbin *et al.*, 1996) and STAT2 (Park *et al.*, 2000) knock out mouse models were shown to have marked susceptibility to viral infection, suggesting the essential and non-redundant role of Type I IFN in antiviral defence mechanisms. However, it has been difficult to validate this in humans. Primary Immunodeficiency Diseases provide the opportunity to learn about the significance of specific genes and pathways in humans. As mentioned in chapter 1, deficiencies of STAT1, TYK2 and STAT2 are known PIDs that impair Type I IFN signalling. These recognised PIDs provide information for understanding the role of IFNα/β in human antiviral immunity.

In this chapter, I will describe the identification of a novel defect in the Type I IFN signalling pathway in a previously healthy patient who presented with fatal illness after receiving the MMR vaccine. MMR vaccine is a live-attenuated combined vaccine that is given intramuscularly to protect from illnesses of measles, mumps and rubella. MMR is a safe vaccine and is administered widely to children around the world. The common mild side effects of MMR vaccine are swelling at the injection site, rash and general feeling of being unwell. Rare side effects such as seizures can also occur. However, a severe illness leading to death of the patient is vanishingly rare and strongly suggests underlying immunodeficiency. Previously, we described a patient with disseminated vaccine-strain measles, who had a STAT2 mutation which caused profoundly defective Type 1 IFN signalling (Hambleton *et al.*, 2013) (albeit several STAT2-deficient individuals had mild phenotypes and led a relatively healthy life). By analogy, we considered that the current patient's susceptibility to live attenuated vaccine viruses indicated an underlying problem in the Type 1 IFN pathway.

3.2 Hypothesis

Enhanced viral susceptibility in a patient with fatal encephalitis following MMR is caused by a defect of type 1 IFN signalling.

3.3 Aims

To identify the putative disease-causing mutation(s) and associated molecular mechanism of viral susceptibility in the patient.

3.4 Results

3.4.1 Clinical case

The proband was well until he received a first dose of a live-attenuated MMR vaccine at 13 months old. Six days after his vaccination, he was admitted to hospital with a complaint of swelling at the vaccination site, fever, irritability and generalized morbilliform skin rash. He was thought to suffer from common complications of post-MMR vaccination but was treated with antibiotics for possible cellulitis. The patient had received all the routine childhood immunizations such as BCG and oral live attenuated rotavirus without

complication. His condition improved and he was discharged home, but readmitted on day 15 due to fever, misery, prominent purulent tonsillitis and widespread erythematous rashes. Investigations showed mild anaemia, lymphopenia, hepatitis and small pericardial effusion. He was suspected of Kawasaki's disease which was excluded later after his angiography showed normal coronary arteries.

While in the ward, at day 21 after first presentation, he developed seizure activity that was highly refractory to medical management. Brain MRI was performed and demonstrated bilateral hippocampal abnormalities, suggestive of meningeal inflammation. He was transferred to the paediatric intensive care unit for intubation and ventilation, and was treated with intravenous Immunoglobulin followed by high dose of corticosteroid for a suspected autoimmune/autoinflammatory disorder. Later on, his condition improved somewhat and he was extubated at day 30; however, he did not recover neurologically, showing persistent encephalopathy and temporal lobe seizure activity. His condition appeared to worsen and he became systematically unwell, with fever, hepatosplenomegaly, worsening rash and electrolyte disturbances. Bone marrow aspirate confirmed the diagnosis of Haemophagocytic Lymphohistiocytosis (HLH), a dysregulated immune response to his persistent viral infection. At this stage, the patient was supported with palliative care and subsequently, he died on day 81.

Throughout the period, thorough investigations were carried out to establish the diagnosis. Standard immunological testing was taken at day 21 and was shown to be normal (Table 3.1).

Parameter	Result	Normal range
Immunoglobulins (g/L)		
IgG	5.52	3.1-13.8
IgA	0.35	0.3-1.2
IgM	1.86	0.5-2.2
Leucocytes (10^9/L):		
Total	7.7	6.0-18.0

Lymphocytes	3.6	
Neutrophils	2.0	
Monocytes	1.3	
Eosinophils	0.8	
Basophils	0.0	
Bone marrow trephine:		
Myelopoesis	Normal	
Megakaryocytes	Normal	
Lymphocytes (cells/µL):		
T cells	1683	1200 – 3000
CD4	1229	850 – 1800
Naïve $CD4^+$ T cells	56 %	N/A
CD8	436	650 – 1500
B cells	349	600 – 1300
NK cells	260	180 – 600

Table 3.1: Patient immunological parameters. Standard immunological testing was normal in the patient. NK cells = Natural killer cells. N/A= Not available

Human Herpes Virus 6 (HHV6) was detected consistently at similar levels throughout the illness in nasopharyngeal aspirate, plasma, oral fluid and CSF by PCR (Table 2). Measles (confirmed as genotype A vaccine-strain), rubella and mumps viruses were detected by PCR on nasopharyngeal aspirate, oral fluid and plasma (Table 2). Mumps virus was also detected in the patient urine at day 29 (Table 3.2).

Day	PCR detection				Viral culture
	Measles	Mumps	Rubella	HHV6	
20	NPA	NPA	NPA	NPA	ND
21	Oral fluid, plasma (Vaccine genotype)	Oral fluid, plasma (Vaccine genotype)	Oral fluid	Oral fluid, plasma	NT
23	ND	NT	NT	CSF	NT
29	NT	NT	NT	NT	Mumps (urine)
35	ND	Serum, CSF	NT	Serum	NT
44	ND	Plasma	Plasma	Plasma	NT
50	NPA	Plasma,	NPA, swab, Plasma,	NPA, swab, Plasma,	ND

Table 3.2 : Vaccine strain measles, mumps and rubella and HHV6 were detected by PCR or viral culture in the patient. NPA= nasopharyngeal aspirate, CSF= Cerebrospinal fluid, NT= Not tested, ND= Not detected**.**

This occurred despite an appropriate serological response to MMR, with IgM and IgG to both measles and mumps and IgG to rubella (Table 3.3)

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Day	Sample	Antibody isotype		
		Measles	Mumps	Rubella
21	Plasma	IgM	IgM/IgG	IgG
30	CSF	IgM	NT	NT
44	Serum	IgG	IgM/IgG	NT
48	CSF	IgM	NT	NT

Table 3.3: Antibody response to measles, mumps and rubella. NT= not tested, ND=not detected

Interestingly, viruses that commonly cause infection in humans such as EBV, VZV, enterovirus and CMV were not identified in any of the samples taken. As the patient had persistent encephalopathy and persistent temporal lobe activity,

brain biopsy was done on day 50 and confirmed histological appearances of viral encephalitis (Figure 3.2).

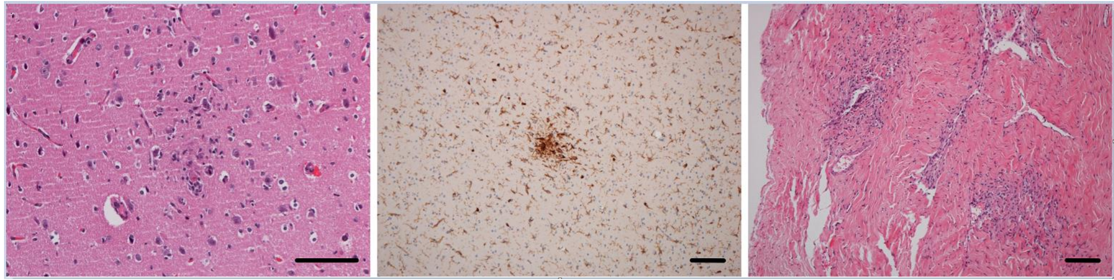

Figure 3.2:Brain biopsy showed the histological appearances of meningoencephalitis. Cortical inflammatory cell nodular infiltrate (left) with marked microglial activation (CD45 staining, middle) and patchy dural inflammation (right). This work was performed and provided by Dr Thomas Jacques, Department of Histopathology, Great Ormond Street Hospital, London.

In addition, vaccine-strain rubella and HHV6 were detected by PCR in cerebral cortex, arachnoid and dura as well as mumps in cerebral cortex; these results were confirmed by RNA sequencing carried out in the laboratory of Prof Judith Breuer. However, immunostaining for HSV1 and HSV2 were negative in the brain. Based on patient history and laboratory findings, it appeared highly likely that the fatal illness was due to the vaccine viruses mumps and rubella.

Recently, the mother gave birth to a second child, P2, who is currently healthy with normal growth and development.

3.4.2 Increased susceptibility to viral infection and lack of protection from IFNα in patient fibroblasts

To investigate the apparently increased susceptibility to viral infection, a viral plaque assay was performed on a cell monolayer, comparing the ability of patient and control fibroblasts to support viral plaque formation in vitro. Viral plaques are localized areas of cells killed by the replication of a viable virus (Mocé-Llivina *et al.*, 2004). This work was done by Professor Richard Randall's lab, St. Andrews University. Patient and control fibroblasts were infected with highly attenuated recombinant strains of Parainfluenza Virus 5 (PIV5ΔC) and Bunyamwera virus (BUNΔNSs). Because these viruses lack defined functional IFN antagonists, the IFN system is readily upregulated in normal cells and

protects against viral replication. Thus, few viral plaques were formed on control fibroblasts.

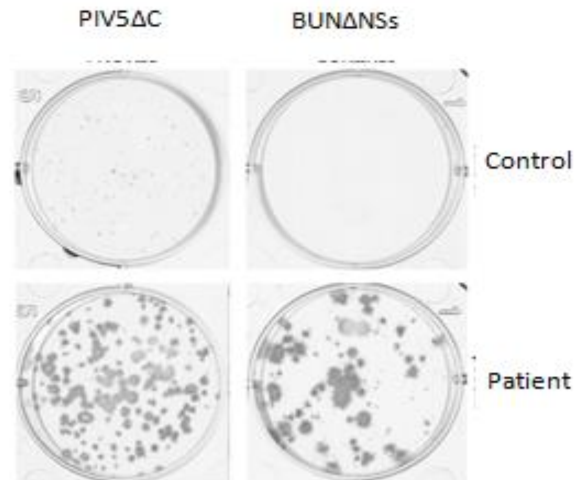

Figure 3.3 Increased susceptibility to viral infection in patient fibroblasts. Patient and control fibroblasts were infected with PIV5ΔC and BUNΔNSs viruses. Plaques were visualized by immunostaining. Figure was provided by Professor Richard Randall, St. Andrews University.

However, in patient cells, there was formation of large plaques of PIV5ΔC and BUNΔNSs, suggesting a complete failure of the type 1 IFN response in the cells (Figure 3.3). To investigate whether patient cells were able to develop an antiviral state in response to exogenous IFNα, the cells were infected with Parainfluenza virus 5 (PIV5) and the viral replication was visualized by immunofluorescence. This work was also done by Professor Richard Randall's lab, St. Andrews University. This time, wild type viruses were used for the experiment, meaning that the viruses were able to interfere with the IFN response. Therefore exogenous IFNα was added so that IFN response is induced in the cells and it is anticipated that the cells will be protected against viral replication.

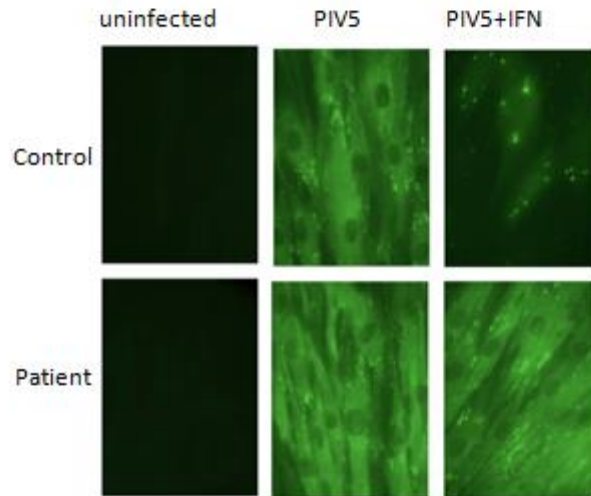

Figure 3.4: Lack of protection from IFNα in patient fibroblasts. Patient and control fibroblasts were untreated or treated with IFNα for 15 hours before infection with PIV5. At 48h.p.i, virus-infected cells were visualized by immunofluorescence. Figure was provided by Professor Richard Randall, St. Andrews University.

Control cells were protected by IFN against viral replication, whereas patient cells demonstrated a remarkable lack of protection, being unable to develop an antiviral state even in the presence of exogenous IFNα. This result showed that the cellular response to IFNα is defective in patient cells (Figure 3.4).

3.4.3 Failure of ISG upregulation in response to IFNα in patient fibroblasts, despite preserved expression of STAT1 and STAT2.

To understand this impaired IFN response, I next investigated whether antiviral ISGs such as MxA and ISG56 were upregulated in response to IFNα in patient fibroblasts. Both MxA and ISG56 are members of the viral stress-inducible genes which are strongly induced by Type 1 IFN (Haller *et al.*, 2006). In addition, I also examined the integrity of STAT1 and STAT2, which are two of the components that form the heterotrimeric complex, ISGF3. Patient and control fibroblasts were left untreated or treated with IFNα and protein expression was examined by Western Blotting.

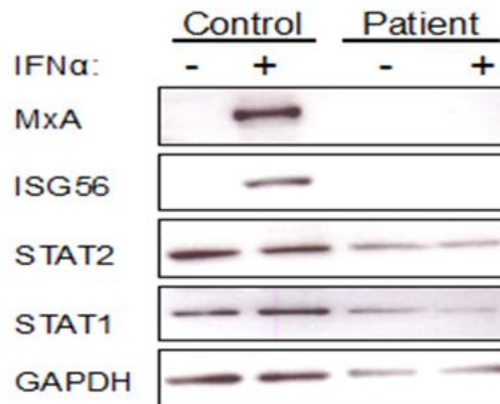

Figure 3.5 : Failure of ISG upregulation in response to IFNα in patient fibroblasts despite preserved expression of STAT1 and STAT2 . Patient and control fibroblasts were analysed by Western Blotting using antibodies directed against the indicated proteins. The cells were left untreated (-) or were treated (+) with IFNα at the concentration of 100IU/ml overnight. GAPDH was used as loading control. Data are representative of two separate experiments.

In the control cells, MxA and ISG56 proteins were upregulated, in contrast to patient cells, which completely failed to upregulate the ISGs (Figure 3.5), suggesting a defect within the Type 1 IFN signalling pathway. As for the ISGF3 complex components, STAT1 and STAT2 were expressed in control and patient cells (Figure 3.5).

3.4.4 Absence of antiviral transcriptional responses to IFNα and IFNβ but preserved transcriptional responses to IFNγ in patient cells

As it was shown that the antiviral response was impaired in patient cells, whole genome microarray analysis by Illumina HT-12 v4 Expression Ebead Chips was performed using RNA that was extracted from 3 technical replicates of patient and 3 different control fibroblast lines, to further support and determine the degree of the impaired antiviral responses.

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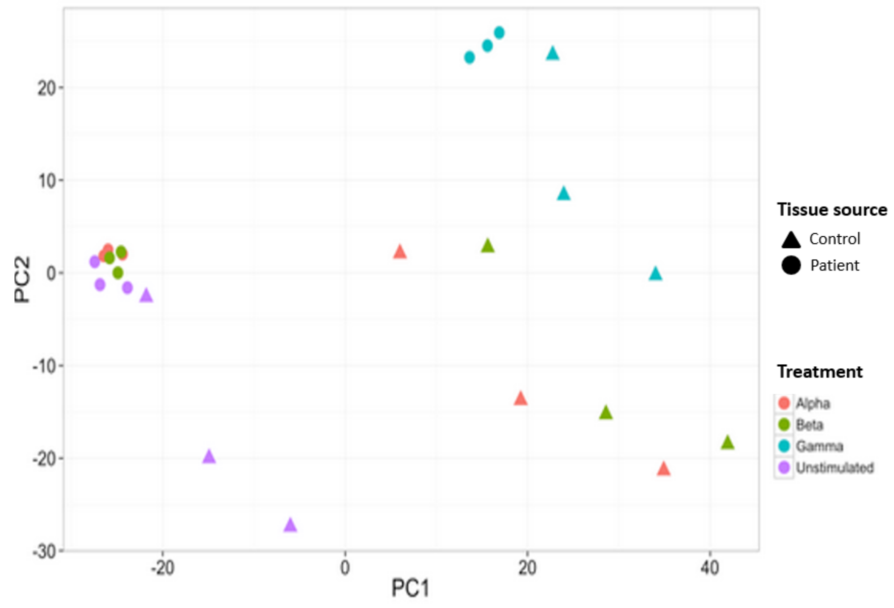

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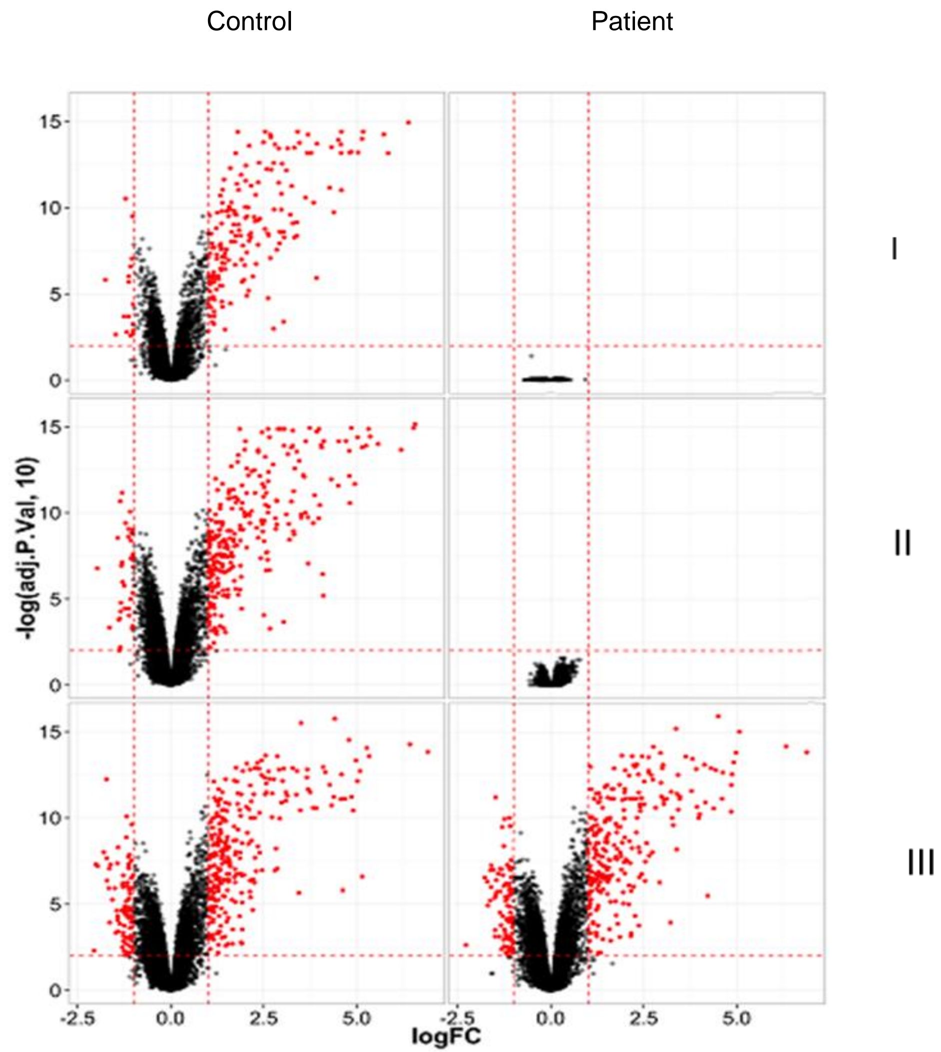

Figure 3.6 : Absence of transcriptional responses to IFNα and IFNβ but preserved transcriptional response to IFNγ by microarray. (A) Principle Component Analysis (PCA) plot of patient and control cells untreated (unstimulated) or treated with IFNα (Alpha), IFNβ (Beta) and IFNγ (Gamma) (B) Volcano plot of patient (right) and control (left) treated with IFNα (I), IFNβ (II) and IFNγ (III). Shown in red are differentially expressed genes (>= 2 fold change up or down and p ≤ 0.01) in patient cells, n = 3 technical replicates and control cells, n = 3. Data was analysed using Lumi and Limma Bioconductor packages in R and performed by Andrew Skelton, Bioinformatics Unit, Newcastle University.

All the samples that were used for the array were of high quality where the RNA Integrity Number (RIN) value was more than 9. RIN is measured using a Bioanalyzer (Agilent Technologies) which evaluates the integrity of the RNA samples using software algorithm (Schroeder *et al.*, 2006). To make the samples comparable to one another, a normalization step was done through Variant Stabilising Transformation (VST) and Robust Spline Normalisation (RSN) in Lumi package. After normalization, principle component (PCA) plot (Figure 3.6(A)) was generated to summarise and visualise the variance of the samples. Principle Component Analysis 1 showed that in control cells that were treated with IFNα, IFNβ and IFNγ, the variance was large compared to untreated cells (Figure 3.6(A)). In contrast, patient cells that were treated with IFNα and IFNβ, were not transcriptionally different from untreated cells whereas in cells treated with IFNγ, there were large transcriptional differences compared to untreated cells (Figure 3.6(A)).

To identify differentially expressed genes, a pairwise comparison was performed between IFN-treated and untreated cells (either control or patient), using a multi-level approach through Limma. The genes with an expression level that was changed ≥ 2 fold with p value ≤ 0.01 were considered as significantly differentially expressed genes. To visualize the significant differentially expressed genes, a volcano plot was generated for each comparison (Figure 3.6(B)). There were 157 and 230 upregulated genes in IFNα- and IFNβ- treated control cells, respectively, however, no changes in gene expression were observed upon IFNα or IFNβ treatment of patient cells (Figure 3.6(B)(I) & (II)). This result demonstrated the striking failure of transcriptional response to IFNα and IFNβ, consistent with all the results of the

impaired antiviral responses that were shown earlier. Nevertheless, when treated with IFNγ, both control and patient showed 250 and 241 upregulated genes respectively (Figure 3.6(B)(III), indicating preserved transcriptional response to IFNγ in the patient.

Detailed comparison revealed that both the identity of genes upregulated by IFNγ and the magnitude of their induction were similar between patient and control cells (Table 3.4).

Genes	Fold Change			
	Control			Patient
	IFNα	IFNβ	IFNγ	IFNγ
MX1	83.67944	94.74192	38.53125	31.58689
IFIT1	56.96109	72.63143	7.10684	6.270381
IFIT2	52.60496	92.00999	31.67814	21.61149
ISG15	35.94936	39.67155	12.61514	17.57555
OAS2	35.12103	40.8928	17.82121	19.83077
IFIT3	32.62033	37.817	24.0788	15.24897
MX2	28.74235	35.12128	5.080561	3.725809
HERC6	23.9643	28.6527	6.603215	5.31487
OAS1	23.94958	28.11519	5.488271	4.314488
IFI44L	23.88935	27.74313	12.97447	11.0865
ISG20	23.72714	32.24204	4.512792	4.95057
IFI6	22.56866	22.86152	8.543254	7.866593
EPSTI1	19.8517	26.05893	17.12185	15.04604
HERC5	19.236	27.88745	not upregulated	not upregulated
IFITM1	15.22598	17.27342	10.51996	14.07765
IFI27	15.07486	16.89267	3.754558	4.442659
IFI35	13.61686	16.62107	12.7105	10.75185
IFI44L	12.86096	27.74313	12.97447	11.0865
HELZ2	12.6438	16.53127	5.220394	5.332532
TNFSF13B	12.254	19.79534	11.16043	9.546272
IRF7	10.98105	15.16052	2.663317	2.18853
TAP1	10.61389	13.20197	21.24851	22.4947
RSAD2	10.48412	16.02325	3.736038	not upregulated
OAS3	10.37657	14.21979	3.779401	4.473941
OASL	9.965741	15.76712	not upregulated	not upregulated
XAF1	9.931357	12.34975	3.314304	2.659994
IDO1	9.719625	11.37384	120.0388	118.1495
SLC15A3	9.474831	11.98493	8.499544	8.671811
GBP1	9.257324	12.26308	33.97603	29.44939
PARP9	8.784777	9.676822	6.275462	6.554662
IL18BP	8.631514	10.92923	27.61209	33.65494
IFIH1	8.276362	12.88486	4.676429	3.856069
CXCL10	8.160909	17.18051	24.64496	9.302818
RNF213	7.944793	10.41398	5.935499	5.377577
TYMP	7.82178	9.435624	2.568863	3.518993

DDX58	7.792168	11.37879	2.106979	not upregulated
WARS	7.618849	9.848175	19.98488	16.02173
UBE2L6	7.440798	8.243137	6.181831	6.183141

Table 3.4 : 40 highest upregulated ISGs in IFNα-stimulated control fibroblasts and extent of induction by alternative IFNs or in IFNγ-treated patient fibroblasts. The genes were identified from the significant differentially expressed genes (>= 2 fold change and p ≤ 0.01) from IFNα, IFNβ and IFNγ - treated fibroblasts of controls and patient.

As expected, the most strongly upregulated genes in IFNα- and IFNβ-treated control cells were the known classical IFNα/β ISGs such as Mx1, IFIT1, IFIT2, ISG15 and OAS2 (Table 3.4) (Liu *et al.*, 2012). Interestingly, it was also shown that many ISGs that are upregulated in IFNα- and IFNβ-treated control cells are also upregulated in IFNγ-treated control and patient cells. However the extent of upregulation was less in IFNγ-treated control and patient cells compared to IFNα-treated control cells. This result demonstrated that IFNγ, although partially, also activates similar ISGs as IFNα, with likely relevance to its antiviral effects.

3.4.5 Absent phosphorylation of STAT1, STAT2 and STAT3 in response to IFNα but intact STAT1 phosphorylation in response to IFNγ

To localize the defect of the type 1 IFN signalling pathway, I stimulated patient and control cells with IFNα and analysed the phosphorylation of STAT1, STAT2 and STAT3 by Western blotting.

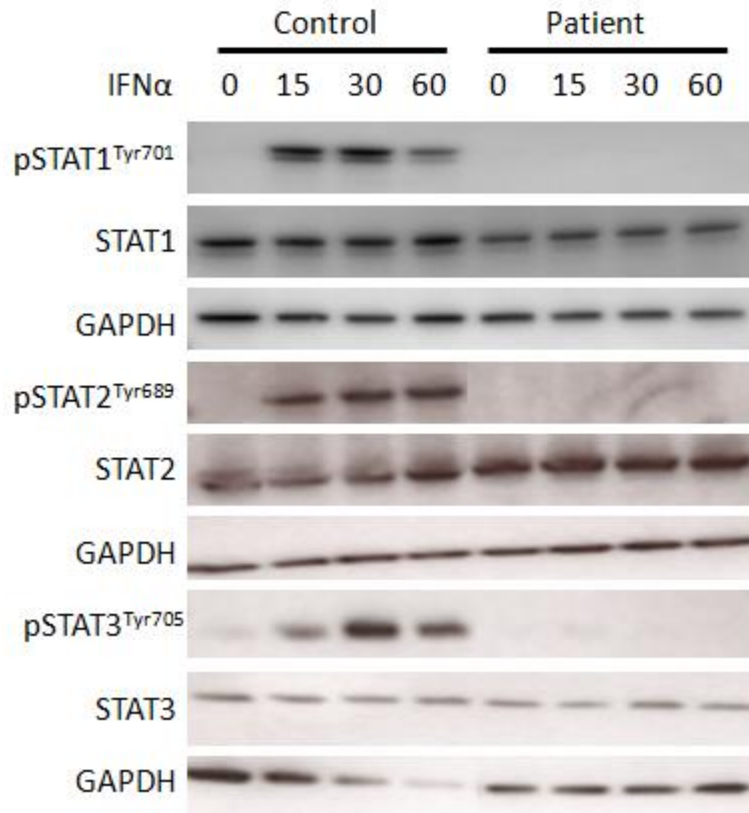

Figure 3.7 : Absence of STAT1, STAT2 and STAT3 phosphorylation in response to IFNα in patient fibroblasts. Control and patient cells were untreated (0 min) or treated with IFNα (1000IU/ml) for 15, 30, and 60 minutes. The cells were analysed by Western blotting using antibodies directed against pSTAT1 ^{Tyr701}, STAT1, pSTAT2 ^{Tyr689} , STAT2, pSTAT3 ^{Tyr705} and STAT3. GAPDH was used as loading control. Data are representative of 3 experiments. Figure of STAT1 phosphorylation and total STAT1 shown was by Dr Christopher Duncan, Newcastle University.

Upon stimulation with IFNα at different time points, control cells showed phosphorylation of STAT1, STAT2 and STAT3 whereas in patient cells, no phosphorylation was observed despite similar levels of total STAT1, STAT2 and STAT3 (Figure 3.7). The failure of STAT1, STAT2 and STAT3 phosphorylation in patient cells indicated a defect upstream of STAT phosphorylation in the IFNα signalling pathway, which includes IFNAR (1 and 2), JAK1 and TYK2. JAK1 phosphorylation can also occur in the IFNγ signalling pathway where it leads to STAT1 phosphorylation and results in the transcription of GAS-element containing genes in the nucleus (Platanias, 2005). To investigate whether the causative variant is in *JAK1*, I therefore stimulated control and patient cells with IFNγ and observed STAT1 phosphorylation by Western blotting.

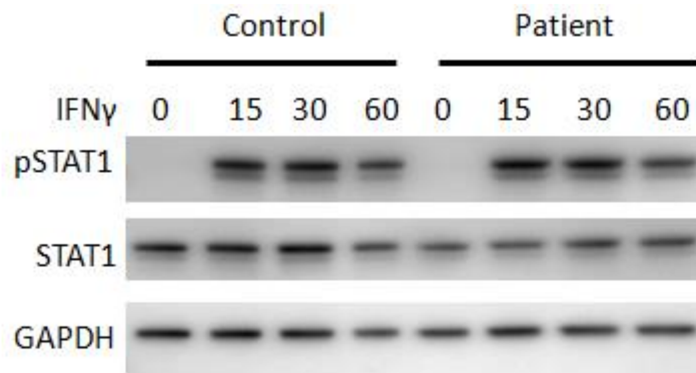

Figure 3.8 : Preserved STAT1 phosphorylation in response to IFNγ in patient fibroblasts. Control and patient cells were left untreated (0) or treated with IFNγ for 15, 30 or 60 minutes. The cells were lysed and analysed by Western Blotting using antibodies directed against total STAT1 or pSTAT1 ^{Tyr701}. GAPDH was used as loading control. Data shown are representative of 3 independent experiments.

In contrast to IFNα, STAT1 phosphorylation was normal in patient fibroblasts upon stimulation with IFNγ (Figure 3.8). This finding appears to exclude JAK1 as harbouring the causative variant (Figure 3.9).

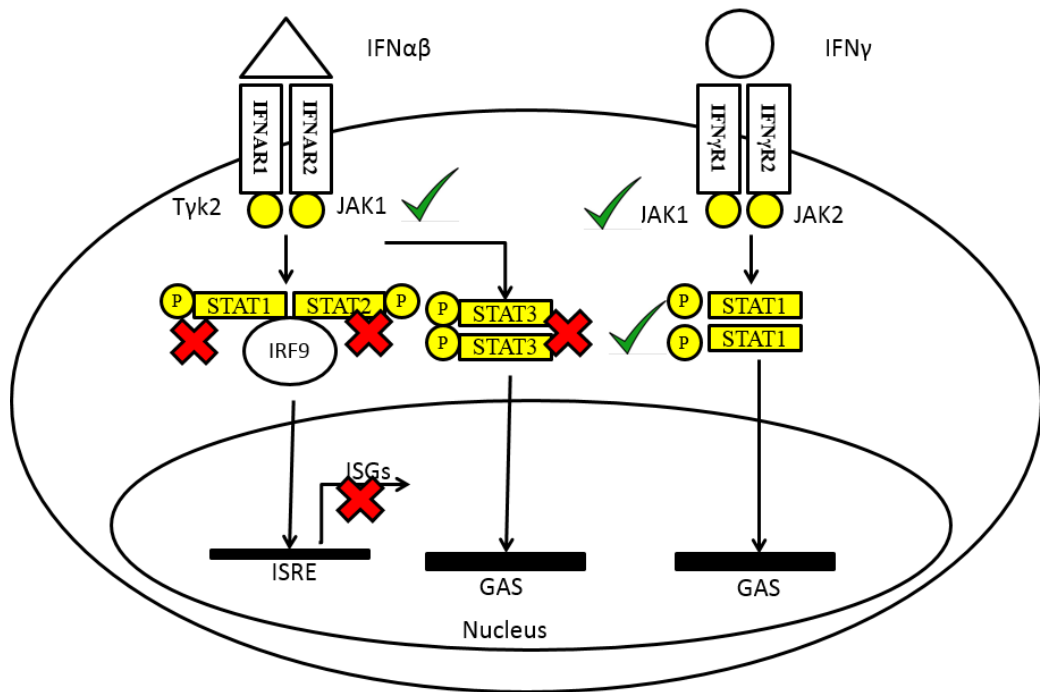

Figure 3.9 : Localization of the causative genetic lesion to *IFNAR1*, *IFNAR2* or *TYK2*. JAK1 phosphorylation occurs through the binding of IFNγ to IFNγ receptors and results in the formation of GAF and transcription of GAS genes in the nucleus. JAK1 is excluded as the disease causative mutation due to preserved STAT1 phosphorylation in response to IFNγ (✓). Failure of upregulation of ISGs (✖) and absence of STAT1, STAT2, STAT3 phosphorylation (✖) in response to IFNα in patient cells suggests the disease causing variant that affects the protein is upstream of STATs , excluding JAK1 in the IFNα signalling pathway.

3.4.6 Homozygous deletion in exon 5 of IFNAR2

By excluding JAK1, I reasoned that the putative genetic lesion(s) must affect *IFNAR1*, *IFNAR2* or possibly *TYK2,* which encode the molecules upstream of the STATs in the IFNα signalling pathway. To investigate this I performed Sanger sequencing of *IFNAR1, IFNAR2* and *TYK2* using PCR-amplified patient cDNA as template.

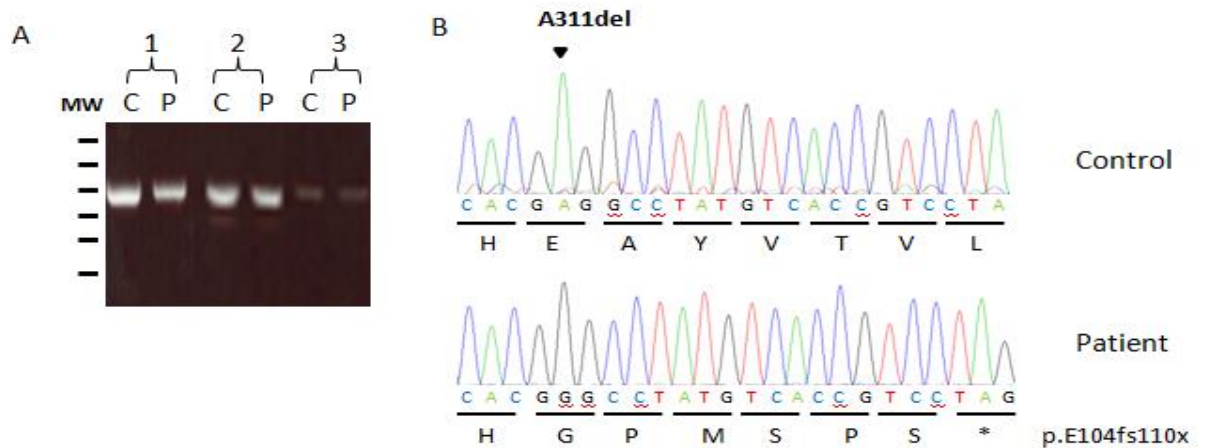

Figure 3.10: Deletion of A311 (c.A311del) in exon 5 in *IFNAR2* gene using patient cDNA. (A) Amplification of *IFNAR2* gene using three pairs of primers (1- pair 1, 2- pair 2, 3-pair 3) by PCR in control (C) and patient cDNA (P) and visualisation by agarose gel electrophoresis. (B) Sequences of patient cDNA compared to control revealed A311del of *IFNAR2*, resulting in frameshift mutation, p.E104fs110x.

By comparing and aligning patient sequences to healthy controls from public databases of genomic variation such as dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and Ensembl release 78 (http://www.ensembl.org), no variants were observed in *IFNAR1* or *TYK2*. However, there was a rare single nucleotide deletion identified in patient cDNA that was amplified and sequenced using primer pair 2 of IFNAR2 (Figure 3.10). This single base deletion is in exon 5 at position c.A311. To confirm the deletion, I sequenced the same region in patient genomic DNA.

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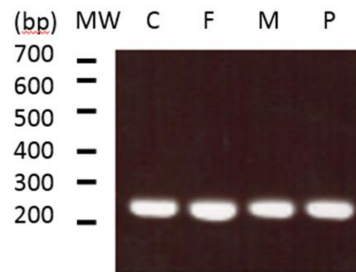

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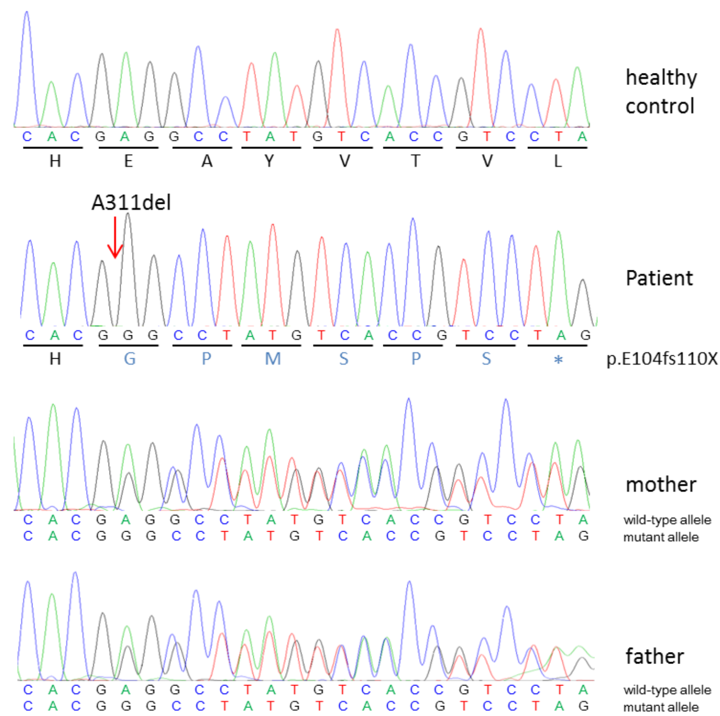


Figure 3.11 : Homozygous deletion of A311 in exon 5 of *IFNAR2* gene in patient and heterozygous deletion of A311 in both father and mother genomic DNA (gDNA). (A) Amplification of *IFNAR2* gene, targeted in exon 5 by PCR in control (C), patient (P), father (F), mother (M) and visualisation by gel electrophoresis. (B) Sequences of patient, father, mother gDNA.

By sequencing of IFNAR2 exon 5, I confirmed that the deletion was homozygous in patient genomic DNA (Figure 3.9). To investigate whether both parents are carriers for the deletion, I sequenced both mother's and father's genomic DNA. The sequencing confirmed that both parents were heterozygous for the deletion (Figure 3.11). The homozygous c.A311del caused a frameshift mutation of glutamic acid to glycine at position 104 in the predicted protein

product, resulting in a premature stop codon, six amino acids after the mutation (p.E104Gfsx6). This variant was therefore predicted to be disease-causing.

3.4.7 Absence of IFNAR2 protein expression in patient fibroblasts

The human *IFNAR2* gene is spliced into three isoforms (short, soluble and long) by exon skipping, alternative splicing and differential usage of polyadenylation sites (de Weerd *et al.*, 2007). The position of the premature stop codon created by c.A311del, p.E104Gfsx6 is predicted to affect all three isoforms of IFNAR2, with loss of the transmembrane and cytoplasmic domains as well as much of the extracellular domain (figure 3.12(A)). To confirm the expected loss of IFNAR2 protein expression, Western blotting was performed using an antibody directed against the C-terminus of IFNAR2.

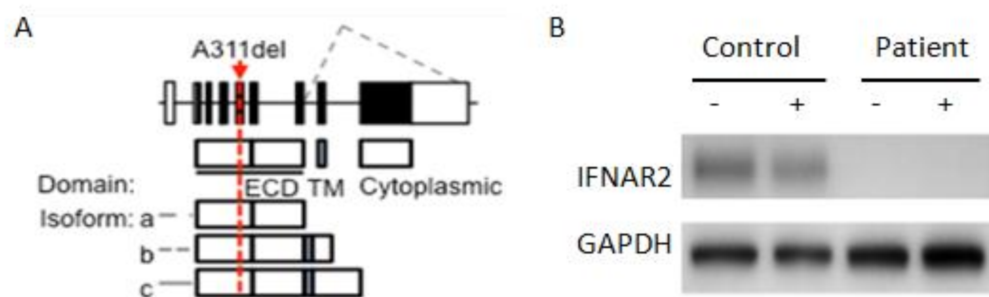


Figure 3.12: Absence of IFNAR2 protein expression in patient fibroblasts. (A) c.A311del results in truncated IFNAR2 protein affecting all three isoforms of IFNAR2. (B) Control and patient fibroblasts were left untreated (-) or treated (+) with IFNα (100IU/ml) overnight. IFNAR2 protein expression was analysed by Western blotting using an antibody directed against the C-terminus of IFNAR2. GAPDH was used as loading control. This experiment was performed by Dr. Christopher Duncan, Newcastle University. Data is representative of three independent experiments.

In keeping with the loss of the full length protein, by immunoblotting against C-terminus of IFNAR2, it was shown that IFNAR2 was completely absent in patient, in contrast to control fibroblasts where IFNAR2 protein expression was normal irrespective of IFNα treatment (Figure 3.12(B)). The absence of IFNAR2 protein in patient cells was consistent with the observed failure of IFN signalling and antiviral response.

3.4.8 Reconstitution of IFNAR2 into patient fibroblasts restores IFNα/β responses

From the sequencing, it is suggested that the disease causing variant is in *IFNAR2*. To prove that the loss of function of IFNAR2 was responsible for the impaired IFNα/β responses, patient fibroblasts were transduced with wild type IFNAR2 by lentiviral transduction (Figure 3.13(A)) and assessed for STAT1 phosphorylation as well as the upregulation of ISGs, ISG56 and MxA in response to IFNα. The commercially obtained lentiviral particles encoded the full length open reading frame of human IFNAR2 transcript variant 1 (NM_207585) under the control of the constitutive promoter EF1a, with a GFP-Puromycin selection marker under an RSV promoter. Particles containing the vector backbone but which lacked the IFNAR2 insert were used as a negative control (denoted Null).

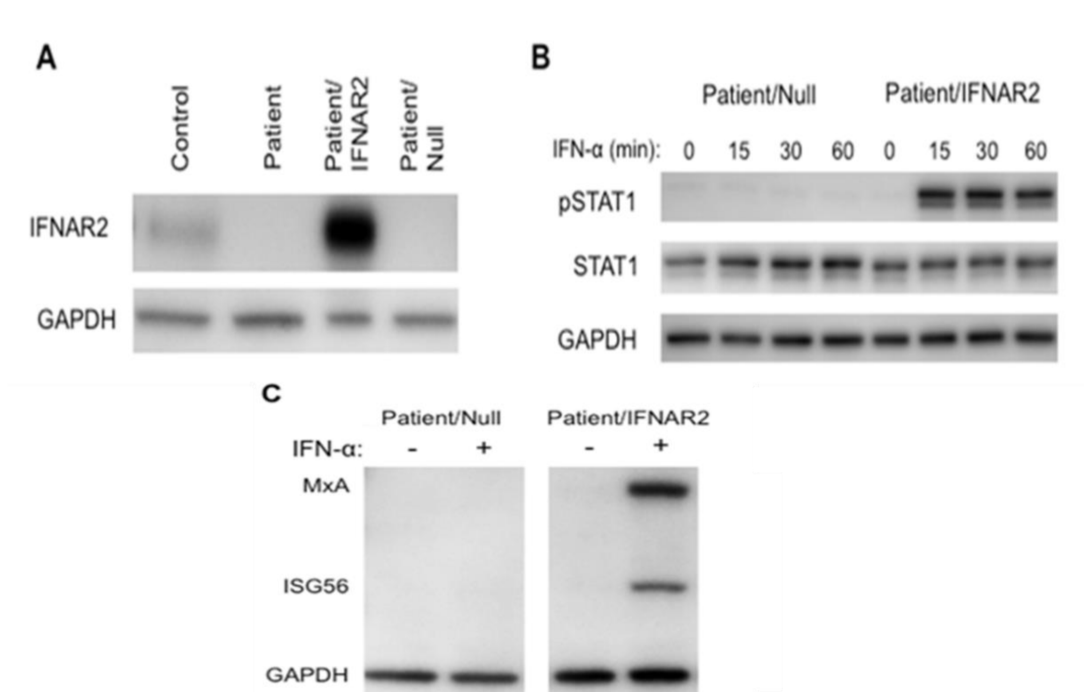


Figure 3.13 : Complementation of IFNAR2 in patient fibroblasts restores responsiveness to IFNα. (A) IFNAR2 expression; (B) STAT1 phosphorylation in response to IFNα; (C) Upregulation of ISGs, ISG56 and MxA in response to IFNα. Patient cells were either untransduced ("patient", transduced with null control ("patient/null") or transduced with lentivirus encoding IFNAR2 ("patient/IFNAR2"). This experiment was performed by Dr Christopher Duncan, Newcastle University. Data are representative of three experiments.

Immunoblotting showed that STAT1 phosphorylation in response to IFNα was restored in patient fibroblasts expressing IFNAR2 (Figure 3.13(B)). The ISGs, ISG56 and MxA were also upregulated by IFNα in patient cells expressing IFNAR2 whereas in patient cells expressing null control, there was no upregulation of ISGs (Figure 3.13(C)). These results demonstrated that reconstitution of wild type IFNAR2 expression in patient cells restores the IFNα response.

Our collaborator, Prof Richard Randall, St. Andrews University, repeated the earlier described tests of viral susceptibility in IFNAR2-reconstituted patient cells.

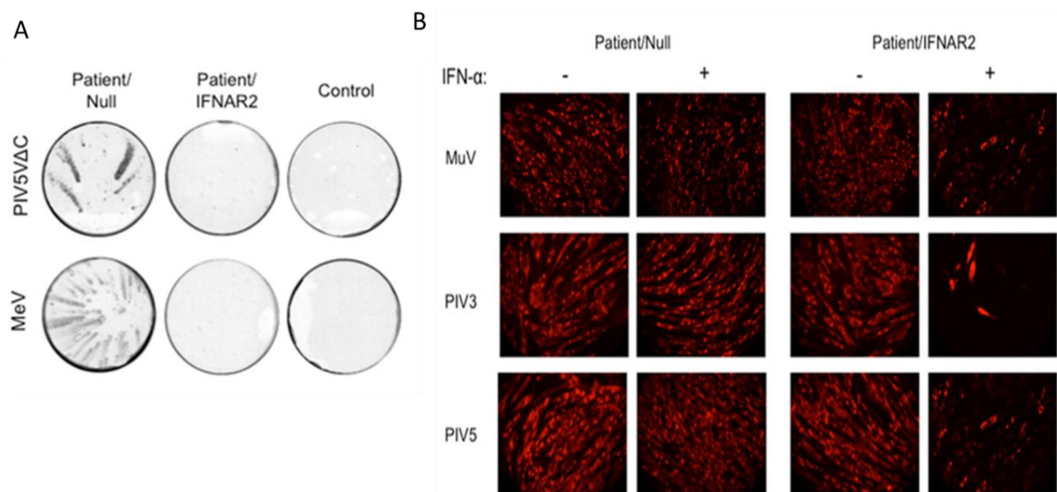


Figure 3.14: Complementation of IFNAR2 protects patient fibroblasts against viral infection. (A) Plaque assays of control, patient expressing null control and patient expressing IFNAR2. (B) Patient expressing null control and patient expressing IFNAR2 fibroblasts were not treated (-) or pre-treated with IFNα (+) overnight and were later infected with Enders mumps vaccine (MuV), Parainfluenza virus 3 (PIV3) and Parainfluenza virus 5 (PIV5). At 24 h p.i., the virus infected cells were visualised by Immunofluorescence staining. Figure was provided by Prof Richard Randall, St.Andrew's University.

In patient cells expressing IFNAR2, no plaque formation was observed which is similar to control cells, whereas in patient cells expressing null control, there was formation of plaques (Figure 3.14(A)). It was also shown by immunofluorescence staining that there was less replication of viruses in patient cells expressing IFNAR2 (Figure 3.14(B)). Both results demonstrated that

IFNAR2 complementation reinstated the ability of the patient cells to control viral infection.

3.4.9 Homozygous deletion in IFNAR2 in exon 5 in newborn sibling of the index patient

Recently, the mother gave birth to a newborn baby. To investigate whether the baby also has the deletion in *IFNAR2*, I have extracted the DNA from the baby cord blood, performed a PCR to amplify the relevant region and sent for Sanger sequencing.

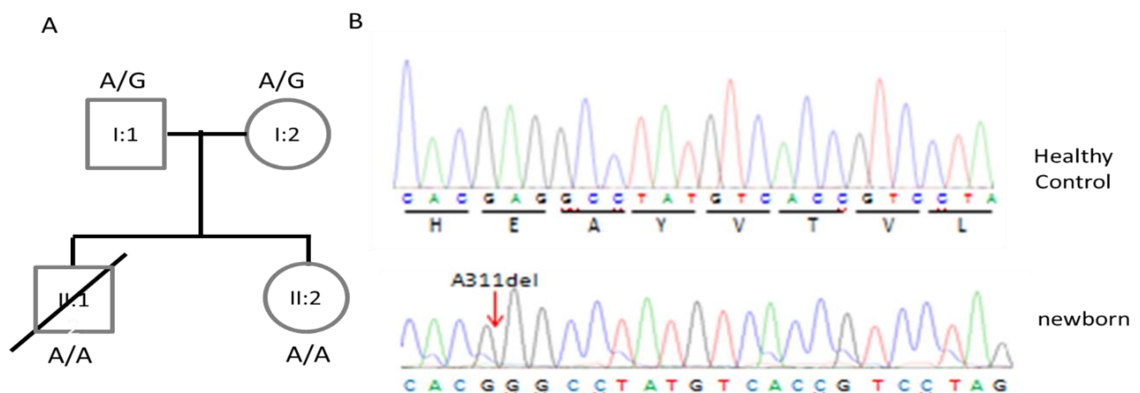


Figure 3.15: Homozygous deletion in *IFNAR2* in exon 5 in newborn baby.
(A) Pedigree of the family, Father : I:1 , Mother : I:2, Patient : II:1. Newborn : II:2
(B) Sequences of healthy control and newborn baby gDNA.

Sequencing of the newborn gDNA showed that she has the same homozygous variant c.A311del as her deceased brother (Figure 3.1). To confirm this result, Western blotting was performed to investigate IFNAR2 expression and IFNα induction of MxA in PBMCs.

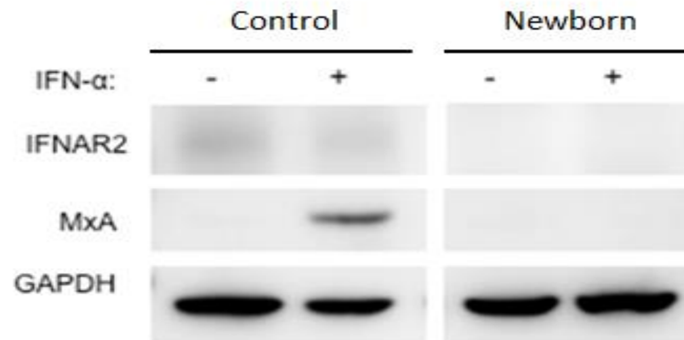


Figure 3.16 : Absence of IFNAR2 expression and MxA induction in newborn PBMCs. Control and patient PBMCs were untreated (-) or stimulated with IFNα (+) at 100IU/ml overnight. Western blotting was performed using antibody directed against IFNAR2 or MxA. GAPDH was used as loading control.

Upon stimulation with IFNα, there was upregulation of MxA in control cells, however, in contrast, absence of upregulation of MxA in the baby PBMCs. In addition, IFNAR2 expression was also absent from baby PBMCs. Both findings supported the sequencing result and confirmed that the baby also has the same defect as the patient.

3.5 Discussion

We clearly demonstrated that the c.A311del, p.E104Gfsx6 frameshift mutation in *IFNAR2* leads to absence of IFNAR2 protein, resulting in a profoundly impaired response to Type I IFN in patient cells. This proves beyond reasonable doubt that the mutation in *IFNAR2* is the cause of the viral susceptibility phenotype observed in the proband. IFNAR2 deficiency has never before been reported in humans and represents a novel Primary Immunodeficiency Disease.

Mouse knock out models provide information and understanding on the importance of IFNα/β response against viral infection. Mice lacking the IFNα receptor were shown to be very susceptible to a wide variety of viruses such as vesicular stomatitis virus (VSV, member of rhabdoviridae family (Roche *et al.*, 2008)), vaccinia virus (member of poxviridae family (Walsh and Dolin, 2011)), Semliki Forest virus (SFV, member of arbovirus family (Willems *et al.*, 1979)), Influenza virus (member of orthomyxoviridae family (Jeffery and David, 2008)) and Lymphocytic choriomeningitis virus (LCMV, member of arenaviridae family

(Buchmeier *et al.*, 1980)) in vivo and in vitro (Muller *et al.*, 1994; Van Den Broek *et al.*, 1995; Goodman *et al.*, 2010), implying that type I IFN signalling is crucial for antiviral immunity. By contrast, the IFNAR2-deficient patient did not develop any unusual illnesses until he was given MMR vaccine, despite the profound defect of IFNα/β signalling and inevitable contact with a variety of viruses during the previous 13 months of life.

Apart from the measles, mumps and rubella viruses, HHV6 was also detected in the patient CNS and other tissues at low level but its significance was unclear. HHV6 is a member of the herpesviridae along with other herpesviruses that are well known to infect humans such as Herpes Simplex Virus Type 1 (HSV1), Herpes Simplex Virus Type 2 (HSV2), Varicella Zoster virus (VZV), Epstein-Barr Virus (EBV) and Cytomegalovirus (CMV) (Yoshikawa and Asano, 2000). HSV1 has been identified to cause encephalitis in humans that had mutations which specifically impaired the viral IFN Type I induction (Casrouge *et al.*, 2006; Zhang *et al.*, 2007). These data and the fact that our IFNAR2-deficient patient also developed encephalitis, supports other studies (Paul *et al.*, 2007) that emphasise the importance of IFNα/β pathway in CNS antiviral immunity. Our patient showed serologic evidence of prior CMV infection, which he had controlled, but had not yet encountered EBV or VZV (data not shown). However susceptibility to herpesviruses might not be expected based on the experience in STAT2 deficiency, in which patients appeared to handle VZV, HSV, CMV and EBV relatively normally. In contrast to inbred mouse models (Muller *et al.*, 1994; Park *et al.*, 2000), these data suggest overall a redundant role for human IFNα/β-mediated antiviral immunity in the natural environment.

Many live attenuated viruses including measles, mumps, small pox and polio are created by passaging the virus in nonhuman cell lines, making the virus less virulent and thereby preventing the occurrence of disease (Lauring *et al.*, 2010). Such attenuation often includes loss of IFN evasion genes, with the result that the live attenuated viruses are efficient IFN inducers, compared to wild type virus strains (Shingai *et al.*, 2007). However, the defect of the IFNα/β response in the patient cells led to widespread dissemination and revealed the underlying pathogenicity of the viruses, which had also been observed in mice with targeted mutation inactivating the α/β interferon receptor (Mrkic *et al.*, 1998).

Similarly, susceptibility to another live attenuated vaccine prepared from attenuated strain of Mycobacterium bovis was observed in patients with a defect of IFNγ receptors who presented with susceptibility to mycobacterial disease (Altare *et al.*, 1998). Thus the occurrence of disseminated disease caused by live attenuated vaccines should be considered to indicate underlying immunodeficiency until proved otherwise.

Type II IFN, IFNγ, also displays an antiviral effect through the induction of ISGs (Liu *et al.*, 2012). To understand the antiviral activity of Type I and type II IFN in vitro, control and patient cells were stimulated with IFNα, IFNβ and IFNγ and expression of the ISGs was assessed by microarray. A study done by Liu et al. demonstrated that both IFNα and IFNγ upregulate a similar set of ISGs, however, IFNα is thought to be a stronger inducer of antiviral ISGs than IFNγ as the number of ISGs that were upregulated by IFNα was higher than IFNγ (Liu *et al.*, 2012). From the microarray analysis, our finding is consistent with Liu et al. showing that both IFNα/β- and IFNγ-stimulated control cells activate a similar set of ISGs. However, genes such as Mx1, IFIT and OAS2 were upregulated to much higher levels in IFNα- than in IFNγ-treated control cells, supporting the notion that they are the classical IFNα/IFNβ-specific genes (Liu *et al.*, 2012), whereas IRF1 was upregulated higher in IFNγ- than IFNα-treated cells, supporting as IFNγ-specific gene (Kimura *et al.*, 1994). Previous studies suggested that IFNγ mediated its antiviral effect via an IFN amplification loop which it is thought to be dependent on IFNAR activity (Park *et al.*, 2000; Takaoka *et al.*, 2000). Therefore, we expected that the upregulation of ISGs in IFNγ-treated patient cells would be lesser compared to control. However, from the microarray result, we found that in the IFNγ-treated patient cells, the upregulation of ISGs was similar to IFNγ-treated control cells. One possible explanation could be that as the cells were stimulated with IFNγ for 10 hours, we think that at this time point the upregulation of ISGs such as Mx1, IFIT and OAS is a truly IFNγ-mediated antiviral effect which is not dependent on the IFNα signalling. Type 1 IFN-mediated amplification of the IFNγ response may only occur later; to prove this, a longer time of IFNγ stimulation would be required to observe a difference in ISG upregulation between control and patient cells.

Furthermore, proteomic studies would be required to establish whether transcriptional upregulation is matched by equivalent induction at protein level.

Another type of IFNs are Type III IFNs which consist of IFNλ 1,2 and 3 (Gough *et al.*, 2012). Type I and type III IFNs were shown to exhibit similar properties in that both were triggered by viral infection and TLR ligands, used similar signal transduction pathways and induced similar sets of ISGs (Onoguchi *et al.*, 2007). However, type III IFN is recognised by a different heterodimeric cell surface receptor consisting of IL-10Rβ and IL28Rα (Sommereyns *et al.*, 2008). A study done by Sommeryns et al. demonstrated that the key difference between type I and type III IFNs is the cell specificity of IFNλ where its receptor is primarily expressed by cells of epithelial origin whereas IFNAR is widely expressed in nucleated cells (the production of IFNλ also contributing somewhat to this tissue-specificity) (Sommereyns *et al.*, 2008). Since epithelial surfaces are the primary site of replication for many viruses, intact IFNλ activity could explain the absence of a more general viral susceptibility in the patient. By contrast, the systemic route of vaccine administration for MMR bypasses the IFNλ system at mucosal surfaces, and evidently allowed viral dissemination in the patient.

Besides their role in antiviral defence, type I IFNs have been implicated in regulating adaptive immunity by modulating the behaviour of T cells and antigen presenting cells (Hambleton *et al.*, 2013; Jennings *et al.*, 2014). For example, a study by Montoya et al. demonstrated that IFNα receptor KO mice show reduced expression of co-stimulatory molecules or MHC Class I and Class II on GM-CSF-derived dendritic cells, together with impaired T cell proliferation (Montoya *et al.*, 2002). Other studies also reported impaired myelopoeisis or defective thymic T cell development in the same KO mice (de Weerd *et al.*, 2007). However, our IFNAR2-deficient patient showed no clinical or laboratory evidence of impaired adaptive immunity, implying evolutionary divergence between species or mouse strains with respect to this interplay between innate and adaptive immunity (Parekh and Crooks, 2013).

As mentioned in the history, recently the mother gave birth to another child. Genomic DNA sequencing showed that she bears the same homozygous IFNAR2 mutation with absence of IFNAR2 protein and no IFNα-inducible MxA

expression by western blotting. It is extremely important to know that the newborn has heightened viral susceptibility so as to be able to manage and treat her. For instance, she must not receive live attenuated vaccines, in order to prevent the occurrence of a similar fatal illness. As our analysis demonstrated that IFNγ can induce an antiviral state in patient cells, it could be considered as a potential therapeutic in the event of overwhelming viral infection. In future work, it would be interesting to assess the surviving patient's adaptive immunity in more detail, for example by examining T cell effector polarisation and responses in vitro. Unfortunately we cannot currently study serologic antiviral responses because the child is receiving immunoglobulin replacement therapy as a means to prevent potentially fatal viral infection.

Haematopoietic stem cell transplantation (HSCT) has been a choice of treatment for many PIDs (Dvorak and Cowan, 2007). The goal of treating PID patients with HSCT is to provide normal cells of haematopoietic origin to replace the genetically defective immune system (Buckley *et al.*, 2013). However, in this case transplantation would appear to be unhelpful as the IFNAR2 defect is intrinsic to all cells and hence correction of the haematopoietic compartment would most likely fail to cure the viral susceptibility phenotype. In addition, the inevitable period of intense iatrogenic immunosuppression would likely be hazardous for the baby, compounding her intrinsic viral susceptibility by transiently removing both adaptive and innate immune cells.

My data clearly show that the Type I IFN signalling pathway is essential for host defence against certain viral pathogens in humans, in agreement with my earlier findings in STAT2 deficiency. This novel PID, IFNAR2 deficiency, helps us in understanding the role of this receptor and the importance of IFNα/β signalling in human antiviral immunity. The recognition of this phenotype provides an important model in defining the significant role of innate IFN and its interaction with viral pathogenesis in humans.

Chapter 4: A novel autoimmune lymphoproliferative disorder

This chapter is structured into three sub chapters :

4.1 Identification of disease causing variant

4.2 Effect of TET2 p. H1382R variant on its functional activity

4.3 Effect of TET2 p. H1382R variant on lymphocyte apoptosis

4.1 Identification of the disease causing variant

4.1.1 Introduction

4.1.1.1 Whole exome sequencing

Whole exome sequencing is a genetic technique that is currently widely used to identify gene defects responsible for Mendelian disorders. Most primary immunodeficiency diseases are Mendelian, meaning that the disease is caused by a DNA mutation in single genes inherited from parents by their offspring (Ochs *et al.*, 1999). Whole exome sequencing uses the approach of targeted sequencing of the protein coding regions known as exons within the human genome (Bamshad *et al.*, 2011). The exome constitutes only 1% (30MB) of the human genome but it is estimated to contain approximately 85% of deleterious variants (Rabbani *et al.*, 2014). Therefore, sequencing these coding regions of the genome provides a high chance of identifying the cause of genetic disorders (Rabbani *et al.*, 2014). Depending on the sequencing platform used, whole exome sequencing is able to identify 20,000 – 35,000 single nucleotide variants per exome by comparison with the reference human genome sequence (Platt *et al.*, 2014). It should be noted that some proportions of the variations is at the level of common polymorphisms whereas smaller proportion is 'private' or rare to the individual which provide the risk of developing certain diseases.

In order to discover the putative disease-causing variant(s) among the many variants identified by whole exome sequencing, several filtering steps are applied including elimination of variants that are synonymous as they will only alter the DNA but not the amino acid sequence (Platt *et al.*, 2014); as such these are very rarely pathogenic. Since by definition in rare diseases the causative variant is novel or rare, common variants (polymorphisms with an allele frequency of more than 1% in public single nucleotide polymorphism

(SNP) databases such as dbSNP and 1000 genome projects or in house exomes) are also eliminated (Bamshad *et al.*, 2011; Platt *et al.*, 2014). Polyphen 2, SIFT, Mutation Taster and MAPP are bioinformatic algorithms that are used to predict whether the variants are benign or deleterious e.g. on the basis of evolutionary conservation and amino acid similarity, and benign variants will be removed from further analysis (Platt *et al.*, 2014). Similar software can be applied to understand the effect of mutations within splice sites. Another filtering step is by removing variants that do not fit with the expected mode of inheritance (Singleton, 2011). For example, in autosomal recessive disorders, two mutated alleles are present in the offspring, resulting in a homozygous mutation due to one of the mutated allele is generally inherited from the mother and the other from the father of a consanguineous couple who are both likely to inherit from one ancestor. Both parents are unaffected as they only carry one mutated allele and one normal allele.

4.1.1.2 Homozygosity mapping

Homozygosity mapping is often applied in parallel with whole exome sequencing in consanguineous families where autosomal recessive inheritance is suspected (Platt *et al.*, 2014). Homozygosity mapping is applied to determine the genomic region linked to the disease, commonly by means of SNP arrays and/or microsatellite markers (Alkuraya, 2010). In autosomal recessive disorders, the affected children are likely to be homozygous at the markers linked to the disease locus because of co-inheritance from a recent common ancestor on both maternal and paternal sides. Homozygosity mapping identifies the chromosomal homozygous regions that are shared by affected family members but are heterozygous or not shared at all in healthy family members. By combining both whole exome sequencing and homozygosity mapping data, attention can be focused on a shorter list of candidate variants that lie within shared regions of homozygosity.

4.1.1.3 Novel autoimmune lymphoproliferative syndrome

In this chapter, I will describe the investigation of two siblings from a consanguineous family who were diagnosed clinically with autoimmune lymphoproliferative syndrome (ALPS) but lacked known molecular defects.

ALPS is a disease that results from failure of the apoptotic mechanisms in maintaining lymphocyte homeostasis (Rao and Oliveira, 2011). Several mutations within the Fas-mediated apoptosis pathway have been described which lead to this clinical syndrome (Rieux-Laucat *et al.*, 1995; Wang *et al.*, 1999; Chun *et al.*, 2002; Del-Rey *et al.*, 2006; Oliveira *et al.*, 2007; Takagi *et al.*, 2011). However, one third of ALPS patients still lack a genetic diagnosis (Oliveira *et al.*, 2010), like our two brothers. The fact that they were born to consanguineous parents increased our suspicion for a novel form of autosomal recessive ALPS, caused by a homozygous disease-causing mutation. Accordingly, whole exome sequencing was applied together with homozygosity mapping to identify the genetic defect.

4.1.2 Hypothesis

The patients' phenotype is caused by a homozygous mutation in a novel disease gene.

4.1.3 Aims

To identify the disease-causing variant in both patients by whole exome sequencing and homozygosity mapping.

4.1.4 Results

4.1.4.1 Patients' history

We investigated two siblings from a consanguineous marriage who were clinically diagnosed with ALPS but lacked a known molecular defect. Patient 1 (P1, the elder brother) presented to hospital at 4 weeks of age due to pneumonia which was caused by Respiratory Syncytial Virus (RSV) and Cytomegalovirus (CMV) infection. He was treated with Ganciclovir and discharged home. Subsequently, P1 showed failure to thrive and developmental delay, and had frequent admissions due to recurrent lower respiratory tract infections. From 18 months old, he developed hepatosplenomegaly, chronic lymphadenopathy and persistent EBV infection. He developed autoimmune phenomena such as immune thrombocytopenia and autoimmune haemolytic anaemia, requiring frequent transfusions. Autoantibodies were present as confirmed by direct antiglobulin test (DAT), and assays for anti-nuclear antibody

(ANA) and Rheumatoid Factor (RF). Since he displayed both lymphoproliferative disease and autoimmunity, he was investigated for ALPS at the age of 3 years. Analysis by the laboratory of Dr Frederic Rieux-Laucat confirmed the diagnosis of ALPS on the basis of defective Fas-mediated apoptosis (Figure 4.1.1), raised double negative (CD4-CD8-) TCRαβ T cells (20%) and a raised level of soluble Fas Ligand (0.9ng/ml) (Table 4.1.1). Throughout this illness, standard lymphocyte subsets were grossly normal. His IgM and IgA levels were normal, but IgG and IgG1 were high for one episode (Table 4.1.2).

P1 was treated with high dose (2g/kg) intravenous immunoglobulin, Rituximab (anti-CD20 antibody) and corticosteroid. His overall condition and lymphadenopathy initially appeared to be responding to this immunomodulatory therapy. However, when he was 4 years old, P1's condition deteriorated markedly, with the development of massive hepatosplenomegaly and evolution of his lymphadenopathy to become hard and 'knobbly'. Further investigations including lymph node biopsy showed lymphoma of a nodular sclerosing, Hodgkin's type (Table 4.1.1). He was started on low intensity chemotherapy but developed the life-threatening complication of tumour lysis syndrome with acute renal failure, requiring prolonged intensive care.

Since the formulation was that his lymphoma had occurred as a complication of an inherited immune defect, P1 was prepared for haematopoietic stem cell transplant (HSCT) as a potentially lifesaving procedure. Because of his gross hepatosplenomegaly, he required a splenectomy prior to transplant; histological examination of the explanted spleen as well as liver biopsy showed clearance of the lymphoma. At 4 years 4 months old, he received a matched sibling donor HSCT after reduced intensity conditioning. One month after BMT, he was at risk of rejecting the graft with evidence of a large population of recipient T cells. However, after receiving alemtuzumab (anti-CD52 antibody) and an unconditioned stem cell top-up, the proportion of donor cells improved to reach 96% T cells but only 14% B cells (mixed chimerism).

P1 was monitored frequently and his condition was clinically stable over the following two years. An important and interesting finding was that starting from 3

months after transplant, P1 developed leucocytosis, monocytosis, neutrophilia and lymphocytosis (Figure 4.1.2). In addition, all his lymphocyte subset absolute counts including CD3, CD4, CD8, CD19, NK cells and HLA-DR+ % were consistently at high levels post-transplant (Figure 4.1.3).

At 26 months post-transplant (6 years 6 months old), he was noted to have recurrent lymphadenopathy, hepatomegaly and hypercalcemia of unknown cause. Lymph node biopsy and bone marrow trephine showed non-caseating granulomata but no evidence of malignancy. In keeping with granuloma formation, an increased serum level of angiotensin converting enzyme (ACE) was detected. At 36 months post-transplant (7 years 4 months old), he developed thrombocytopenia with positive ANA and ongoing widespread lymphadenopathy. During this time, his IgG level, which had been normal throughout the post-transplant period, was noted to be high (29.7g/L), whereas IgM and IgA remained normal. His β2-microglobulin, a tumour marker for blood cell cancer, was also increased (14.6mg/ml, normal range <2.7mg/ml). Based on the recurrent lymphadenopathy, hepatomegaly and autoimmune phenomena, he was diagnosed with recurrent ALPS. The Fas-mediated apoptosis assay was repeated, however, it was shown to be normal (Figure 4.1.1). As the test was performed post-transplant and the patient had 78% donor T cells, it was not surprising that the repeated apoptosis assay was normal. P1 then received a course of rituximab, following which his IgG and β2-microglobulin levels normalised. The CD19 absolute count dropped to 0, indicating that Rituximab was clearing B cells and the CD3 absolute counts were also reduced at the same time.

At 48 months post-transplant (8 years 4 months old), P1 was admitted to hospital with central cyanosis and poor lung function secondary to severe bronchiectasis. The lymphadenopathy and hepatomegaly were reduced in size but new lumps were noted in his scrotum and tongue. Excision biopsy of these lesions demonstrated two granular cell tumours that were completely excised. Subsequently, he developed severe immune thrombocytopenia at 60 months (9 years 4 months old) and anaemia at 62 months (9 years 6 months old) post-transplant, requiring blood product support and a further course of rituximab for presumed autoimmune aetiology. During this time, he also presented with

headache and hypertension and later had a seizure, with brain CT showing a small right frontal bleed. In addition, he developed pleural effusions and ascites of unknown cause which were managed with fluid restriction and diuretics and gradually resolved.

At 82 months post-transplant (11 years 2 months old), another lump was noted at his left upper arm and excision biopsy showed another granular cell tumour. At 84 months post-transplant (11 years 4 months old), he developed another episode of thrombocytopenia and increased lymphadenopathy. So far his last admission to the hospital was at 98 months post-transplant (12 years 6 months old), which was due to respiratory and gut failure secondary to E.coli sepsis complicating a severe febrile diarrheal illness acquired in Pakistan. His stool was positive for cryptosporidium, norovirus and sapovirus. He also had blood transfusion due to thrombocytopenia and anaemia. Care was shifted to a palliative footing as he was severely debilitated and not expected to survive, however he showed a remarkable recovery in the home environment and has since recovered to his usual level of function.

Currently, the patient is 13 years old and 9 years post transplantation. His condition is stable and he attends school 2-3 days per week, where a statement of special educational needs is in place. He has supplemental oxygen at night and his exercise capacity is severely limited. He was recently evaluated for short stature and failure of pubertal development. His history is summarised in Table 4.1.1.

Patient 2 (P2) is the younger brother of P1. His problems started when he developed haematuria (blood in the urine) and proteinuria (protein in the urine) at 4 weeks of age. Renal biopsy was performed and showed granulomatous nephritis which was treated as congenital nephrotic syndrome. He was also noted to have hypothyroidism that was attributed to loss of thyroxine (T4)-binding globulin (TBG) in the urine and was treated with thyroxine. Later on, he was admitted to Paediatric Intensive Care Unit (PICU) due to CMV pneumonitis complicated by pneumothorax. He was started on immunoglobulin supplementation. Subsequently, he developed hepatosplenomegaly and lymphadenopathy at 4 months of age. His lymph node biopsy showed a diffuse

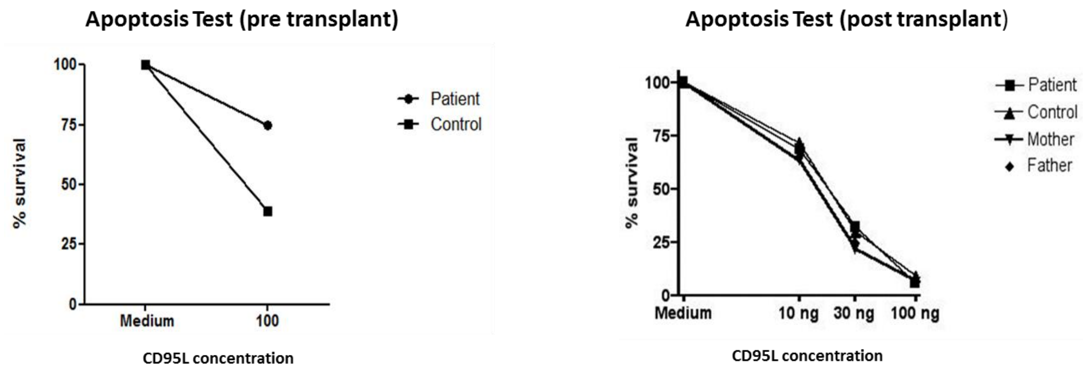
proliferation of T lymphocytes, suggestive of T cell lymphoma (Table 4.1.1). He demonstrated features of autoimmunity such as several episodes of thrombocytopenia but no autoantibody tests were documented. His neurodevelopment was also noted to be delayed for age.

Based on the presence of lymphadenopathy, lymphoma, hepatosplenomegaly and autoimmunity, P2 was clinically diagnosed with ALPS, and diagnosis was confirmed by defective Fas-mediated apoptosis (Figure 4.1.1), increased DN TCRαβ T cells (1.96) (Table 4.1.1) and raised soluble Fas ligand 0.96ng/ml(Table 4.1.1). His lymphocyte subsets and Immunoglobulin levels were within the normal range for age. He was started with cyclophosphamide and methylprednisolone for his lymphoma; however, it was stopped due to deranged liver function. A T cell-depleted paternal haploidentical HSCT was performed at 9 months of age, following modified intensity conditioning. The HSCT was unsuccessful as he developed graft rejection with evidence of 100% of T cells being recipient in origin. Subsequently, 3 months post-transplant, his condition deteriorated when he developed respiratory failure and required intubation for presumed sepsis. The patient died at the age of 13 months, 4 months post-transplant. Patient history is summarised in Table 4.1.1.

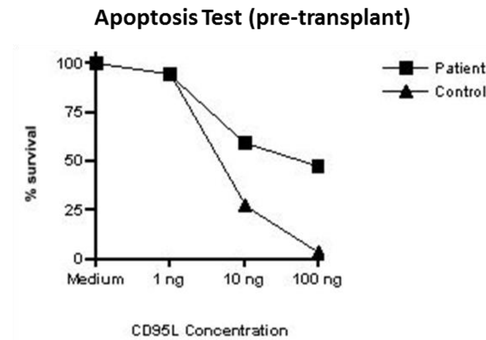
	Patient 1 Pre-transplant	Patient 1 Post-transplant	Patient 2
Age of onset	18 months		4 weeks
CLINICAL SYMPTOMS			
Immunodeficiency	Recurrent lower respiratory infections with established bronchiectasis		Recurrent lower infections
Viral infections	RSV, CMV, persistent EBV	Norovirus, sapovirus*	CMV
Bacterial infections		E.coli, cryptosporidium	
Autoimmunity (cytopenias)	Thrombocytopenia, anaemia	Thrombocytopenia, anaemia	Thrombocytopenia
Early onset nephrotic syndrome	Yes		Yes

Lymphadenopathy	Yes	Yes	Yes
Hepatomegaly	Yes, massive	Yes	Yes
Splenomegaly	Yes		Yes
Malignancy	Nodular sclerosing Hodgkin type lymphoma	Granular cell tumour (Scrotum, tongue and liver)	T-cell lymphoma
Bone marrow aspirate	Marrow is hypercellular with expansion of myelopoeisis		Lymphocytosis not more than 10%
Developmental delay	Yes		Yes
Failure to thrive	Yes		-
Others	-	Seizure due to small right frontal bleed	Congenital nephrotic syndrome, hypothyroidism secondary to nephrotic syndrome
LABORATORY VALUES			
Apoptosis	Impaired	Normal	Impaired
Soluble Fas Ligand	Increased (900pg/ml)		Increased (960pg/ml)
DNT cells	Increased (20%)		Increased (1.96%)
Blood results	thrombocytopenia, anemia	Leucocytosis, lymphocytosis, monocytosis, thrombocytopenia, anemia	Thrombocytopenia
Autoantibodies	DAT +ve, ANA +ve, RF +ve		Not available
TREATMENT			
Transplant	Matched sibling donor HSCT		T cell-depleted paternal haploidentical HSCT
Outcome	Mixed chimerism Alive		Graft rejection, Died 4 months post transplant

Table 4.1.1: Clinical features of patient 1 (pre and post transplant) and patient 2.DAT- direct antiglobulin test, ANA-anti nucleic antibody, RF-Rheumatoid factor, +ve – positive, RSV-respiratory syncytial virus, CMV-cytomegalovirus, EBV- Epstein Barr Virus, HSCT- haematopoietic stem cell transplant. * respiratory and gut failure



Patient 1



Patient 2

Figure 4.1.1 : Fas-mediated apoptosis patient 1 and patient 2. Fas-mediated apoptosis assay pre- and post-transplant of patient 1, and pre-transplant of patient 2. Patients' T cells were left untreated (medium) or treated with 100ng, 30ng, 10ng and 1ng of CD95L (soluble Fas ligand). The % of surviving cells vs CD95L concentration were plotted. Patient 1 Fas-mediated apoptosis assay (pre-transplant) was performed in the laboratory of Frederic Rieux Laucat, Paris, France. Patient 1 (post-transplant) and patient 2 (pre-transplant) Fas-mediated apoptosis assay was done in the laboratory of Stephan Ehl in Freiburg, Germany.

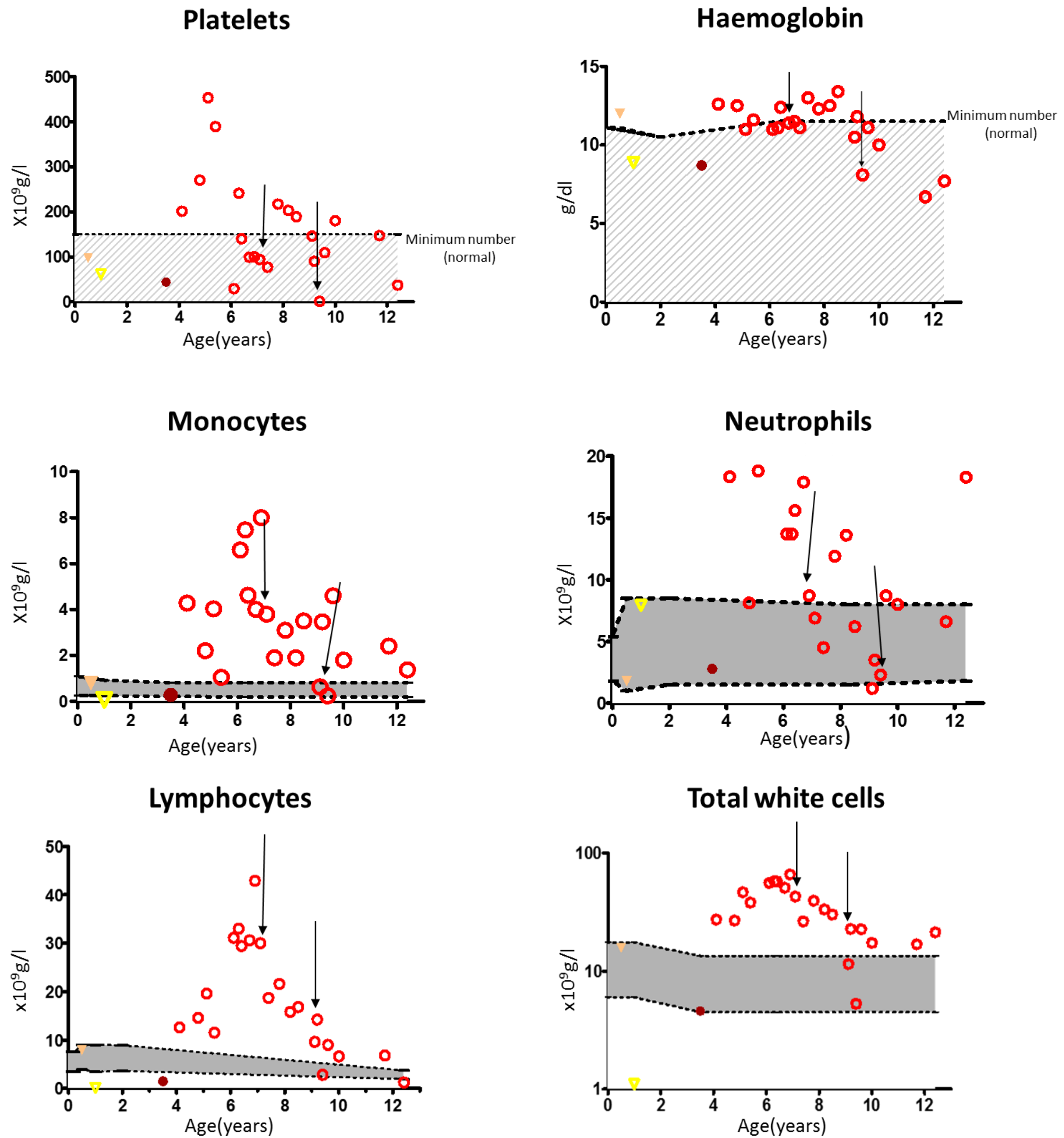


Figure 4.1.2 : Haemoglobin, platelets, total lymphocytes, total white cells, monocytes and neutrophil absolute counts in peripheral blood of patient 1 and patient 2 For haemoglobin and platelets, the hatched areas indicate sub-normal range. Grey areas in total lymphocytes, total white cells, monocytes and neutrophils indicate normal ranges. ↓ indicates post rituximab. Patient 1 pre transplant (●), Patient 1 post transplant (○), Patient 2 pre transplant (▼), Patient 2 post transplant (▽).

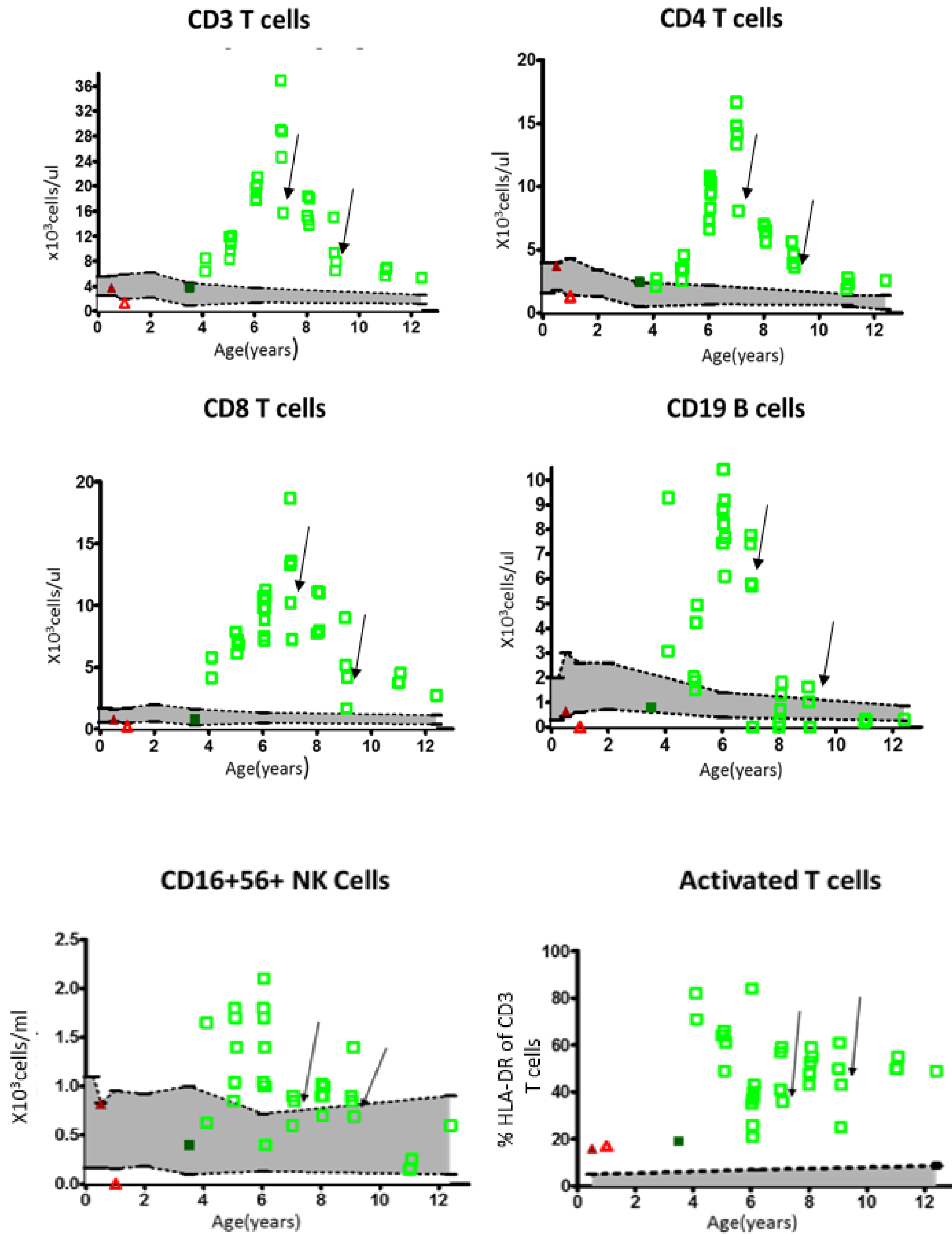


Figure 4.1.3 : Absolute numbers of indicated lymphocyte subsets (CD4, CD3, CD8, CD19 and CD16+56+) and percentages of activated T cells. Grey area indicates normal ranges of the lymphocyte subsets. ↓ indicates post rituximab. Patient 1 pre transplant (■), Patient 1 post transplant (□) Patient 2 pre transplant (▲), Patient 2 post transplant (△ **)**.

	Patient 1				Patient 2			
	Pre-transplant (age 3.5 years)		Post-transplant (age 7 years)*		Pre-transplant (age 5 months)		Post-transplant (age 12 months)*	
	Values (g/l)	Reference range	Values (g/l)	Reference range	Values (g/l)	Reference range	Values (g/l)	Reference range
IgG	**22.4**	3.6-15.2	29.7	4.9-16.1	12.9	Normal	20.4	High
IgA	1.16	0.43-1.90	0.79	0.5-2.4	0.98	Normal	0.86	Normal
IgM	1.24	0.4-2.18	0.72	0.5-2.0	0.22	Normal	0.89	Normal
IgG1	**20.78**	2.5-6.9	NA				NA	
IgG2	2.47	0.7-4.8	NA				NA	
IgG3	2.76	0.1-1.1	NA				NA	

Table 4.1.2 : Immunoglobulin levels of patient 1 and patient 2 pre- and post-transplant. For patient 1, the values were taken from one episode of high IgG level for pre- and post-transplant. * on Iv Ig replacement.

To summarise, both patients presented with clinical features of ALPS including lymphadenopathy, hepatosplenomegaly, lymphoma and autoimmune phenomena such as thrombocytopenia and anaemia. In further support of this diagnosis, both patients demonstrated laboratory features of ALPS including defective Fas-mediated apoptosis, increased DN TCRαβ T cells and increased soluble Fas ligand. In addition, they demonstrated susceptibility to infection including CMV and EBV, which is a less commonly associated feature of ALPS. Since neither patient bore mutations in genes already associated with ALPS, a novel disease causing variant was suspected.

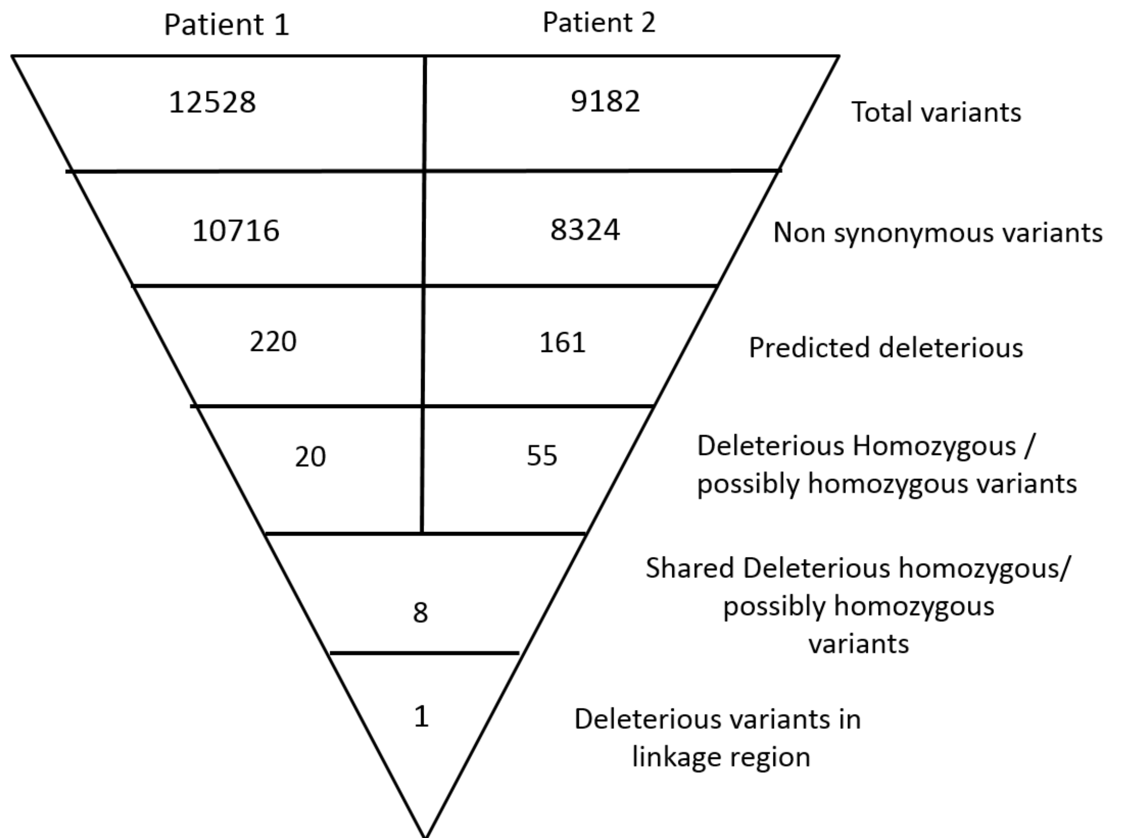
4.1.4.2 Identification of candidate disease causing variants

As we hypothesised that the disease was inherited in an autosomal recessive manner, combinations of two approaches were used to identify the disease-causing variants in both patients: 1) whole exome sequencing, and 2) genome wide linkage analysis by homozygosity mapping to search for homozygous regions shared by both patients.

Whole exome sequencing was performed using both patients' genomic DNA which was extracted from the early passage primary dermal fibroblasts. The genomic DNA was sheared and size-fractionated prior to exonic enrichment

using SureSelect Human All Exon kit (Agilent Technologies) and was subjected to next generation sequencing on an Illumina GA II sequencing platform. The sequences were aligned to the hg19 human genome by NovoAlign. Bioinformatic analysis was performed using an in house pipeline by Dr Helen Griffin and Yaobo Xu (Institute of Genetic Medicine, Newcastle University).

A



B

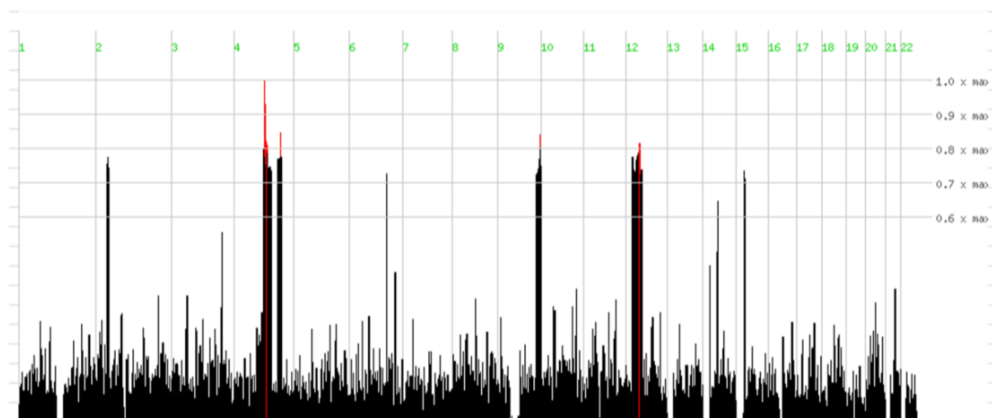


Figure 4.1.4 : Whole exome sequencing and homozygosity mapping of patient 1 and patient 2. A) Whole exome sequencing of both patients showing the numbers of variants as indicated after each filtering step. Possibly homozygous variants are variants that are homozygous in patient 1 but not available in patient 2 or vice versa. This disparity arose because of a change in the exon enrichment kit between sequencing of P1 and P2. B) Homozygosity mapper showing the genome wide homozygosity bar charts; homozygous regions of interest are indicated by red bars. Numbers labelled in green indicate the chromosomes. The homozygosity mapping analysis was performed by Dr Neil Morgan, Birmingham University.

By whole exome sequencing, a total number of 12528 variants was identified in patient 1 and 9182 in patient 2 (Figure 4.1.4(A)). As many variants were identified in both patients, the next step was to identify the variants that cause an alteration to the amino acid sequence. Patient 1 had 10716 and patient 2 had 8324 such non synonymous variants (Figure 4.1.4(A)). These non synonymous variants were further filtered using computational algorithms such as Polyphen-2, Mutation Taster, SIFT and MAPP which predict whether associated amino acid changes are benign or deleterious with respect to protein function. We identified 220 variants in patient 1 and 161 in patient 2 that were predicted to be deleterious. As we hypothesised that the candidate disease causing variant is a homozygous mutation, a further filtering step included eliminating predicted deleterious variants that were heterozygous. We identified 20 homozygous and possibly homozygous variants in patient 1 and 55 homozygous and possibly homozygous variants in patient 2. We then further identified 8 variants that were predicted deleterious homozygous and shared or possibly shared by both patients, (Figure 4.1.4(A)). Of note, owing to a change in exon capture method, coverage was quite uneven between the two siblings. We did not exclude homozygous variants in genes for which analysis was only available in one patient as shared homozygosity could not be ruled out. As we were focusing on rare and novel disease causing variants, it should be noted that variants that are common (more than 0.05% frequency in the population) in dbSNP (http://www.ncbi.nlm.nih.gov/ projects/SNP), 1000 genome project and our in house database were eliminated from further consideration.

In parallel, homozygosity mapping was carried out for this kindred using Affymetrix Genome-Wide Human SNP 5.0 microarrays. The homozygosity

mapping analysis was performed by Dr Neil Morgan, Birmingham University. Therefore, this method was applied to search for the homozygous chromosomal regions that were shared by both patients but not by their unaffected relatives. To analyse and map these homozygous regions, SNP array data were uploaded into a programme called Homozygosity mapper (http://www. Homozygositymapper.org/) (Seelow *et al.*, 2009). For data visualisation, the homozygosity mapper plots genome wide homozygosity as bar charts and highlights in red the homozygous regions that are shared by both patients but are heterozygous or not shared at all by the healthy family members. The homozygosity mapper identified three such regions on chromosomes 4, 9 and 12 (Figure 4.1.4(B) and Table 4.1.3. Microsatellite markers were further used to confirm and refine the borders of these regions. The homozygosity mapping analysis was performed by Dr Neil Morgan, Birmingham University.

Chromosome	From (bp)	To (bp)
4	98135713	100285148
4	101889130	102980397
4	151137804	152489087
4	104744735	105794365
4	106488663	107423164
4	105831847	106482887
9	135738813	136720821
12	45155399	45849255

Table 4.1.3 : Shared homozygous regions that were detected by homozygosity mapper in patient 1 and patient 2. Six regions were homozygous on chromosome 4, one region on chromosome 9 and chromosome 12. The position, from bp to bp, is indicated for each region. bp = base pairs.

When we cross-referenced the results of WES with the output from the homozygosity mapping, 639 variants lay within the three homozygous regions. However, we narrowed down the candidate variants to only 1 predicted deleterious variant which mapped to a region of homozygosity, on chromosome 4. This variant, which was among the 8 deleterious variants identified by WES, is a homozygous missense mutation (c.4115A>G) changing the amino acid histidine to arginine at position 1382 (p.H1382R) in exon 9 of the gene *Ten Eleven Translocation 2* (*TET2*) localized on chromosome 4

Aligned reads covering the candidate variant of patient 1 in *TET2* were visualised with the Integrated Genome Viewer (IGV), a computational tool which aligns the sequence reads to the reference genome, in this case hg19 (Figure 4.1.5). IGV confirmed that the *TET2* variant is a homozygous missense mutation, substituting A>G (bp:g. 106190867) in exon 9.

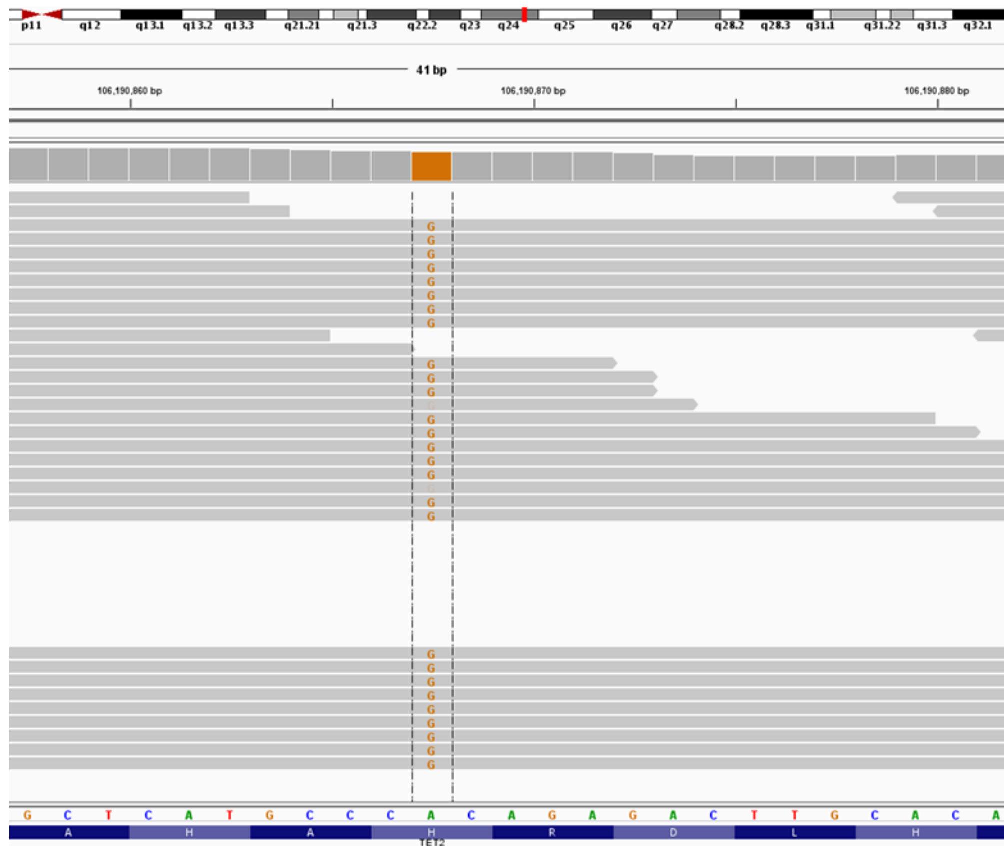


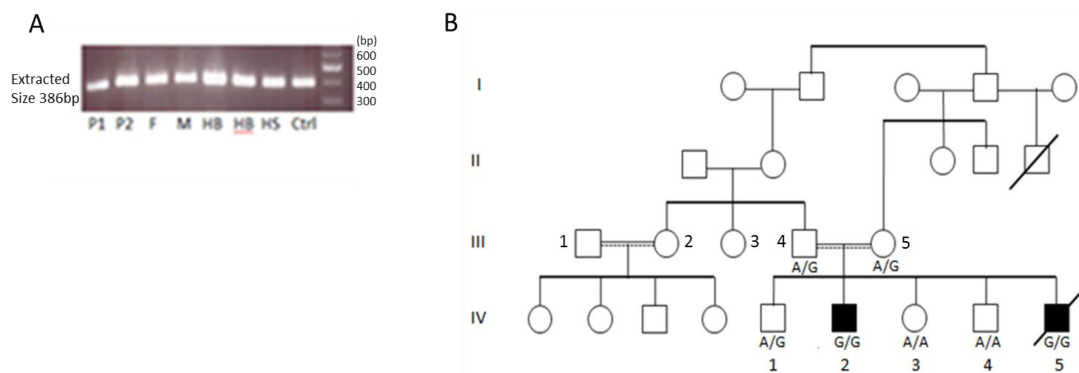
Figure 4.1.5: Homozygous *TET2* mutation in Patient 1. Overlapping sequencing reads of exon 9 of *TET2* show the homozygous A>G base pair substitution on Integrated Genome Viewer (IGV). The nucleotides and amino acids were indicated at the bottom.

For patient 2, whole exome sequencing data for exon 9 of *TET2* were not available because this region was poorly covered among sequence reads.

4.1.4.3 Segregation of TET2 mutation in both patients and family members

To validate this putative disease causing variant, the next step was to perform dideoxy sequencing of PCR amplicons, (1) to confirm that the mutation is present in both patients and was not a false- positive result of whole exome sequencing, and (2) to investigate the segregation of the *TET2* mutation with disease within the family.

Genomic DNA was extracted from patients' fibroblasts and family members' peripheral blood. Exon 9 of *TET2* was amplified by PCR using specific forward and reverse primers (see method chapters), which were designed using the NCBI/Primer blast tools (http://www.ncbi.nlm. gov/tools/primerblasts/). Agarose gel electrophoresis confirmed a PCR product of the correct size, 386bp (Figure 4.1.6(A)), which was submitted for Sanger sequencing by a commercial provider.



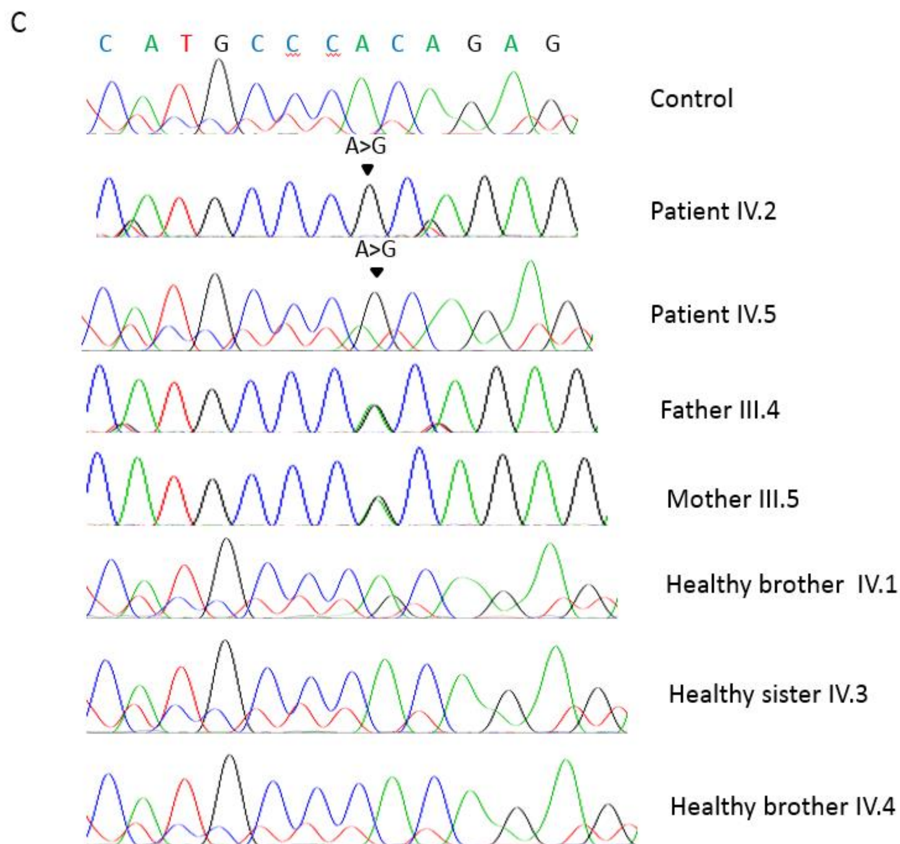


Figure 4.1.6 : PCR gel electrophoresis, pedigree of the family and sequencing of patients and family members. (A) Amplification of *TET2* gene, targeting exon 9 by PCR in patient 1 (P1), patient 2 (P2), father (F), mother (M), healthy brothers (HB), healthy sister (HS) and control and visualised by gel electrophoresis. (B) Pedigree of the family. Patient 1 (IV.2), patient 2 (IV.5). (C) Sequencing of the patients, patient 1 (patient IV.2), patient 2 (patient IV.5) and family members.

The sequencing results confirmed that both patients are homozygous for the *TET2* mutation (Figure 4.1.6(C)). They also confirmed that the mutation segregates as expected with disease; both parents and one healthy sibling are heterozygous for the mutation whereas two other healthy siblings are wild type (Figure 4.1.6(B)). This is compatible with autosomal recessive inheritance.

Chapter 4: A novel autoimmune lymphoproliferative disorder

4.2 : Effect of TET2 p.H1382 variant on its functional activity

4.2.1 Introduction

The genetic data presented in chapter 4.1 suggested TET2 c.4115A>G, p.H1382R as the disease-causing variant in the two siblings with ALPS. However, to confirm this, an exploration of the functional role of TET2 in humans is required. TET2 turns out to be a key enzyme for DNA hydroxymethylation, one of the important mechanisms in epigenetic regulation of gene expression (Nakajima and Kunimoto, 2014).

4.2.1.1 Epigenetics

Epigenetics, in general, is understood as a study of the heritable changes of gene expression or cellular phenotypes that occur without changing the underlying DNA sequence (Goldberg *et al.*, 2007). Epigenetic mechanisms are fundamental in many cellular processes such as differentiation, genomic imprinting, X inactivation, and embryogenesis (Portela and Esteller, 2010). Epigenetic studies have been useful and helpful in understanding how genetically identical cells can generate different cell types and phenotypes as well as maintaining alternative cellular states (Esteller, 2008). In recent years, the mechanistic study of epigenetics has increased tremendously. Disruption of the balance of epigenetic networks can lead to inappropriate expression or silencing of genes which can contribute to the development of human diseases including cancer, syndromes involving chromosomal instability and mental retardation (Egger *et al.*, 2004). Two major mechanisms of epigenetic regulation are 1) histone modification and 2) DNA methylation and demethylation. Significant crosstalk occurs between these two mechanisms in which they interact and stabilize each other in regulating the cellular processes (Egger *et al.*, 2004).

4.2.1.1.1 Histone modification

Nucleosomes are formed by the packaging of DNA around a protein core which consists of a tetramer of histone 3 (H3), histone 4 (H4) and two dimers of histone 2, H2A and H2B (Sakabe *et al.*, 2010; MacRae, 2011). The core

histones are tightly packed in a globular formation and protruding from the nucleosomes are the N-terminal histone tails (Sakabe *et al.*, 2010). The N-terminal histone tails are subject to majority of the post-translational modifications including phosphorylation, methylation, acetylation, sumoylation and GlcNAcylation. These modifications regulate their ability to remodel the chromatin structure for DNA-related processes such as transcription, DNA replication and repair, recombination and chromosomal organization (Sakabe *et al.*, 2010). Specific enzymes such as histone acetylases (HATs) and deacetylases (HDACs) are known to catalyse the histone post-translational modification such as acetylation and deacetylation at the lysine residue of the N terminus of histones tails (Ruthenburg *et al.*, 2007). Unmodified lysine residues are positively charged and tightly bind to the negative charge of the DNA, forming condensed nucleosomes. Conversely, acetylation reduces the binding affinity between histone and DNA, resulting in open-chromatin structure, allowing access of transcription factors (Handy *et al.*, 2011). Many studies report that HATs are mainly linked to transcriptional activation whereas HDACs, in contrast, are involved in transcriptional co-repressor complexes (Portela and Esteller, 2010). Another common post -translational modification is histone methylation where one, two or three methyl groups are added to a different site of the lysine residue catalysed by the enzyme histone methyltransferase (Kouzarides, 2007). Histone methylation has different effects on gene activity, including transcriptional activation, inactivation or silencing of the genomic regions, depending on the specific lysine residue and the degree of methylation. For example, enrichment of histone methylation at H3K4me,H3K36me or H3K79me is associated with transcriptional activation (Kouzarides, 2007), whereas enrichment of histone methylation at a different site, H3K9me, H3K27me or H4K20me is implicated in gene inactivation or silencing (Kouzarides, 2007).

4.2.1.1.2 DNA methylation

Another well-characterised epigenetic modification that has a profound impact on genome stability, gene expression and normal development is DNA methylation (Wu and Zhang, 2014). DNA methylation occurs in a cell type-specific manner that is established during mammalian development and

maintained in adult somatic cells (Chen and Riggs, 2011). It is a process by which a methyl group is added at the 5'position of cytosine to create 5-methylcytosine (5mC). In human somatic cells, 70-80% of 5mC occurs at CpG sites (Chen and Riggs, 2011). In addition, methylation also occurs in non CpG context in cells such as oocytes, pluripotent embryonic stem cells and mature neurons (Lister *et al.*, 2009; Wu and Zhang, 2011). By using a methylated DNA immunoprecipitation (MeDIP) method combined with high throughput sequencing, it was demonstrated that cytosine methylation occurs throughout the genome, including genes, transposons and intergenic DNA(Suzuki and Bird, 2008). The addition of the methyl group is mediated by a family of enzymes known as DNA Methyltransferases (DNMT) including DNMT1, DNMT2 and DNMT3 which are required for the establishment and maintenance of DNA methylation. Aberrant methylation is associated with silencing of tumour suppressor genes as observed in cancers (Esteller, 2005).

4.2.1.1.3 DNA demethylation

DNA methylation is a dynamic process in equilibrium with demethylation. DNA demethylation is essential in regulating gene expression during mammalian development and differentiation. It occurs either actively or passively. Active demethylation refers to the enzymatic process that removes or modifies the methyl group from 5mC. In passive demethylation, the methylation is omitted from newly synthesised DNA strands during replication. For example, it is known to occur in the absence of the functional DNA methylation maintenance machinery in the maternal genome during preimplantation growth (Chen and Riggs, 2011; Wu and Zhang, 2011; Kohli and Zhang, 2013). Ten Eleven Translocation 2, TET2, along with other family members of TET proteins, TET1 and TET3, have been discovered as the enzymes that mediated the active demethylation.

4.2.1.2 TET2 protein structure and enzymatic function in promoting DNA demethylation

TET1 is highly expressed in embryonic stem cells whereas TET2 and TET3 are more ubiquitously expressed (Solary *et al.*, 2013). TET2 expression predominates in a variety of differentiated tissues, particularly in haematopoietic

and neuronal lineages (Solary *et al.*, 2013). The human *TET2* gene is found on chromosome 4q24 and contains 11 exons. Three isoforms are produced from the *TET2* gene by alternative splicing. The enzymatic role of TET2 (as well as TET1 and TET3) was discovered to catalyse oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (Tahiliani *et al.*, 2009). The global 5hmC level was found to be the highest in the brain (Kriaucionis and Heintz, 2009) and embryonic stem cells (Tahiliani *et al.*, 2009). Different 5hmC distribution in the genome among cell types may be due to the differences in the underlying distribution of 5mC and TET proteins (Tsagaratou *et al.*, 2014). By genome wide mapping, several groups identified the presence of 5hmC in gene bodies which correlates with gene expression in mouse and human embryonic stem cells (Ficz *et al.*, 2011; Pastor *et al.*, 2011) and human T cells (Tsagaratou *et al.*, 2014). Their findings implied that TET2 was responsible for depositing 5hmC in the gene bodies in these cells, whereas other groups demonstrated that TET1 regulated 5hmC levels at the promoter and transcription start sites (Huang *et al.*, 2014). Later on, several studies identified that TET2 can also further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) in DNA at low levels (Ito *et al.*, 2011). As one of the enzymes that converts 5mC to 5hmC, 5fC and 5caC, thus TET2 is important in tuning the epigenetic status of the cells.

The catalytic domain of TET2 comprises cysteine rich and double stranded beta helix (DSBH) regions. Structural analysis shows that the DSBH domain consists of antiparallel β strands that face each other and coordinate sequestering of a central Fe(II) ion and N-oxalylglycine (NOG, a 2-OG analogue) molecule (Figure 4.2.1.(B)). If oxidation occurs, NOG is converted to 2-OG under physiological conditions (Hu *et al.*, 2013). The Fe(II) is coordinated by three highly conserved amino acid residues, also known as the signature HxD motif (Ko *et al.*, 2010), H1382, D1384 and H1881 whereas NOG is coordinated by four highly conserved residues, R1261, H1416, R1896 and S1891 of TET2 (Hu *et al.*, 2013). Interestingly, the highly conserved residue H1382 is mutated in our two patients with ALPS.

All TET proteins possess a catalytic domain, however only TET1 appears to contain the CXXC domain, which has been predicted maybe responsible for the

DNA binding activity (Pastor *et al.*, 2013). It is suggested that TET2 had the CXXC domain but due to the chromosomal inversion during the vertebrate evolution split the TET2 gene into distinct segments encoding the catalytic domain of TET2 and CXXC domain which is now separately encoded by a neighbouring gene named CXXC4 (also known as IDAX4) (Ko *et al.*, 2013; Pastor *et al.*, 2013; Solary *et al.*, 2013) Figure 4.2.1(A)) .

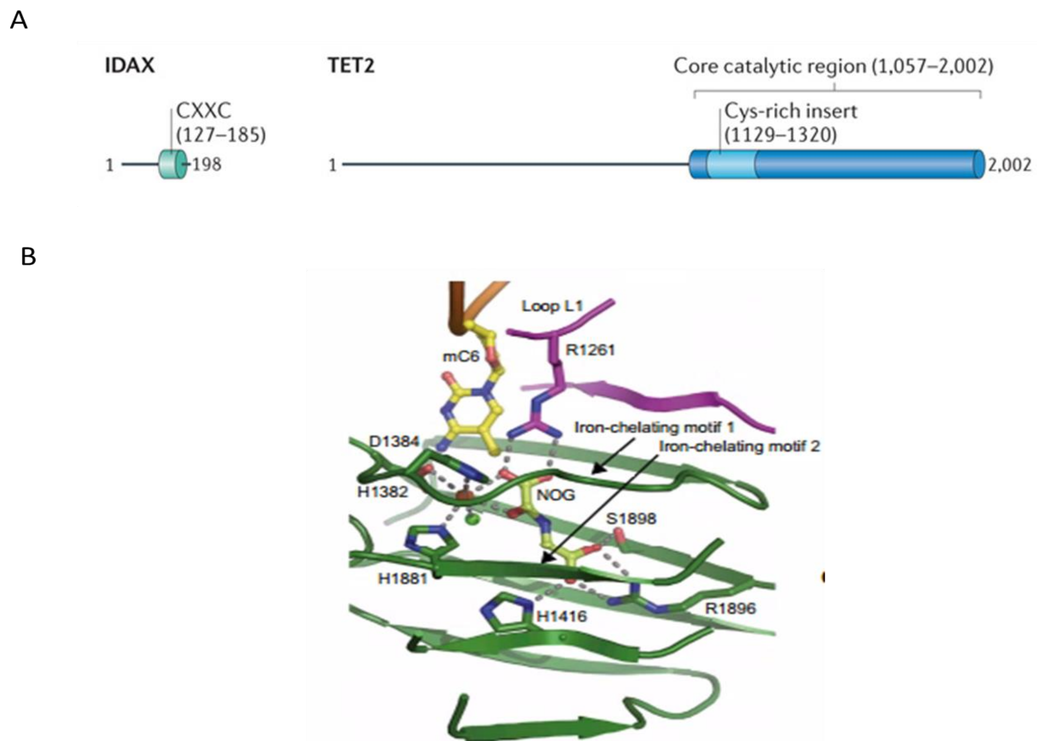


Figure 4.2.1 : Structure of TET2 protein and DSBH Core. A) The TET2 protein consists of the core catalytic region including the cysteine rich insert and DSBH domain. A chromosomal inversion detached the TET2 catalytic domain from its CXXC domain, which became a separate gene, IDAX. The number of amino acid is indicated. B) Ribbon presentation of the DSBH core. The highly conserved residues that coordinates both Fe(II) and NOG are indicated as dashed line. Figure (A) is reproduced from Pastor et al. (Pastor *et al.*, 2013) and Figure (B) is reproduced from Hu et al.(Hu *et al.*, 2013).

4.2.1.3 Active demethylation mechanism

It was suggested that TET proteins including TET2 mediate active DNA demethylation through DNA repair of which three mechanisms were proposed (Pastor *et al.*, 2013). In the first mechanism, TET proteins oxidize 5mC to 5hmC and then further oxidize 5hmC to generate 5fC and 5caC. 5fC and 5caC will then be excised by thymine DNA glycosylase (TDG) (He *et al.*, 2011; Maiti and

Drohat, 2011) via base excision repair, and their replacement with cytosine results in demethylation (Figure 4.2.2). An alternative mechanism was proposed where 5hmC is deaminated to 5-hydroxyuracil (5hmU) by AID (activation induced cytidine deaminase) and APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide) family enzymes (Guo *et al.*, 2011a) then targeted by SMUG1 (single-strand selective monofunctional uracil DNA glycosylase 1) and TDG glycosylases via base excision repair and ultimately replaced by cytosine (Figure 4.2.2) (Pastor *et al.*, 2013) Another proposed mechanism is the decarboxylation of 5caC to cytosine, however until now the factors that catalyse this decarboxylation reaction are still unknown (Pastor *et al.*, 2013) (Figure 4.2.2). Although some studies provided evidence to support a role for many of these DNA modifying pathways, how effectively these pathways occur in vivo in mammalian cells is still unclear and controversial. Part of this controversy was due to the fact that the active demethylation that uses 5hmC as an intermediate involves several different enzymes and pathways, and it is unclear whether all of them contribute to gene regulation in different stages of development or different cell types (Pastor *et al.*, 2013). However, newly developed technology enabling the mapping of oxidised methylcytosine at a single base resolution in small numbers of differentiating cells (Pastor *et al.*, 2013) could provide better understanding on active demethylation mechanisms.

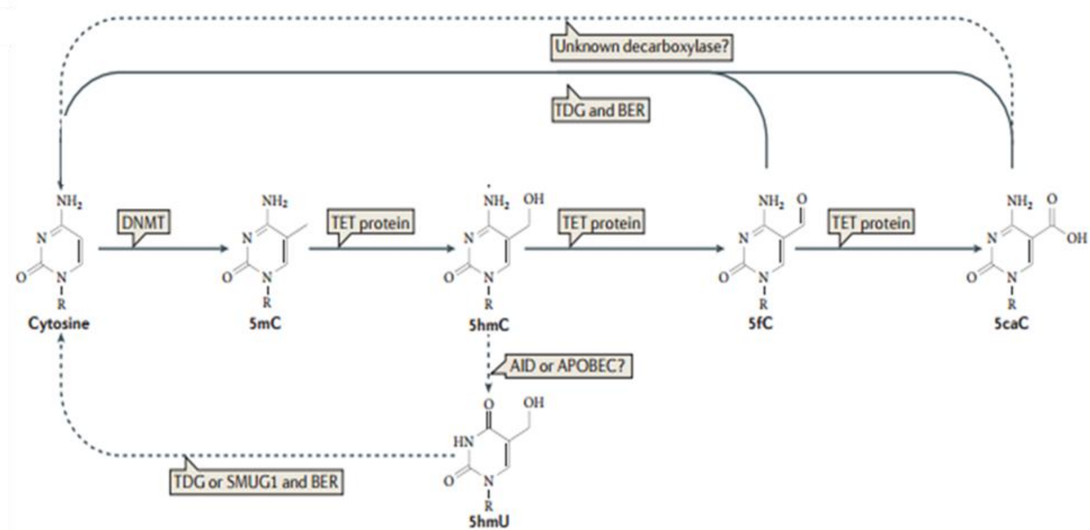


Figure 4.2.2 : Mechanisms of DNA demethylation involving TET proteins: DNMT add a methyl group to the 5’ position of cytosine and produce 5mC. TET proteins then oxidize 5mC to 5hmC, 5fC and 5caC. 5fC and 5caC will be

removed by TDG via base excision repair and replaced by cytosine. 5caC can also be removed by decarboxylation. 5hmC is deaminated by AID/APOBEC family of enzymes, to 5hmU, which will then be converted to cytosine by SMUG1 or TDG via the base excision repair pathway (Pastor *et al.*, 2013).

4.2.1.4 TET2 mutations in haematological malignancies

As mentioned earlier, TET2 is highly expressed in haematopoietic cells including lymphocytes, monocytes, NK cells and particularly granulocytes (Langemeijer *et al.*, 2009). The high expression of TET2 in haematopoietic cells reflects its role in regulating haematopoiesis, including stem cell self-renewal, lineage commitment and terminal differentiation of specific lineages (Solary *et al.*, 2013). It is well documented that somatic TET2 mutations are frequent in haematological malignancies (Ko *et al.*, 2015). This relationship was first observed in Myelodysplastic syndrome (MDS) and Myeloproliferative neoplasms (MPN) with chromosome 4q24 abnormalities, which includes the TET2 locus (Delhommeau *et al.*, 2009; Langemeijer *et al.*, 2009). Both MDS and MPN are clonal disorders that arise in haematopoietic stem cells and the main features are ineffective haematopoiesis that affects one or several lineages and an increased risk of transformation to Acute Myeloid Leukemia (AML) (Delhommeau *et al.*, 2009). Following these findings, several other studies identified somatic TET2 mutations in other MDS and MPN patients as well as other myeloid malignancies such as chronic myelomonocytic leukemia (CMML), and AML (Abdel-Wahab *et al.*, 2009; Jankowska *et al.*, 2009; Kosmider *et al.*, 2009; Mullighan, 2009; Tefferi *et al.*, 2009; Weissmann *et al.*, 2012). All these studies have established TET2 as one of the common mutated genes in myeloid malignancies. TET2 mutations were distributed between exon 3 and exon 11 (Weissmann *et al.*, 2012). Studies done by Weissmann et al. showed that various types of somatic TET2 mutations including missense, nonsense, deletion, insertion and splice site mutations were observed in acute myeloid cancer patients (Figure 4.2.3). Interestingly, all the missense mutations occurred within either of two conserved domains of TET2, spanning from amino acid 1104 to 1478 (first conserved domain, CD1) and amino acids 1845-2002 (second conserved domain, CD2) (Weissmann *et al.*, 2012).

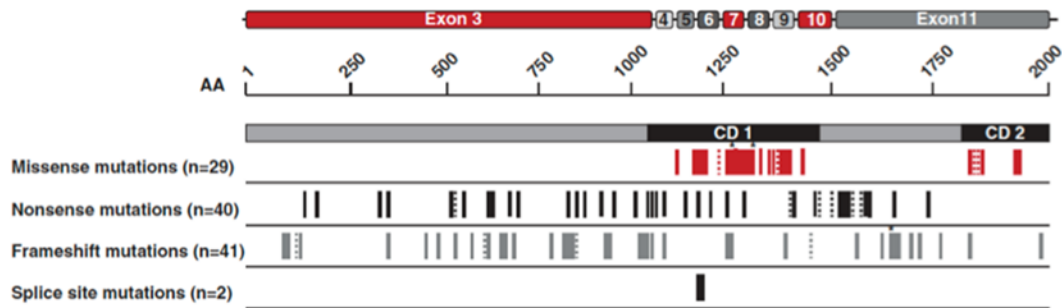


Figure 4.2.3: The localization and types of TET2 mutations that had been discovered in acute myeloid cancers patients by Weissmann et al.(Weissmann *et al.*, 2012). The complete region of TET2, the conserved domains (CD1 and CD2) and the amino acid positions are indicated. Figure reproduced from Weissmann et al.(Weissmann *et al.*, 2012).

Recently, an observation in Tet2-deficient mice led to the identification of TET2 mutations in human lymphoid malignancies. A study by Quivoron et al. in Tet2-deficient mice demonstrated altered T and B lineage development, prompting a further investigation in patients with lymphoma. Sequencing of TET2 confirmed the presence of somatic mutations in B and T lymphomas (Quivoron *et al.*, 2011). Subsequently, more TET2 mutations were identified in other types of lymphoma such as angioimmunoblastic T cell lymphoma (Odejide *et al.*, 2014), peripheral T cell lymphoma (Lemonnier *et al.*, 2012), and more recently mantle cell lymphoma (Meissner *et al.*, 2013) and diffuse large B cell lymphoma (Asmar *et al.*, 2013).

A study conducted by Ko et al. demonstrated that the genomic level of 5hmC was significantly reduced in patients with myeloid malignancies who had TET2 mutations, consistent with loss of TET2 enzymatic activity in these patients (Ko *et al.*, 2010). The same group also performed a study in which they transfected leukaemia-associated mutations, R1896S and R18996S, as well as substitutions in the HxD motif such as H1802Y in mouse (similar to human H1382 residue), which coordinates the Fe(II) in the highly conserved CD domain, into HEK293 T cells and the results also confirmed that these mutations result in impaired catalytic activity (Ko *et al.*, 2010). It was expected that the TET2 mutations would lead to an accumulation of 5mC in the genome. However, this idea still remains controversial as two studies demonstrated two different results. In agreement, the first study done by Figueroa et al. showed

global hypermethylation at the CpG methylated sites in patients with AML (Figueroa *et al.*, 2010). However, in contrast, Ko et al. demonstrated that TET2 mutations were associated with global hypomethylation at differentially-methylated site in patients with various myeloid malignancies (Ko *et al.*, 2010) The possible differences in these results might be due to cancer subtypes examined and the tools used for 5mC quantification and statistical analysis (Ko and Rao, 2011).

Interestingly, other common genetic mutations in haematological malignancies were discovered in the isocitrate dehydrogenase 1 (IDH1) and IDH2 genes (Figueroa *et al.*, 2010). IDH1 and IDH2 form a key enzymes in the tricarboxylic acid (TCA) cycle which catalyses the conversion of isocitrate to alpha-ketoglutarate, an essential cofactor for TET2 (Nakajima and Kunimoto, 2014). The absence of IDH1 and IDH2 results in the formation of 2-hydroxyglutarate (2-HG), which inhibits α-KG-dependant enzymes such as TET2 by competing with α-KG (Figueroa *et al.*, 2010; Nakajima and Kunimoto, 2014). Therefore, in AML with IDH1/IDH2 mutation, the increase of 2-HG results in loss of TET2 function (Figueroa *et al.*, 2010; Ward *et al.*, 2010). Collectively, all these findings emphasise the critical role of TET2 in preventing aberrant haematopoiesis and leukemogenesis.

4.2.1.5 TET2 mutations in non-haematological malignancies

TET2 loss of function has also been discovered to contribute to the development of a small number of solid tumours (Solary *et al.*, 2013). It was recently proposed that TET2 plays a role in defining the subset of metastatic tumours in castration-resistant prostate cancers (Nickerson *et al.*, 2013). Interestingly, it was demonstrated that the levels of 5hmC were dramatically reduced in human breast, liver, lung, pancreatic and prostate cancers compared with the matched surrounding normal tissue and the decreased 5hmC is associated with the downregulation of TET2 and other TET proteins (Yang *et al.*, 2013). The decrease of 5hmC in these solid tumours indicates that 5hmC could be an epigenetic hallmark of tumour development. In agreement, loss of 5hmC has also been observed in melanoma (Lian *et al.*, 2012). Reintroduction of TET2 or IDH2 in melanoma cells increased the 5hmC levels and suppressed

melanoma growth as well as increasing the survival in the animal model, suggesting the function of 5hmC in melanoma development and linking the TET enzymatic pathway to 5hmC–mediated suppression of melanoma (Lian *et al.*, 2012).

4.2.1.6 Disruption of Tet2 in mouse models

In order to understand the role of TET2 in regulating haematopoietic development and cellular transformation, in vivo studies using TET2-disrupted mice were generated. Li et al. generated a gene-trap mouse strain in which a β-galactosidase-GFP cassette was inserted into exon 3 of the TET2 locus, disrupting the endogenous start codon and inducing premature termination of transcription (Ko and Rao, 2011; Li *et al.*, 2011). The $TET2^{-/-}$ mice were viable, fertile, developed grossly normally and did not show any lethality. However, as they aged, they developed diverse myeloid malignancies: 1) 57% were found to have MDS with erythroid predominance and depletion of haematopoietic stem/progenitor, LSK (Lin^{-}c-Kit+Sca-1^{+}) ; 2) 20% had myeloproliferative disorder (MPD) or CMML-like phenotypes with increased LSK and granulocyte/macrophage precursor (GMP) populations, as assessed by increased monocytosis, leucocytosis, bone marrow hypercellularity and extramedullary haematopoiesis with evidence of hepatomegaly and splenomegaly (Ko and Rao, 2011; Li *et al.*, 2011). TET2 deficiency also conferred a significant advantage on these cells in repopulating heterogenous hematopoietic lineages in a cell-autonomous manner. The TET2-/- LSK cells were highly proliferative and they displayed an enhanced capacity for clonal expansion. In addition, the $TET2^{-/-}$ mice demonstrated that the level of genomic 5hmC was diminished in bone marrow cells, in agreement that loss of TET2 function compromised its enzymatic activity as shown previously in patients with myeloid cancers and in vitro studies by Ko et al. (Ko *et al.*, 2010).

Several other groups generated alternative Tet2 knock out mouse models (Ko *et al.*, 2011; Moran-Crusio *et al.*, 2011a; Quivoron *et al.*, 2011). Their Tet2-/- mouse phenotypes were consistent with the phenotypes described by Li et al. (Li *et al.*, 2011). Moran Crusio et al. (Moran-Crusio *et al.*, 2011b) generated conditional Tet2-deficient mice targeting exon 3 containing the start codons in

the Tet2 locus, whereas Ko et al. (Ko *et al.*, 2011) generated conditional Tet2-disrupted mice targeting exons 8-11. Another group, Quivoron et al. (Quivoron *et al.*, 2011), generated two different strains of Tet2-deficient mice; in the first strain, a β-galactosidase –neomycin was inserted into exon 9 and in the other strain, exon 11 was conditionally deleted. Of note, exons 7-11 in the *Tet2* gene encode the double stranded β-helix domain, meaning that their deletion disrupts the core region of the catalytic domain. All these Tet2-/- mice showed phenotypes consistent with that described by Li et al. They had normal growth and organ development in their early life. Later on, they developed CMML-like disease. The Tet2-/- LSK cells demonstrated enhanced self-renewal and replating capacity in vitro. The deletion of Tet2 resulted in an increase of HSCs in a cell intrinsic manner and increased HSCs' self-renewal capacity. In addition, Quivoron et al. demonstrated that lymphoid lineage development was also affected with an expansion of an aberrant ($CD19^{+}B220^{low}$) lymphoid population, decrease in B cell lineages in the bone marrow and an increase in CD4-CD8-double negative T cells (DNT) progenitors in thymus (Quivoron *et al.*, 2011; Solary *et al.*, 2013). The Tet2-deficient mice phenotypes are summarised in Table 4.2.1.

	Quivoron et al.	**Moran-Crusio et al.**	**Ko et al.**	**Li et al.**
Models	Exon 9 (gene trap) Exons 10-11 (conditional deletion)	Exon 3 (conditional deletion)	Exons 8-10 (conditional deletion)	Tet2 disruption 6bp upstream of the transcription start
Development	Normal development and growth			
5-hmC levels	Decreased			
$Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}$ (LSK)	Increased and increased serial replating			
Bone marrow progenitors	Increased CMP, MEP	Increased CMP	Increased CMP	Increased GMP

Phenotypes	Hepatomegaly, splenomegaly, leucocytosis, anaemia, thrombocytopenia	Splenomegaly, leukocytosis	Splenomegaly, monocytosis	Hepatomegaly, splenomegaly, leucocytosis, Monocytosis
Lymphoid lineages	Expansion of an aberrant (CD19+B220 low) lymphoid population, decrease in cell lineages in the bone marrow and an increase in CD4-CD8- double negative T cells (DNT) progenitors in thymus	unchanged	unchanged	Unchanged
Diseases	CMML-like	CMML-like	CMML-like	CMML-like, MDS , MPD

Table 4.2.1 : Animal models and phenotypes of TET2-deficient mice. CMML - chronic myelomonocytic leukemia, CMP- Common Myeloid progenitors, MEP- Megakaryocyte-erythroid progenitors

These Tet2-deficient mouse models are consistent with a pathogenic role for TET2 loss of function mutations in myeloid and lymphoid malignancies. Indeed, TET2 function is required to restrict the function and expansion of HSCs by regulating the gene transcription in HSCs (Solary *et al.*, 2013). Aberrant methylation at CpG islands in specific gene promoters is often observed to lead to silencing of the tumour suppresser genes and development of cancer (Herman and Baylin, 2003). As TET2 function is to oxidise 5-methylcytosine, it is appealing to assume that the loss of TET2 contributes to gene-specific hypermethylation and results in silencing or activation of genes involved in HSCs development. However, based on the conflicting results of previous studies Ko et al. (Ko *et al.*, 2010) and Figueroa et al. (Figueroa *et al.*, 2010), mentioned earlier in Section 4.2.1.1.6, this assumption requires to be tested. Thus, more in vivo and in vitro studies of the epigenetic landscape in TET2

mutated patients with haematological malignancies are needed in order to advance our understanding on how loss of TET2 activity leads to disease development.

4.2.1.7 Role of TET2 in embryonic stem cells and induced pluripotent stem cells

TET1 and TET2 play an important role in maintaining the pluripotency, self-renewal capacity and lineage specification of embryonic stem cells. Embryonic stem cells are derived from the inner cell mass of the developing blastocyst and they are pluripotent and self-renewing (Koh *et al.*, 2011). Both TET1 and TET2 are upregulated in mouse embryonic stem cells and downregulated after the cells differentiate; similarly the 5hmC level is increased in embryonic stem cells and downregulated during development. The binding of TET2 to the pluripotency factor Oct4 links TET2 to the pluripotency machinery (Koh *et al.*, 2011). In addition, it has been shown that TET2 interacts with pluripotency loci such as Esrrb and Nanog, where it creates 5hmC, suggesting the involvement of both TET2 and 5hmC in the pluripotency mechanism in mouse embryonic stem cells (Koh *et al.*, 2011). Similar findings have also been observed in studies of reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) using pluripotency factors. Doege et al. showed that TET2 is involved in somatic cell reprogramming by demonstrating that both Parp1 and TET2 were recruited to the loci of Nanog and Esrrb in iPSCs (Doege *et al.*, 2012). In addition, Mikkelsen et al. showed that TET2 was upregulated at early reprogramming stages and in iPSCs formed from somatic cells that were transformed with pluripotency factors (Mikkelsen *et al.*, 2008). Moreover, it was also reported that upon downregulation of TET2 in iPSCs, 5hmC was reduced while methylation changes varied at the pluripotency loci. This suggests that TET2 was needed for inducing 5hmC for pluripotency as well as showing that 5hmC potentially serves as a distinct epigenetic mark from 5mC (Doege *et al.*, 2012). Although many studies demonstrate the involvement of TET2 in promoting pluripotency in mouse embryonic stem cells and iPSCS, nevertheless, how TET2 regulates the pluripotency in human embryonic stem cells remains confusing and controversial (Solary *et al.*, 2013) and requires further exploration.

4.2.1.8 Role of TET2 in histone modification

Besides DNA demethylation, it has been shown that TET2 is associated with histone modifications. TET2 recruits O-GlcNAc transferase (OGT) to chromatin, promoting histone GlcNAcylation which influences gene transcription (Chen *et al.*, 2013). OGT is an enzyme that catalyses the addition of O-GlcNAc to Ser and Thr residues of proteins (Sakabe *et al.*, 2010). It was also demonstrated that the TET-OGT complex occupies the CpG islands at transcriptional start sites where high levels of O-GlcNAc modified histones but low levels of 5mC and 5hmC exist, further supporting that TETs are not only involved in DNA hydroxylation but also in mediating the recruitment of OGT to genomic loci to modify histones (Deplus *et al.*, 2013). Furthermore, TET2 also recruits OGT together with HCF1 (Host Cell Factor 1), a component that is required for the recruitment of SET1/COMPASS and H3K4 methylation (Deplus *et al.*, 2013). However, the TET2-OGT interaction is independent of TET2's enzymatic activity (Chen *et al.*, 2013) and was preserved in the enzymatically dead HxD mutant. The involvement of TET2 with histone modification via OGT suggests another role of TET2 in regulating gene transcription.

4.2.1.9 TET2 regulates cytokine expression in T cells

Most previous work on TET2 addresses its function in regulating haematopoiesis and cellular transformation specifically in myeloid cells. So far, few studies address TET2's functions in lymphocytes and the immune system. However, very recently, Ichiyama et al. demonstrated the important role of TET2 in T cell function, particularly in cytokine expression (Ichiyama *et al.*, 2015). The recruitment of TET2 promotes DNA demethylation of regulatory elements of cytokine genes and is dependent on the expression of key transcriptions factors such as Tbet, RorγT and STAT3 (Ichiyama *et al.*, 2015; Zhong and Zhu, 2015). The interaction between TET2 and these transcription factors influences the expression of cytokine genes which are important for T cell differentiation, such as *ifng* for Th1, and *IL17* and *IL10* for Th17 cells.

In addition, this group demonstrated that TET2 regulates autoimmune responses by controlling the production of IL-10 in vivo. They found that the development of Type 1 regulatory T (Tr1) cells, which express IFNγ and IL-10,

were inhibited in the absence of TET2. Furthermore, they also demonstrated that in TET2-deficient mice, IL-10 production was reduced which leads to exacerbation of symptoms of autoimmune encephalomyelitis (EAE), a prototype for T cell-mediated automimune disease. From this finding, it is believed that TET2 plays an important role in regulating IL-10 expression and in regulating the T cell-mediated autoimmune response. This study by Ichiyama et al. suggests another role of TET2 in the immune system.

4.2.2 Summary

Based on previous literature, TET2 plays an important role in DNA demethylation as well as histone modification. As mentioned earlier, TET2 has been identified as the disease-causing gene in the two patients described in Chapter 4.1, who have a homozygous missense mutation, c.4115A>G, p.H1382R, in *TET2*. The residue H1382 is within the catalytic domain and is a highly conserved residue that coordinates Fe(II). For this reason, its mutation is predicted to disrupt TET2 enzymatic activity which we hypothesise is responsible for the disease phenotype. In order to test this hypothesis, I set out to investigate the enzymatic function of TET2 using patient material and a recombinant system.

4.2.3 Hypothesis

A homozygous missense mutation, c.4115A>G, p.H1382R, in *TET2* leads to impairment of TET2 enzymatic activity and thereby perturbs epigenetic regulation.

4.2.4 Aims

To investigate the effect of the TET2 missense mutation, c.4115A>G, p.H1382R, on

- TET2 protein expression
- Hydroxymethylating activity towards DNA (patient cells and recombinant system)

4.2.5 Results

4.2.5.1 TET2 protein is expressed in both patients fibroblasts

The first experiment that I did was to investigate the effect of TET2 missense mutation on the protein expression in the patients. As TET2 is ubiquitously expressed in various cells and tissues (Solary *et al.*, 2013) and we had access to patients fibroblasts, therefore these cells were used to look at the protein expression. Both patients and control fibroblasts were lysed with RIPA (radioimmunoprecipitation assay) buffer. RIPA buffer was used in lysing the cells because TET2 is a nuclear protein and this buffer contains the ionic detergent SDS and sodium deoxycholate as active constituents which are useful for disruption of nuclear membranes.

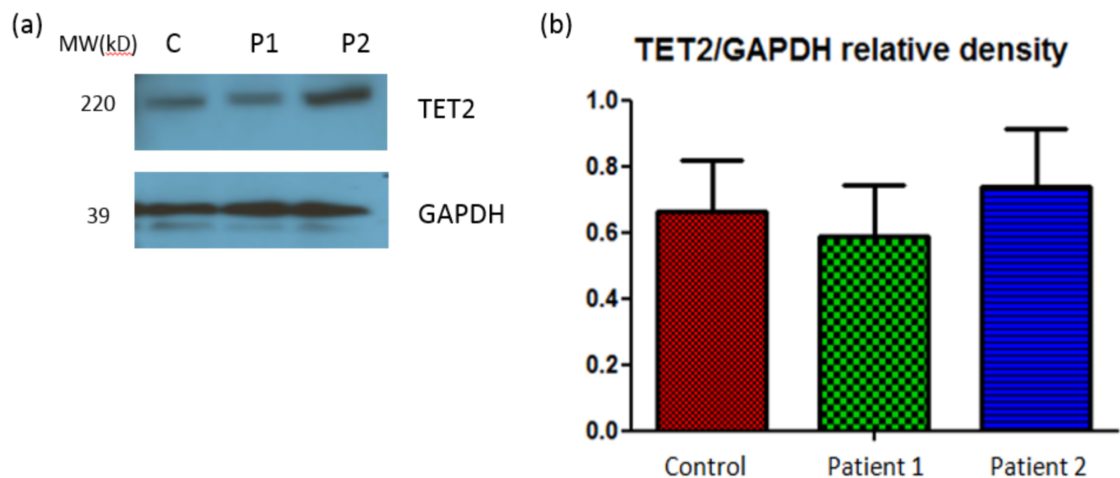


Figure 4.2.4 : TET2 protein expression in patients and control fibroblasts. Patient (P1, P2) and control (C) fibroblasts were analysed by Western Blotting using a mouse monoclonal antibody directed against TET2 and an anti-GAPDH antibody as loading control. The bands were quantitated from the Western Blot using densitometry and the signals were standardized to GAPDH. The result is representative of three separate experiments.

By Western Blotting it was shown that TET2 protein was expressed in both patients' fibroblasts at a level similar to control fibroblasts (Figure 4.2.4). This finding is consistent with a study done by Ko et al. showing that the homologous HxD variant, H1302Y, was stable when expressed in a recombinant system (Ko *et al.*, 2010).

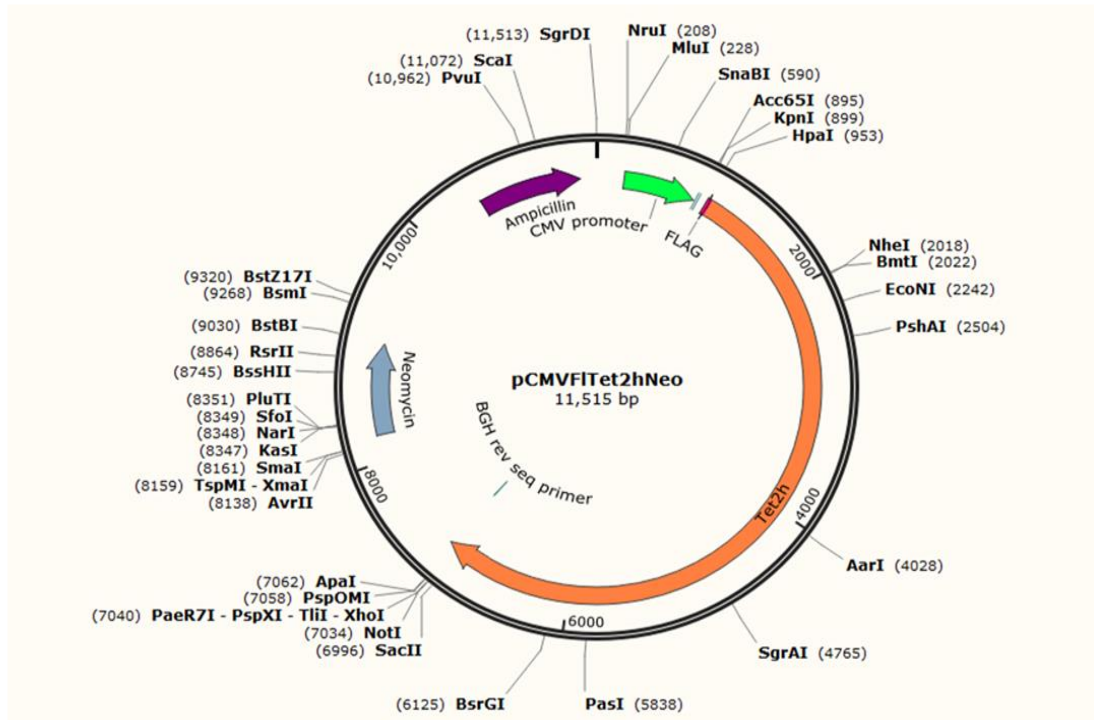
4.2.5.2 TET2 p.H1382R mutation impairs the enzymatic activity of TET2

Ko et al. found a significant decrease of 5hmC levels in cells that were transfected with mutant TET2 (H1302Y) compared to wild type, demonstrating that the catalytic activity of TET2 was impaired (Ko *et al.*, 2010). Interestingly, the mutant TET2 in the study, H1302Y, in mouse, is similar to the mutation in our patients, H1382R. Therefore, the next experiment to perform is to investigate the effect of the H1382R mutation on the enzymatic activity. It is predicted that the TET2 enzymatic activity will be impaired as H1382R is in a highly conserved CD domain, disrupting the Fe(II) binding motif.

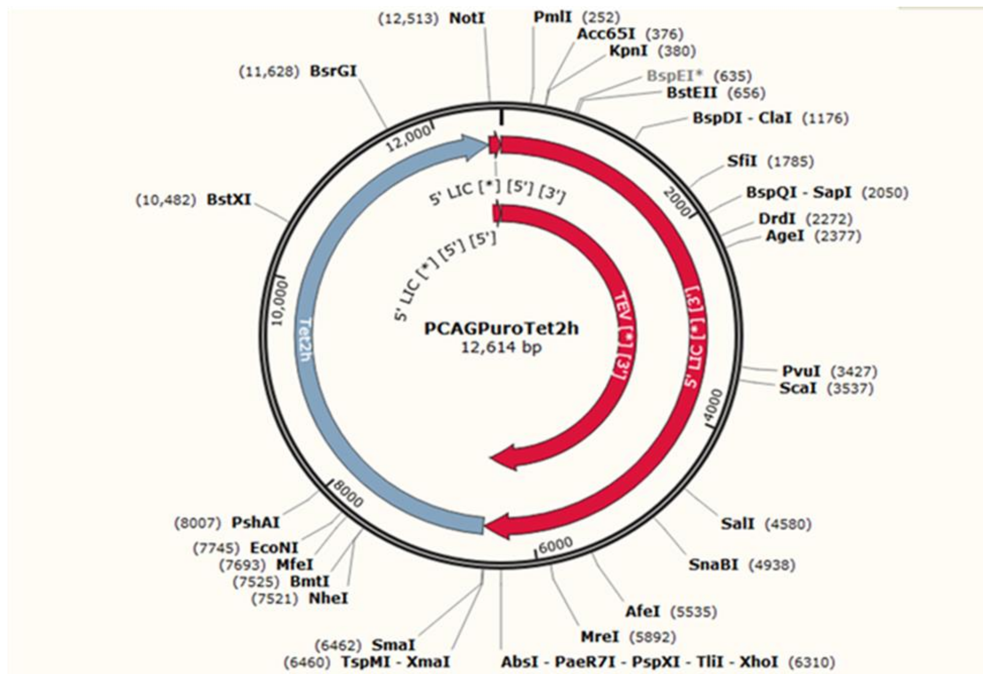
To investigate the effect of TET2 p.H1382R mutation on the enzymatic function, my first approach was to use a recombinant system similar to Ko et al. (Ko *et al.*, 2010). HEK293T cells were transfected with an expression plasmid encoding wild type or mutant TET2. HEK293T cells were chosen because firstly, they had been used to assess the enzymatic activity of TET2 in the study by Ko et al. (Ko *et al.*, 2010), and secondly, they are known to be easily transfected.

Three TET2 expression plasmids were provided by our collaborator, Dr Skirmantas Kraucionis and Melania Zauri, Oxford University, UK (Figure 4.2.5).

A



B



C

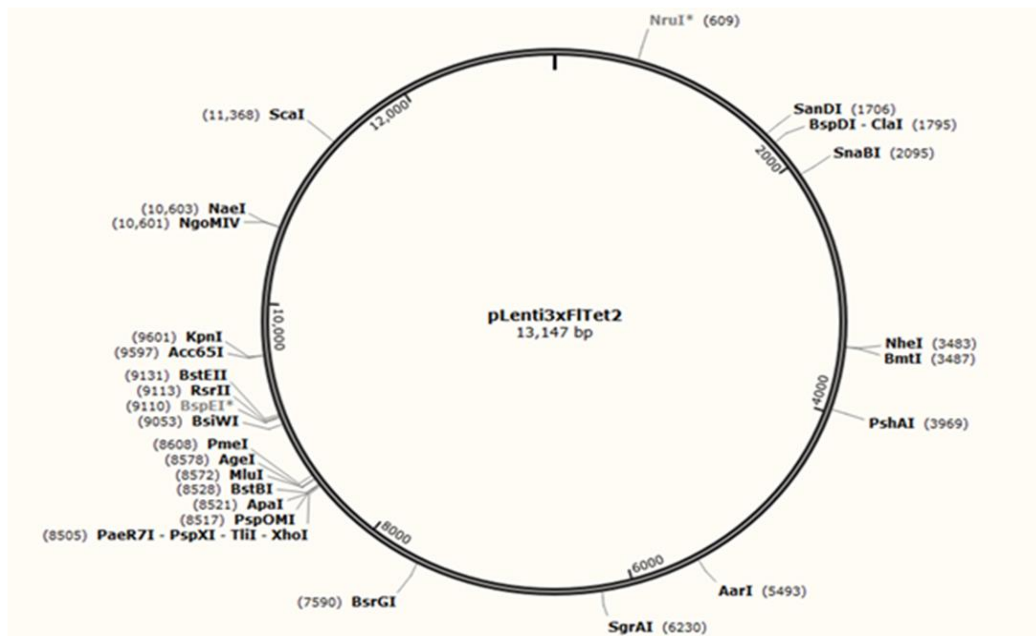


Figure 4.2.5 : Three TET2 plasmids for the recombinant system. A) pCMV TET2 plasmid; B) pCAG TET2 plasmid; C) pLenti TET2 plasmid. All plasmids are flag-tagged and carry an ampicillin-resistance gene. The plasmids were generously given by Dr Skirmantas Kraucionis and Melania Zauri, Oxford University.

To generate the mutant TET2 plasmid, a Site-Directed Mutagenesis QuickChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, USA) was used where histidine (H) 1382 was substituted with arginine (R) by introducing the A>G mutation into the specific plasmid DNA sequence at position c.4115 in TET2 wild type plasmids using a PCR-based technique. This technique was unsuccessful for pCAG TET2 but successful for pCMV TET2. Unfortunately however, no TET2 expression was achieved with the wt and mutant pCMV TET2 plasmids. A subcloning technique was thus attempted, where wild type TET2 in the pCAG plasmid was replaced with mutant TET2 from the pCMV plasmid. Unfortunately this method also failed. We then received the pLent TET2 plasmid and used it for successful site directed mutagenesis. The A>G mutation in pLent mutant TET2 was confirmed by Sanger sequencing (Geneius Laboratories, UK) (Figure 4.2.6(A)).

To investigate the effect of the TET2 p.H1382R mutation on enzymatic function, HEK293 T cells were transfected with either wild type TET2 or mutant TET2

pLent plasmid using Fugene HD transfection reagent. After 48 hours, the transfected cells were fixed with 4 % paraformaldehyde. Subsequently, DNA was denatured with acid hydrochloric (HCL) and neutralised with Tris-HCl buffer. The DNA denaturing step is required for the 5hmC epitopes to be exposed. Then the cells were co-stained with anti-Flag and anti-5hmc antibodies and analysed by Immunofluorescence.

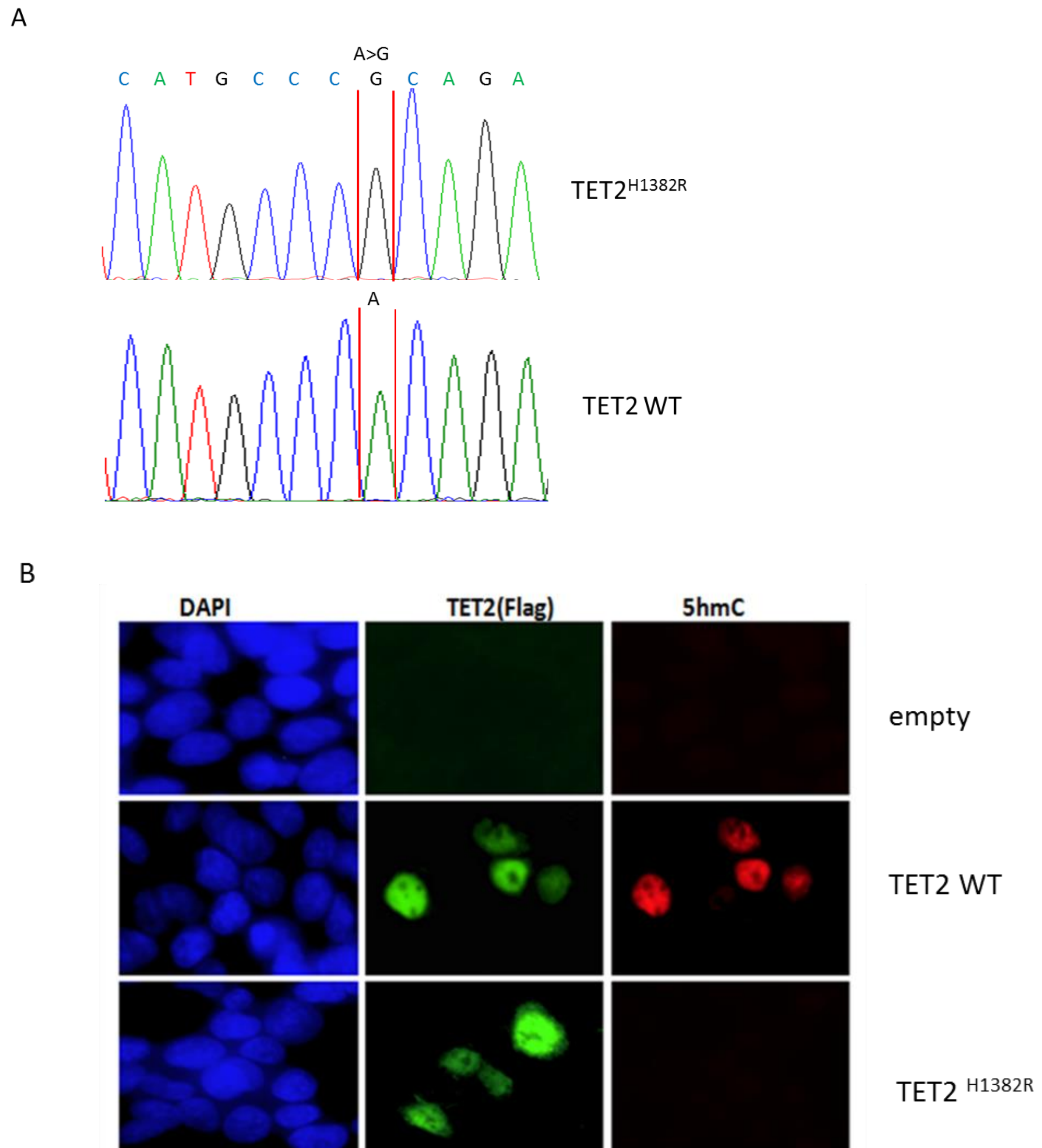


Figure 4.2.6 : The catalytic activity of TET2 is compromised by TET2 p.H1382R mutation. A) Sequences of successful mutant TET2 that was generated using the pLent TET2 plasmid. The A>G mutation was successfully introduced into the DNA and confirmed by Sanger sequencing. B) The catalytic activity is assessed by Immunofluorescence. HEK293 T cells transfected with empty vector (empty), Flag-tagged wild type TET2 (TET2 WT) and mutant TET2

(TET2 H1382R) pLent and were stained with antibodies against 5hmC (red) and the Flag epitope (green). DAPI (blue) indicates nuclear staining. Result is representative of three independent experiments.

Immunofluorescence results showed that in HEK293T cells expressing wild type TET2, 5hmC staining was significantly increased whereas in cells expressing TET2 mutant H1382R, the 5hmC staining was absent (Figure 4.2.6(B)). This result indicates the role of this residue, H1382, in the catalytic activity and confirms that mutation of this residue results in impaired enzymatic activity.

As mentioned earlier, TET2 and conversion of 5mC to 5hmC were shown to play an important role in pluripotency and differentiation of embryonic stem cells and iPSCs. To investigate the effect of our patients' TET2 p.H1382R mutation on the pluripotency and differentiation mechanisms, a pilot study was performed by my colleague, Dr Katarzyna Tilgner at Institute of Genetic Medicine, Newcastle University, using iPSCs that were generated from patients' fibroblasts. Briefly, to generate iPSCs, patient fibroblasts were transduced with Sendai virus vectors that carry the pluripotency factors Oct4, Sox2, Klf4 and c-Myc for 7 days and later transferred onto mouse embryonic fibroblasts (MEFs). After 14 - 21 days the transduced cells produced well-formed colonies. Several of these colonies were picked and transferred onto new feeder plates for expansion. The cells were passaged several times to remove the Sendai virus and by this time the cells were established undifferentiated iPSCs that express all the pluripotency markers. These undifferentiated iPSCs could further be differentiated into specific cell types as required. For the pilot study, our hypothesis was that TET2 is important in maintaining the 5hmC level in undifferentiated iPSCs and in early differentiation stages. Therefore, to confirm our hypothesis, Dr Katarzyna Tilgner assessed the 5hmC level in the established undifferentiated, early differentiated, late differentiated iPSCs and fully differentiated somatic cells, fibroblasts of both patients and controls as well as human embryonic stem cells as positive control by Immunofluorescence.

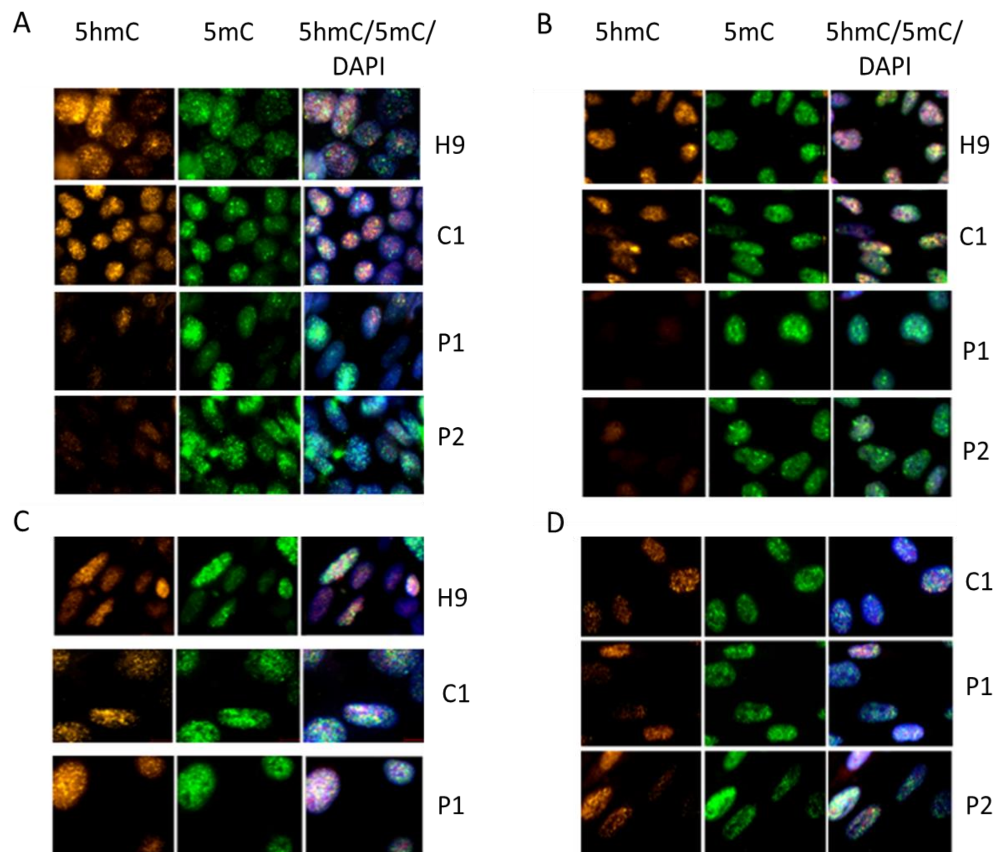


Figure 4.2.7 : 5hmC level in a human embryonic stem cell line (H9), in iPSCS from healthy control and in patient iPSCs analysed by Immunofluorescence. 5hmC (brown) and 5mC (green) levels in H9, control (C1) and patients (P1, P2) A) undifferentiated iPSCs B) early undifferentiated iPSCS (less than 10 days) C) late undifferentiated iPSCS (more than 10 days) D) fibroblasts. DAPI is used for nuclear staining. This experiment was performed by Dr Katarzyna Tilgner. Result of one experiment.

Figure 4.2.7 shows that 5hmC levels were decreased in both undifferentiated and early differentiated patient iPSCs compared to controls and hESC. At later time points, when cells had fully differentiated, the 5hmC levels were similar in both patient and control.These results demonstrate that TET2 p.H1382R mutation does affect the level of 5hmC, consistent with an active role for TET2 in maintaining the 5hmC level in undifferentiated and early differentiated cells. By contrast, in the late and fully differentiated somatic cells (for example fibroblasts) the 5hmC level is similar in patients cells to the controls, suggesting that maybe other TETs such as TET3 play a role in normalising the global 5hmC level in the cells. So far, the recombinant system and iPSCs were

consistent with our hypothesis that the TET2 p.H1382R mutation impairs its enzymatic activity. To investigate further the effect of TET2 p.H1382R, the next experiment was to assess the global 5hmC level in patient material.

4.2.5.3 : Normal 5hmC level in patient B cells

To further explore the effect of TET2 p.H1382R mutation in the patients, patient B-cell DNA was chosen to assess the 5hmC level in vivo. As mentioned in the patients' history, P2 died after transplantation whereas P1 underwent haematopoietic stem cell transplantation and survived. After transplantation, the P1 developed split chimerism with donor T cells and recipient B cells. Therefore, his B cells represent a source of freshly available patient DNA, suitable in assessing the 5hmC level. Unfortunately, we do not have any fresh material from P2.

The first method that was chosen to assess the global 5hmC level in the patient B cells was dot blot assay as it was widely used in previous studies to evaluate the global 5hmc level in mammalian cells (Ko *et al.*, 2010; Quivoron *et al.*, 2011; Kunimoto *et al.*, 2012; Hu *et al.*, 2013; Ko *et al.*, 2013; Yang *et al.*, 2013; Huang *et al.*, 2014). In summary, dot blot is an assay where denatured DNA is blotted onto a nylon membrane using dot blot apparatus, air-dried and immobilised on the membrane by UV cross linking. The membrane is then probed with an anti-5hmC antibody and later with a secondary antibody. Washing steps are required after probing with the anti-5hmC antibody overnight, before and after probing with secondary antibody. Finally, 5hmC in DNA is detected by an enhanced chemiluminescence system. The 5hmC level was quantitated directly by analysing the density of single dots on the membrane. Although many optimisations including

1) titrating the amount of DNA using control DNA or different control DNA (Figure 4.2.8(A) &(B))
2) same amount of DNA were blotted onto the membrane into several wells, to make sure that the dots were equal in each well (Figure 4.2.8 (C)
3) using different antibodies
4) optimising the dot blot technique such as different times of air drying the membrane and UV cross linking exposure

were performed using control DNA, this method was shown to be unreliable as each optimisation experiment showed variabilities in detecting the 5hmC level. Furthermore, by performing two-fold serial dilutions of the amount of DNA, no dots were detected on the membrane for both control and patients B cells DNA, further suggesting that this method was not working successfully.

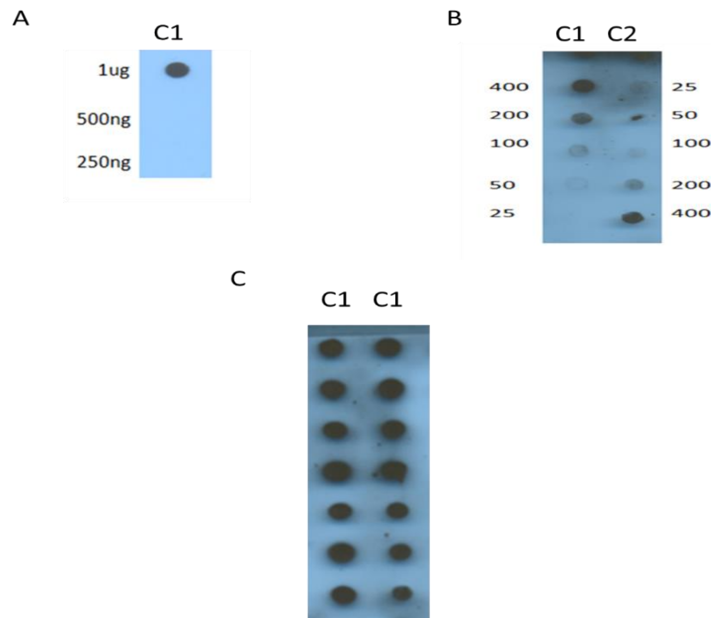


Figure 4.2.8 : Optimisation of the dot blot assay for measuring the 5hmC level in control DNA. 5hmC level in control DNA was detected using anti-5hmC antibody and quantified by dot blot. A) Different amounts of control DNA (C1) (1ug, 500ng, 250ng) were spotted onto the membrane B) Two-fold serial dilutions of different control DNA (C1, C2) (400ng, 200ng, 100ng, 50ng, 25ng) were loaded into each well. C) Equal amount of control DNA (C1) was loaded into each well (16 wells).

Therefore, another method, the ELISA 5hmC kit by Zymo Research was chosen to assess the 5hmC level in vivo. This method was chosen because it requires a small quantity of DNA to perform this experiment as the patient DNA was very limited. However, this method was not commonly used. This ELISA 5hmC kit uses a sandwich-based ELISA format where firstly, the anti-5hmC antibody is coated at the bottom of the well. Then, sheared DNA is added and the single stranded 5hmC-containing DNA binds to anti-5hmC which is then recognised by anti-DNA HRP Antibody. Finally the addition of HRP developer will produce a greenish colour in the wells containing 5hmC DNA, due to the enzymatic reaction of the anti-DNA HRP antibody to the chemical substances in the HRP

developer. The intensity of the colour change is analysed by plate reader. Included with the kit is a control DNA set that is needed to generate a standard curve to calculate the percentage of 5hmC in the DNA samples using the absorbance reading by the plate reader.

To confirm that this method is reliable and sensitive in detecting the 5hmC in DNA, several optimisation experiments were performed such as :

1) Titrating the amount of DNA, expecting to observe an increased amount of 5hmC, correlating with the increased amount of DNA
2) Reading the absorbance with a plate reader at later time points (20 and 60 minutes) to allow more time for generation of the colour signal
3) Titrating the concentration of anti-5hmC antibody.

After ensuring that the assay was working within its dynamic range, aliquots of DNA extracted from the iPSCs that were previously examined by Immunofluorescence (Figure 4.2.7) were used to validate the results obtained by ELISA.

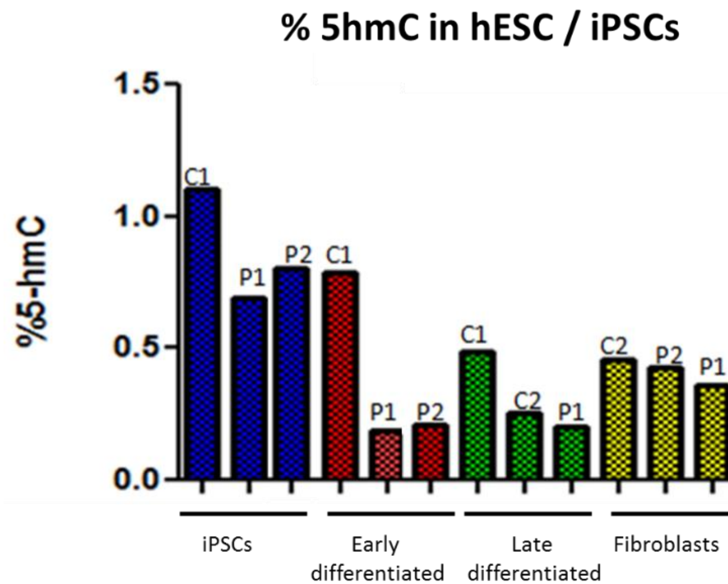


Figure 4.2.9 : Percentage of 5hmC in patients and controls iPScs/hESCs quantified by ELISA. The percentage of 5hmC was measured in human embryonic stem cells line (C1) as well as in undifferentiated iPSCs, differentiated iPSCs and fibroblasts from control (C2) and patients (P1, P2). Result is representative of one experiment.

The result (Figure 4.2.9) showed a reduction in 5hmC levels in both undifferentiated and early differentiated patients iPSCs compared to hESC (C1). When the iPSCS were fully differentiated, the 5hmC levels were similar in both patients and controls iPSCs, while late differentiated cells showed an intermediate pattern. This result correlates with the Immunofluorescence findings, thus this method was thought to be reliable and sensitive to detect 5hmC in DNA.

Further ELISA experiment was performed, to investigate the 5hmC level in patient compared to control B cell DNA.

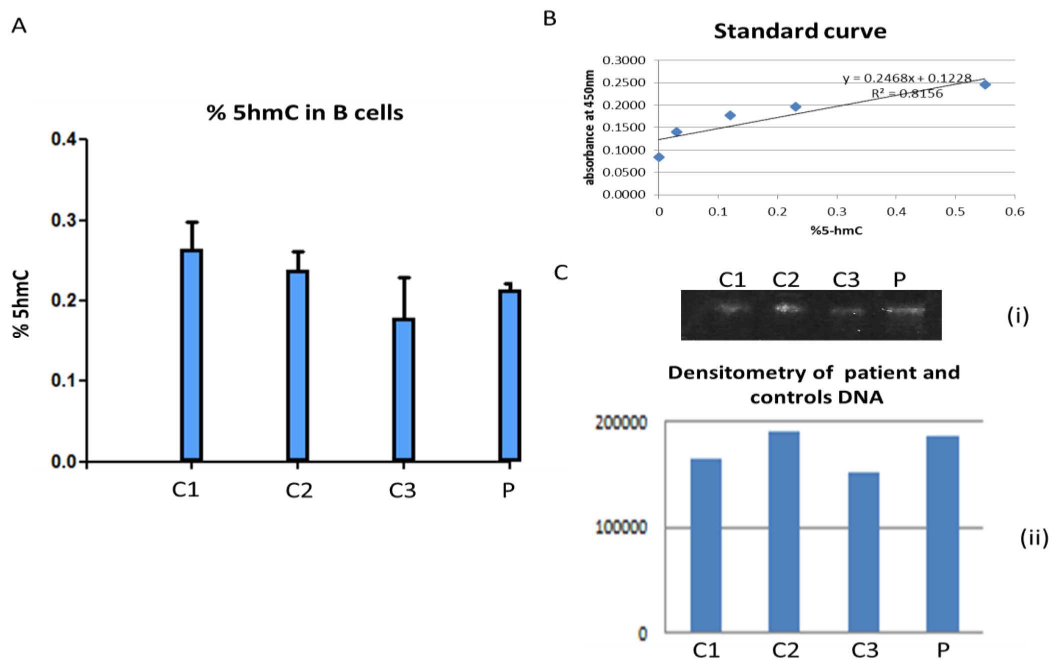


Figure 4.2.10 : Percentages of global 5hmC in DNA from patient and control B cells quantified by ELISA. A) Percentage of 5hmC in controls (C1, C2, C3) and patient (P) were calculated using the equation determined by the standard curve (B). (B) A standard curve was generated from the control DNA set, provided by the manufacturer. C) (i) Aliquots of controls (C1, C2, C3) and patient (P) from the experiment (A) were loaded and run on an agarose gel ii) Densitometry of the bands that were shown in (C(i)), to quantify the amount of each DNA. Result (A) is a representative of two independent experiments. Result (B) and (C) shown is one of the result from experiment (A).

The percentage of global 5hmC in controls and patient is shown in Figure 4.2.10 (A). The 5hmC percentage was calculated using the standard curve (Figure 4.2.10(B)) that was generated from the control DNA set that was provided by the manufacturer. From the ELISA experiment, it was demonstrated

that the percentage of 5hmC in patient B-cell DNA was similar to that of controls (Figure 4.2.10(A)). To check that the same total amount of DNA was used for each sample in the experiment, aliquots of each DNA sample were loaded and run on an agarose gel (Figure 4.2.10(C)(i)). To quantify the amount of DNA, densitometry (Figure 4.2.10(C)(ii)) was generated from Figure 4.2.10(C)(i)). Densitometry showed that similar amounts of DNA were present in each sample used in the ELISA experiment. Hence, this result indicates that the global 5hmC level is normal in patient B cells.

4.2.5.4 TET1, TET2 and TET3 are expressed in healthy control B cells

Since the TET family members consist of TET1, TET2 and TET3, and all are involved in conversion of 5mC to 5hmC, we hypothesised that the normalised global 5hmC level in the patient B cells was due to compensation by other TETs. To explore this possibility, qRTPCR was performed, to measure TET1, TET2 and TET3 mRNA expression in normal B cells. H9, a human embryonic stem cells line was used as a positive control as it was shown in previous studies that TET1 expression was the highest there, followed by TET2 and very low expression of TET3 in mouse embryonic stem cells (Koh *et al.*, 2011),suggesting that the 5hmC levels in embryonic stem cells is oxidised mainly by TET1 and TET2.

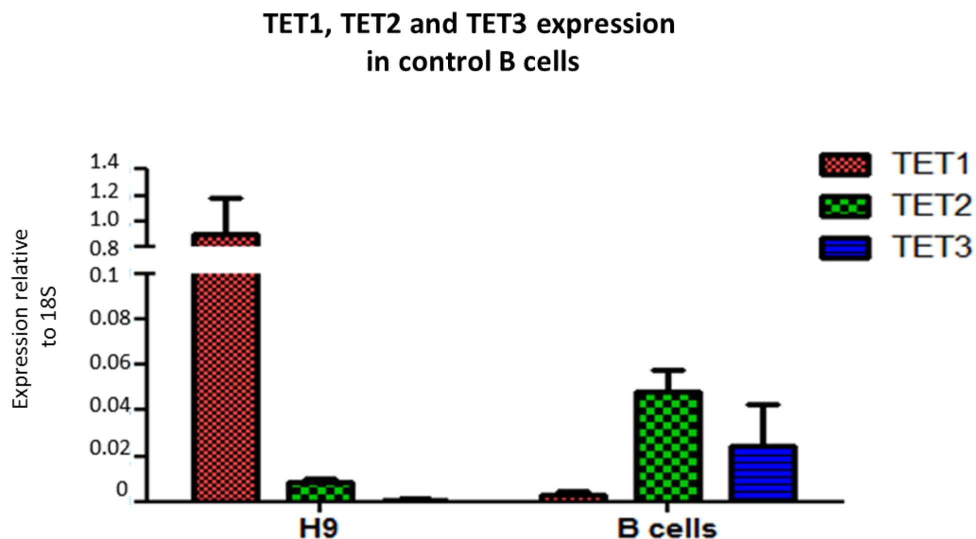


Figure 4.2.11 : TET1, TET2 and TET3 expression levels in control B cells. H9 was used as positive control. The TET1, TET2 and TET3 expression in both cells were relative to 18S. Result is representative of three independent experiments.

The results showed that expression of all three TETs was detectable in control B cells (Figure 4.2.11). This result raises the possibility that TET3 or TET1 might compensate for TET2 in the global 5hmC level in patient B cells which was shown before (Figure 4.2.10(A)). To explain the disease phenotypes, we can postulate epigenetic differences other than crude changes in global hydroxymethylation. While global 5hmC levels might be normal, patients might show reduced 5hmC modifications at transcription start sites or other specific genomic loci that could results in the disease phenotypes observed in the patients. Furthermore the mutant TET2 may behave aberrantly with respect to other mechanisms of transcriptional regulation such as its interaction with transcription factors.

4.2.6 Discussion

Based on the molecular data described in chapter 4.1, the TET2 missense mutation c.A4115A>G, p.H1382R was identified as a candidate disease-causing variant in both patients. Several findings from previous literature support this hypothesis. First, TET2 is highly expressed in the haematopoietic compartment compared to other tissues. Second, somatic TET2 mutations are commonly observed in haematological malignancies including myeloid and lymphoid malignancies, consistent with the occurrence of lymphoma in both patients. It is interesting to highlight that all TET2 missense mutations in acute myeloid leukaemia, reported by Weissman et al. (Weissmann *et al.*, 2012), occurred within the core catalytic region (amino acid 1104 to 2002) with both patients' mutation also being in the same region at amino acid position 1382.

The most striking evidence comes from the $Tet2^{-/-}$ mouse models (Ko *et al.*, 2011; Li *et al.*, 2011; Moran-Crusio *et al.*, 2011a; Quivoron *et al.*, 2011). All Tet2-deficient mice presented with hepatomegaly and splenomegaly which was also one of the features observed in both patients. In addition, before transplant, P1's bone marrow aspirate showed increased myelopoeisis which was also observed in the Tet2-deficient mice. Other shared features were leucocytosis and monocytosis, observed in P1 after transplant and also in Tet2-deficient mice.

As has been mentioned earlier, Quivoron et al. identified altered B and T cell differentiation in the Tet2 knock-out mice (Quivoron *et al.*, 2011). TET2 loss affects development of the lymphoid lineage with increased double negative (DN) CD4-CD8 T cell progenitors in the thymus and increased lymphoid population. Furthermore, apart from the lymphoid malignant transformation, there were other non-malignant phenotypes that were observed such as massive hepatosplenomegaly, anaemia and thrombocytopenia which also resemble those in our two patients. These phenotypes are observed in ALPS patients and are believed to be related to an apoptotic defect through the development of autoimmune cytopenias. However, neither autoimmunity nor apoptosis have been investigated before in the Tet2-deficient mice and remain yet to be explored. The similar phenotypes observed in both patients and all Tet2-deficient mice are compared in Table 4.2.2.

Phenotypes	**Patients**	**TET2-deficient mice**
Hepatomegaly, splenomegaly	Yes	Yes
Increased myelopoiesis	BMA showed increased myelopoiesis in P1 before transplant	Yes
Leucocytosis and monocytosis	Developed in P1 after transplant associated with relapse of ALPS	Yes
Lymphoid malignant transformation	P1 – EBV-positive nodular sclerosing Hodgkin's lymphoma P2 – T lymphoma	Quivoron et al. demonstrated Tet2-deficient mice – increase population of B lymphoid cells
Anaemia, thrombocytopenia	Yes- autoimmune	Yes in Tet2-deficient mice generated by Quivoron et al.

Table 4.2.2: Similar phenotypes in both patients and TET2-deficient mice. BMA- Bone marrow aspirate, EBV- Epstein-Barr virus

A missense mutation is a point mutation in which substitution of a single base pair results in the translation of a different amino acid at that position. Our patients' missense mutation is a single substitution of nucleotide A to G, resulting in changing the amino acid histidine to arginine. By Western blotting, it

was demonstrated that the TET2 protein was expressed in both patients. Although the protein was expressed in patients similarly to control, we show that the mutation results in loss of enzymatic function which results in the disease phenotypes observed in the patients.

TET2 promotes DNA demethylation and regulates gene transcription by converting 5mC to 5hmC (Ko *et al.*, 2010; Hu *et al.*, 2013). Mounting evidence demonstrate that mutations within the catalytic domains or reduced expression of TET2 results in loss of global 5hmC in patients bone marrow cells (Ko *et al.*, 2010) and these were found in patients with haematological malignancies including myeloid and lymphoid malignancies (Solary *et al.*, 2013) as well as in non-haematological malignancies such as prostate cancers, melanoma breast and lung cancers (Solary *et al.*, 2013). In our patient, the mutation is at H1382, a highly conserved residue involved in Fe(II) coordination which is important for its enzymatic activity. Therefore, it was predicted that the TET2 missense mutation affects the TET2 enzymatic function, perturbs the epigenetic regulation and results in the disease phenotypes. By overexpressing wild type and mutant p.H1382R TET2 gene in HEK293 T cells and analysing by Immunofluorescence, I confirmed that the p.H1382R mutation results in impaired enzymatic activity of TET2. In addition, Immunofluorescence also confirmed that this mutation leads to decreased 5hmC staining in patient iPSCs. However, in contrast, by ELISA, patient B cells showed similar global 5hmC levels compared to control, indicating that the global 5hmC level was normal. This result is not in conformance with the loss of global 5hmC observed in patients with myeloid and lymphoid malignancies who have TET2 loss of function. In these patients, bone marrow cells were used for the analysis, whereas here I used B cells. The different results suggest that whereas TET2's essential role in regulating HSCs function and differentiation is reflected by global 5hmC levels, in peripheral B cells, which are differentiated cells, global 5hmC levels don't embody the importance of TET2 function. In addition, as mentioned earlier, it could be that the normalised 5hmC level in B cells is compensated by other TET family members. By qRT-PCR it was shown that TET1 and TET3 are also expressed in normal B cells, suggesting the possibility that 5mC is converted to 5hmC by TET1 and TET3, hence the normal global 5hmC level in patient B cells.

Another possibility is that maybe the ELISA technique is not that accurate in detecting the global 5hmC level, despite performing many optimisation experiments. At the time the experiment was performed, the method was not being described in any previous studies before. Recently, one study had used this method where they quantified the 5hmC level in patients with renal cell carcinoma and demonstrated a significant difference of 5hmC level in patients compared to control (Shim *et al.*, 2014). However, the 5hmC level was also being confirmed by dot blot assay, implying that an established method is required to validate the 5hmC result by ELISA. As shown in this chapter's results section, the optimisation of the dot blot assay using the 5hmC antibody was unsuccessful; therefore this method was not being done to quantify the 5hmC level in patient B cells. Apart from dot blot, there are other established methods that had been shown to quantify the global 5hmC level including thin-layer chromatography (TLC) (Kriaucionis and Heintz, 2009; Tahiliani *et al.*, 2009) and high performance liquid chromatography (HPLC) (Kriaucionis and Heintz, 2009; Liutkeviciute *et al.*, 2009). Both TLC and HPLC are chemical techniques that involve separation of the sample components by chromatography which is described as a mass transfer process involving adsorption. These techniques were shown to be a reliable and sensitive technique but their disadvantage is that they require a high amount of DNA, a minimum of 1ug. As the patient is a child, on rituximab, a drug that is used to suppress the B cells and his condition was unwell during the time the blood was taken, therefore the number of B cells that were isolated from the patient was very few, resulting in a small amount of DNA extracted from the cells. The amount of extracted DNA was very limited and not enough to use for these techniques.

Since the 5hmC level was shown to be normal in patient B cells, it does not explain the development of lymphoma, apoptosis defect and autoimmunity that was observed in the patient. As the ELISA method measured the global 5hmC level, another approach is required such as mapping the 5hmC distribution in the genome of patient and control, to provide an explanation on the phenotypes observed in the patient. With new and advanced technologies emerging, more specific and sensitive techniques can be used to map the 5hmC distribution in the genome including hydroxymethylcytosine-DNA immunoprecipitation

(hMEDIP) followed by next generation sequencing, DNA Array or PCR (Ficz *et al.*, 2011; Stroud *et al.*, 2011; Wu *et al.*, 2011; Kinney and Pradhan, 2013). Another technique that can be used is single molecule real-time (SMRT) sequencing which can discriminate between unmodified cytosine, 5mC and 5hmC due to variations in polymerase kinetics during the sequence reaction (Kinney and Pradhan, 2013). The hMEDIP coupled with deep sequencing approach had been used in a study by Lian et al. who identified a reduction in the 5hmC level at promoters and gene bodies of patients with melanomas (a type of aggressive cancer of the skin) compared to patients with benign nevus (which is a type of benign tumour of the skin) (Lian *et al.*, 2012). So far, no mapping of 5hmC distribution is done in human or mouse B cells. This method, hMeDIP with deep sequencing was thought to be one of the approaches that can be used to elucidate the differences between patient and control at the level of specific genomic loci. However, due to limited fresh patient material, this method could not be performed using patient B cells.

Besides DNA demethylation, TET2 is also involved in recruiting OGT to chromatin and promotes histone GlcNAcylation which influences gene transcription (Chen *et al.*, 2013). Since the only other material that was available from the patients were fibroblasts, therefore I used these cells to explore the TET2-OGT interaction mechanism. Immunoprecipitation technique was chosen to investigate the interaction as this technique was used in a previous study by Chen et al. (Chen *et al.*, 2013). Before using patient cells, I used control cells to optimise the experiments. However, I could not demonstrate any TET2-OGT interaction in control fibroblasts. This was confirmed by three independent experiments. In previous studies, the TET2-OGT interaction was observed in human embryonic stem cells (Chen *et al.*, 2013) and a recombinant system where TET2 was overexpressed in HEK293 T cells (Chen *et al.*, 2013; Deplus *et al.*, 2013). Apart from the cell type, another possibility is the technique itself and the buffer that was used in the experiment. Although the protocol and the buffer preparation were referred from a previous study (Chen *et al.*, 2013) it still results in failure to observe the TET2-OGT interaction. It should be noted that in the study by Chen et al., they demonstrated that by overexpressing the TET2 enzymatic dead mutant,

H1382Y, in HEK293 T cells, the histone GlcNAcylation was also increased, implying that the enzymatic activity of TET2 is not required for histone GlcNAcylation. Due to the possibility that TET2-OGT interaction is reduced in fibroblasts, difficulties in optimising the immunoprecipitation methods and furthermore with the findings by Chen et al., I abandoned attempts to study TET2-OGT interaction.

So far, we concluded that the p.H1382R mutation affects the TET2 enzymatic activity, as it was shown by the recombinant system experiment and patient iPSCs. In contrast, by using patient B cells the 5hmC level was normal. Nonetheless, we believe that impaired enzymatic activity results in the development of lymphoma, defective apoptosis and resultant autoimmunity in our patient and that mapping the 5hmC in the genome by investigating local changes of 5hmc, e.g. in promoter regions, rather than global levels, will explain this. Previous studies had demonstrated that impaired TET2 enzymatic activity results in the development of lymphoma (Couronne *et al.*, 2012; Lemonnier *et al.*, 2012) and very recently in mouse studies, it was demonstrated that TET2 plays an important role in controlling autoimmunity (Ichiyama *et al.*, 2015). However, until now no study has shown the effect of TET2 mutations on apoptosis in lymphocytes, although features such as massive hepatosplenomegaly, anaemia and thrombocytopenia that were thought to be related to apoptotic defects were observed in TET2 knock out mice (Quivoron *et al.*, 2011). The subsequent chapter will discuss the effect of the TET2 p.H1382R mutation on the lymphocyte apoptosis in our patients.

Chapter 4: A novel autoimmune lymphoproliferative disorder

4.3 Effect of TET2 p.H1382R variant on lymphocyte apoptosis

4.3.1 Introduction

Apoptosis is a mode of 'programmed' cell death which plays a critical role at several checkpoints in lymphocyte development and homeostasis (Opferman and Korsmeyer, 2003), (Rathmell and Thompson, 2002), as has been described in Chapter 1. Increased lymphocyte apoptosis can lead to immunodeficiency through cell loss whereas failure of apoptosis can be associated with lymphoproliferative disease and autoimmunity, classically in patients with ALPS (Rathmell and Thompson, 2002). Two major pathways of lymphocyte apoptosis in humans are the extrinsic pathway, which is controlled by death receptor signalling, and the intrinsic pathway, controlled by the Bcl-2 family of pro-and anti-apoptotic proteins.

4.3.1.1 Extrinsic cell death pathway

The extrinsic cell death pathway is initiated when an otherwise healthy cell receives an extrinsic signal that instructs it to enter apoptosis (Rathmell and Thompson, 2002). This signal is provided by death ligands that interact with specific death receptors on the target cell surface. Death receptors form a subset of the Tumor Necrosis Factor Superfamily Receptor (TNFSR) group of proteins (Locksley *et al.*, 2001), and are distinguished by possession of a death domain (DD) in their cytoplasmic tail. Three major death ligand-receptor pairs that have been described are: 1) Fas Ligand, which binds to Fas; 2) TNF-related apoptosis inducing ligand (TRAIL), which binds death receptors DR4 and DR5 and 3) TNF, which binds the TNFα receptor (TNFR1) (Xu and Shi, 2007).

The apoptotic pathway involving Fas and FasL is the most extensively studied in the immune system (Zhang *et al.*, 2005). The binding of Fas to FasL causes Fas trimerisation and leads to the recruitment of Fas-associated death domain (FADD) protein and procaspase 8 (inactive), together forming the death inducing signal complex (DISC). This subsequently activates the self-cleavage of procaspase-8 and -10 to active caspase-8 and -10, followed by the cleavage

of procaspase-3, -6 and -7 to form active effector caspases. Following this, caspase-mediated cleavage of structural proteins and “destruction” enzymes, such as the DNA-fragmenting caspase-activated DNase (CAD) (Strasser, 2005), result in the destruction of the cells. Ligation of DR4 or DR5 by TRAIL leads to a similar chain of events. Binding of TNF to TNFRI initiates a more complex set of downstream signals including activation of the caspases 8 and 10 through a TRADD-containing DISC (Rathmell and Thompson, 2002).

The extrinsic cell death pathway is negatively regulated by several inhibitors, including the cellular - FLICE-inhibitory protein (c-FLIP) and the X-linked inhibitor of apoptosis (XIAP) protein. C-FLIP is a homologue of caspase 8 and acts by inhibiting the recruitment of caspase 8 to the DISC complex (Rathmell and Thompson, 2002). XIAP contains the baculovirus IAP repeat (BIR) domains; through these domains, it binds directly to and inhibits the activity of caspase-3 and -7 (Yang and Li, 2000). The extrinsic signalling pathway is shown in Figure 4.3.1.

4.3.1.2 Intrinsic cell death pathway

In the immune system, the intrinsic pathway is controlled by BCL-2 family members and triggered by various stimuli such as cytokine withdrawal and genomic toxicity (Lenardo *et al.*, 1999; Zhang *et al.*, 2005). The key event of intrinsic signalling is mitochondrial outer membrane permeabilization (MOMP), in which the release of certain proteins from the mitochondrial intermembrane space triggers the activation of caspases and results in cell apoptosis (Zhang *et al.*, 2005). The MOMP is regulated by pro- and anti-apoptotic proteins of the BCL-2 family. Pro-apoptotic BCL-2 family members can be further subdivided into BAX/BAK-like and BH3-only proteins (Strasser, 2005). To date, 25 members of the BCL-2 family have been described (Elmore, 2007); among the pro-apoptotic proteins are BAX, BAK and BAD, whereas anti-apoptotic proteins include Bcl-2 and MCL-1 (Chao and Korsmeyer, 1998; Elmore, 2007).

In healthy cells, BH3-only molecules are in an inactive form and get activated by death stimuli, such as cytokine withdrawal, developmental cues or intracellular signals, to initiate apoptosis (Rathmell and Thompson, 2002; Strasser, 2005). The pro-apoptotic proteins of the BCL-2 family such as BAX and BAK then

permeabilize the outer membrane of mitochondria, resulting in the release of pro-apoptotic factors such as cytochrome C and second mitochondria-derived activator of caspase (SMAC, also known as Diablo) and high temperature requirement serine peptidase 2 (HTRA2, also known as OMI) from the intermembrane space into the cytosol (Elmore, 2007). Cytochrome C binds to and activates apoptotic protease activating factor-1 (APAF-1) and pro-caspase 9, forming a complex known as the apoptosome (Chinnaiyan, 1999). This result in the auto-cleavage of pro-caspase 9, giving rise to active caspase 9 which then cleaves the effector caspases such as caspase 3 and caspase 7, leading to their activation (Xu and Shi, 2007).

Anti-apoptotic proteins of the BCL-2 family inhibit the activation of BAX and BAK by sequestering these proteins, resulting in inhibition of cytochrome C release into the cytosol. Similarly, in the extrinsic pathway, XIAP proteins act as anti-apoptotic proteins by inhibiting the activation of caspase-3 and -7. However, the release of SMAC/Diablo into the cytosol upon activation antagonizes the XIAP activity, leading to caspase 3 activation, allowing cell death to occur (Xu and Shi, 2007).

Amplification and cross talk between the extrinsic and intrinsic pathways occur through the activation of caspase 8, which cleaves the BH3-only protein BH3-interacting domain death agonist (BID). This results in the pro-apoptotic truncated BID (tBID) fragment that induces the release of cytochrome C into the cytosol, leading to activation of caspase 9 (Luo *et al.*, 1998). The intrinsic cell death pathway is shown in Figure 4.3.1.

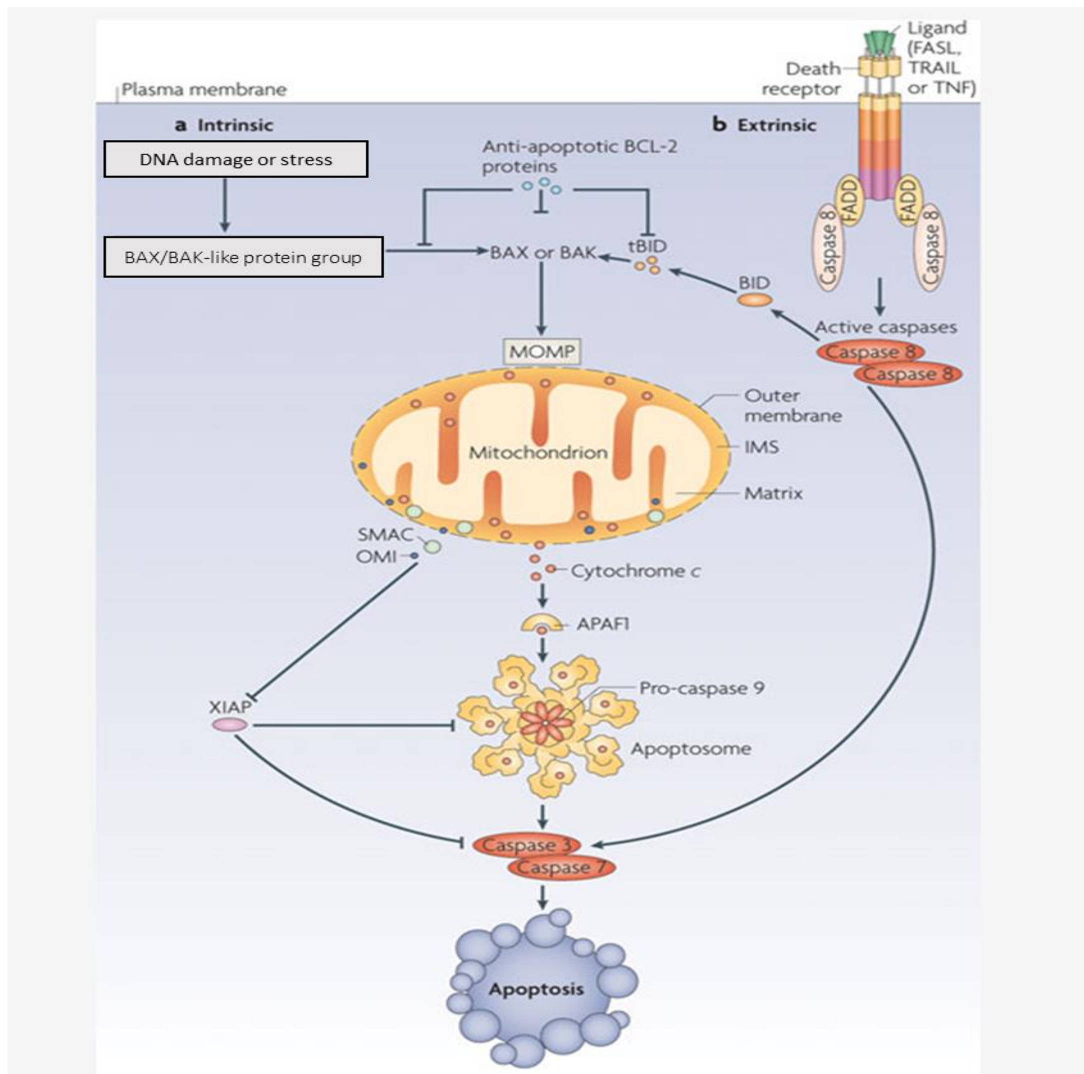


Figure 4.3.1 : The intrinsic and extrinsic pathways of apoptosis. a) Intrinsic pathway. Intracellular signals such as DNA damage and Endoplasmic Reticulum (ER) stress lead to the activation of the pro-apoptotic proteins BAX or BAK. In addition, cytokine withdrawal and antigen receptors such as BCR and TCR can also activate the intrinsic pathway. These trigger the MOMP, resulting in the release of cytochrome C and SMAC/Diablo from the mitochondrial intermembrane space into the cytosol. The activation of BAX and BAK is controlled by the anti-apoptotic protein BCL-2. Once released into the cytosol, cytochrome C binds to and activates APAF1 and pro-caspase 9, forming the apoptosome complex. This results in activation of caspase 9 and subsequently caspase-3 and -7, finally leading to cell apoptosis. The activation of caspase 3 and caspase 7 can be inhibited by XIAP. However, XIAP activity is antagonised by SMAC and OMI. b) Extrinsic pathway. The binding of death ligand to death receptors results in the recruitment of FADD and pro-caspase 8 to FAS, forming a DISC. This leads to the activation of caspase-8 and -10, followed by caspase-3 and -7 which trigger apoptosis. Crosstalk between extrinsic and intrinsic pathway occurs through the activation of caspase 8 which then cleaves BID, producing the active fragment tBID. This leads to activation of BAX or BAK and finally apoptosis. This figure is reproduced from Tait et al (Tait and Green, 2010)

4.3.1.3 Other genes and pathways involved in cell death and survival

In addition to the well-studied pathways described above (Xu and Shi, 2007), several other pathways have also been linked to apoptosis and survival. For example, the interaction of Ras superfamily GTPases NRAS and KRAS with RAS effectors , the Raf family of serine/threonine kinases which include A-Raf, B-Raf, and Raf-1 leads to the activation of the mitogen activated protein kinase kinase (MEKK)/ MAP kinase extracellular signal-regulated (ERK)-signalling cascade, resulting in phosphorylation of downstream targets that either negatively or positively regulate apoptosis (Kang and Pervaiz, 2013). Furthermore, the p53 –mediated pathway leads to cell cycle arrest and finally apoptosis via p21, a protein encoded by the cyclin-dependant kinase inhibitor 1A (*CDKN1A*) gene (Gartel and Tyner, 2002). In contrast, TNF family ligands such as B cell activating factor (BAFF) and activated TRAF family members including TRAF1,TRAF2 and TRAF5 deliver a pro-survival signal via NFκB (Kern *et al.*, 2004). Other mechanisms, such as regulation of apoptotic gene expression, have also been described to influence cell apoptosis. One example is the protein known as T cell-restricted-intracellular Antigen 1 (TIA1) which acts as pro-apoptotic factor by regulating pre-mRNA splicing of the Fas gene (Förch *et al.*, 2000). TGF-β-mediated signalling can induce apoptosis through the activation of SMAD3-regulated pro-apoptotic genes (Jang *et al.*, 2002). Similarly, transcription factors of the Forkhead Box O family exert pro-apoptotic effects by various mechanisms including upregulation of pro-apoptotic BCL-2 family members and death ligands such as TRAIL (Fu and Tindall, 0000).

4.3.1.4 Autoimmune lymphoproliferative syndrome in P1 and P2

ALPS is a disorder characterized by immune dysregulation due to disrupted lymphocyte homeostasis and it is caused by defects in the Fas-induced apoptotic pathway (Li *et al.*, 2015). Fas surface expression is high on activated T and B lymphocytes (Rao and Oliveira, 2011). The role of Fas in maintaining lymphocyte homeostasis was initially observed in Fas-deficient MRL/lpr-/- mice. These mice demonstrated hypergammaglobulinemia, glomerulonephritis, lymphadenopathy and expansion of DNT cells (Watanabe-Fukunaga *et al.*, 1992). Later, two groups, Rieux-Laucat et al. (Rieux-Laucat *et al.*, 1995) and Fisher et al. (Fisher *et al.*, 1995) identified heterozygous germline Fas mutations in patients who presented with autoimmunity and lymphoproliferative disease.

Subsequently, more somatic or germline mutations of Fas were identified and shown to account for the majority of ALPS cases (Rao and Oliveira, 2011). In addition, other proteins within the Fas-mediated apoptosis pathway such as Fas ligand (Del-Rey *et al.*, 2006), caspase 8 (Chun *et al.*, 2002) and caspase 10 (Wang *et al.*, 1999) were found to harbour mutations in patients with ALPS. Other genetic aetiologies have been described in patients with ALPS-like disorders which include germline mutation of NRAS (Oliveira *et al.*, 2007) and somatic mutation of KRAS (Takagi *et al.*, 2011). Nevertheless, there are still 20-30% of patients with ALPS who have an unidentified defect (Li *et al.*, 2015).

Mutations within TET2, such as the p.H1382R variant that we identified in our patients, have never previously been linked to any apoptotic defect, despite their association with haematopoietic malignancy. Functional assays of patient T cell blasts, as well as their clinical phenotype, indicated an apoptotic defect in both affected patients. Several features observed in Tet2-deficient mice, including thrombocytopenia, anaemia and increased double negative T cells, recall the clinical phenotype of ALPS and would be compatible with an apoptotic defect but this disease mechanism had not been considered. I hypothesised that impaired TET2 function led to epigenetically dysregulated expression of genes relevant to apoptosis, providing a mechanistic link to the occurrence of autoimmunity and lymphoproliferation in both humans and mice. Therefore I set out to investigate the effect of the TET2 missense mutation on the lymphocyte apoptosis pathway, using two approaches: 1) transcriptional microarray to investigate the expression of genes relevant to apoptosis in patient cells; and 2) a Tet2-deficient mouse model to investigate the integrity of Fas- mediated apoptosis in lymphocytes.

4.3.3 Aims

1) To investigate the effect of TET2 mutation on expression of genes involved in apoptosis

2) To investigate whether lymphocytes from a Tet2 knock out mouse are defective for Fas-mediated apoptosis

4.3.2 Hypothesis

TET2 mutation leads to dysregulated expression of genes relevant to apoptosis/survival.

4.3.4 Results

4.3.4.1 Upregulation and downregulation of pro- and anti-apoptotic gene expression by microarray

To investigate the expression of genes relevant to apoptosis and survival, microarray analysis was performed using patient and control B-cell RNA. As mentioned before, the surviving patient P1 developed mixed chimerism post-transplant in which the T cells were donor and B cells were recipient, therefore B cells were the only source of patient lymphocytes available for this experiment. Furthermore we knew that P1 developed recurrent ALPS after bone marrow transplant in the context of mixed chimerism, with T cells being donor and B cells being recipient. Therefore it was plausible that the disease phenotype could be driven by apoptotically defective B cells, as has been shown in a mouse model of B-cell-specific Fas deficiency (Hao *et al.*, 2008).

Two biological replicates of RNA were extracted from P1's B cells, which were taken at two different time points. For comparison, 10 samples of RNA were extracted from B cells of 10 different healthy controls. Both patient and control B cells were isolated using the whole blood B cell isolation kit (Miltenyi) and an autoMacs separation machine, described in Chapter 2: Materials and methods. The B cell purity for each sample ranged between 80-98% measured by flow cytometry. Patient and control RNA was extracted using the ReliaPrep RNA Cell MiniPrep System (Promega) and quantified using NanoDrop 2000 Spectrophotometer.

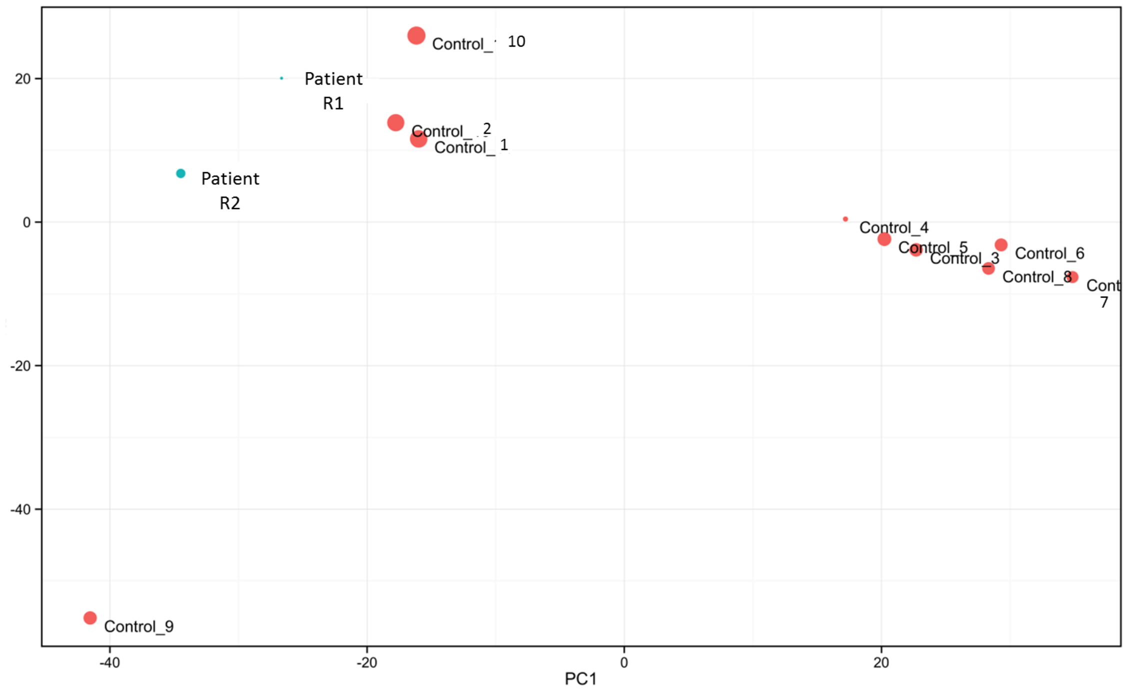
The quality and RIN of patient and healthy control samples were analysed by ServiceXS (Netherlands) using Agilent BioAnalyzer. For the two replicate samples of the patient, the RIN were 6.10 and 6.30, whereas for healthy controls, all 10 samples, the RIN were above 7. As the total amount of RNA per sample was low (less than 200ng), a specialised protocol, NuGEN Ovation PicoSL, was used for preparing biotinylated single stranded cDNA (ss-cDNA).

The biotinylated ss-cDNA was hybridized onto the Illumina Human HT-12 v4 Expression Bead Chip (Illumina Inc, San Diego, USA). The Illumina Human HT12v4 Expression BeadChip data were background corrected in Illumina Beadstudio. Subsequent analysis was undertaken using the Lumi and Limma Bioconductor packages in R.

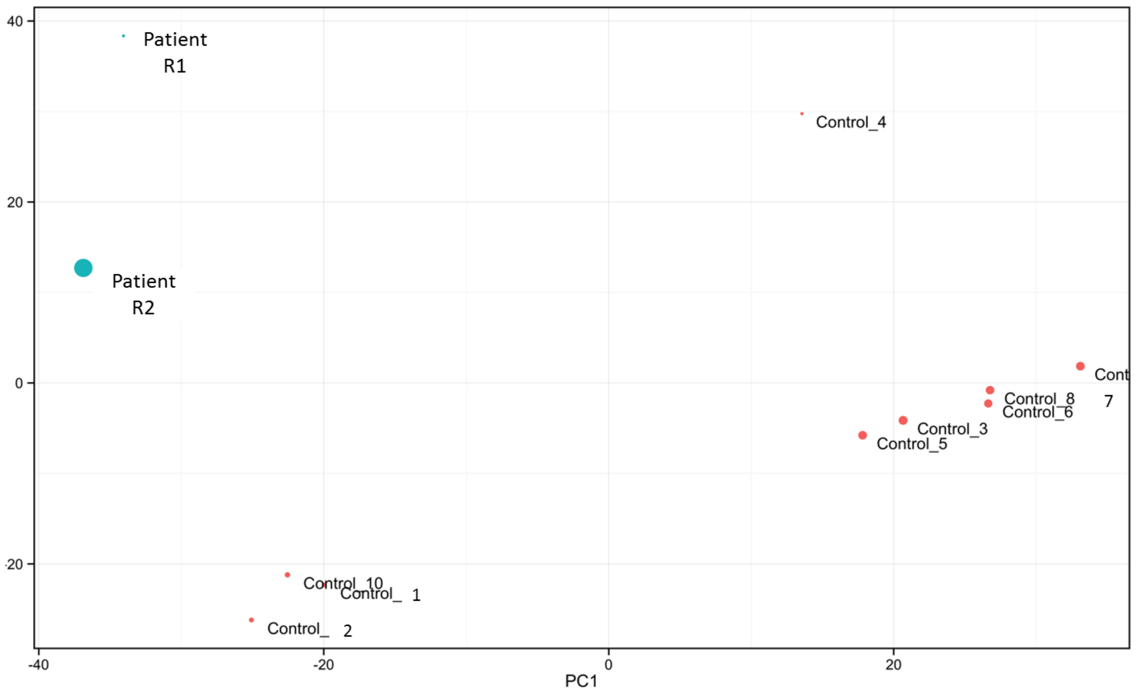
The first analysis performed using the Lumi package was Variant Stabilising Transformation (VST) which takes advantage of technical replicates per probe on the array when transforming the data and outperforms log2 based transformation (Lin *et al.*, 2008). Using VST improves the detection of differentially expressed genes and reduces false-positive identifications (Lin *et al.*, 2008). The next step was to normalise the samples using Robust Spline Normalisation (RSN), included in the Lumi package. This step was performed to ensure that the values of intensity across the different samples have the same distribution.

A Principle component analysis (PCA) plot was generated using Lumi package to show the variance in the data on a two dimensional graph. This was performed by Andrew Skelton, Bioinformatics Unit, Newcastle University. It is a useful plot in spotting the samples that may have gone awry and need to be removed from further analysis. The main concept behind PCA plot is that samples whose gene expression is similar will be clustered together in space.

A



B



C

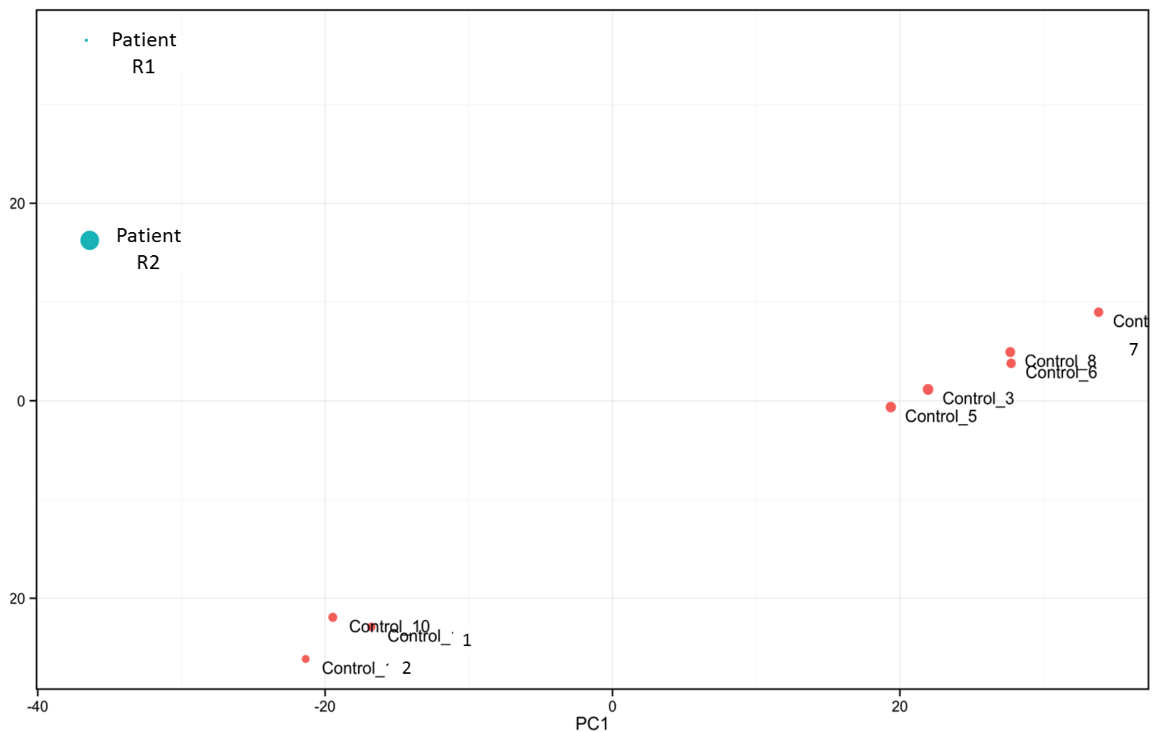


Figure 4.3.2 : Principal Component Analysis (PCA) plot. A) All 12 samples were included in the plot. B) 11 samples were included in the plot as healthy control 9 was removed due to being an outlier as shown in (A). C) Only 10 samples were included in the plot as healthy control 9 and 4 were removed. Patient_R1- Patient replicate 1 sample, Patient_R2 – Patient replicate 2 sample . This analysis is performed by Andrew Skelton, Bioinformatics Unit, Newcastle University.

When all 12 samples were plotted in the PCA plot, it was demonstrated that healthy control 9 was an outlier as it was not in the two groups that were clustered together (Figure 4.3.2 (A)). As healthy control 9 was not responding like other samples and may have a problem, it was removed from further analysis. This is similar to healthy control 4, as it was also shown to be an outlier when PCA was re-plotted without healthy control 9 (Figure 4.3.2 (B)). By removing both healthy control 9 and 4, PCA was re-plotted and demonstrated that control 1, 2 and 10 were clustered together and control 3, 5, 6, 7 and 8 were in the same group, whereas patient R1 and R2 were at the top of the plot (Figure 4.3.2 (C)). There were several clustered groups because the arrays were performed in two batches at different times; batch 1 consists of control 3, 5, 6, 7 and 8, and batch 2 consists of control 1, 2, 10, patient replicate 1 and

replicate 2. However, these differences can be corrected using a batch correction, the ComBat function from the SVA package.

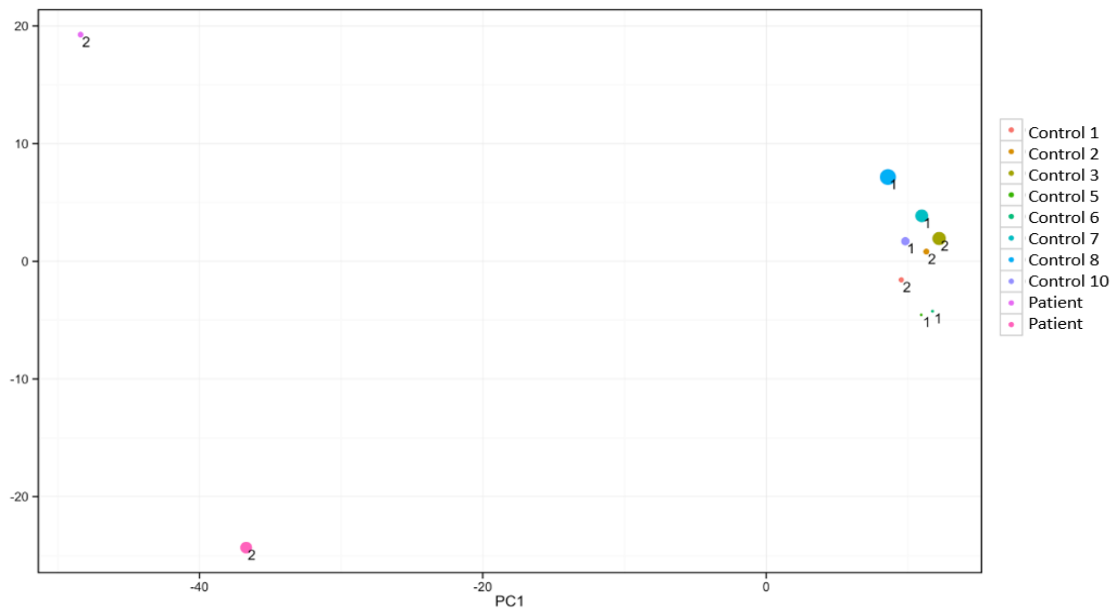


Figure 4.3.3 Principal Component Analysis (PCA) plot after batch correction. After correction, all the controls samples are clustered together whereas patient replicates (2 samples) were not clustered together with the controls. 1 indicates batch 1 and 2 indicates batch 2.

After batch correction, it was demonstrated that all controls were clustered together whereas patient replicates samples were not clustered together with the controls (Figure 4.3.3), implying that patient samples were different and the gene expression was not similar to controls. Patient replicate 1 and replicate 2 were not clustered together most probably because the B cell RNAs were extracted at different times.

To identify the differentially expressed genes between patient and control, Limma Bioconductor package in R was used. The genes with an expression level that was ≥ 2-fold different and had an adjusted P-value less than 0.01 were considered as significant differentially expressed genes.

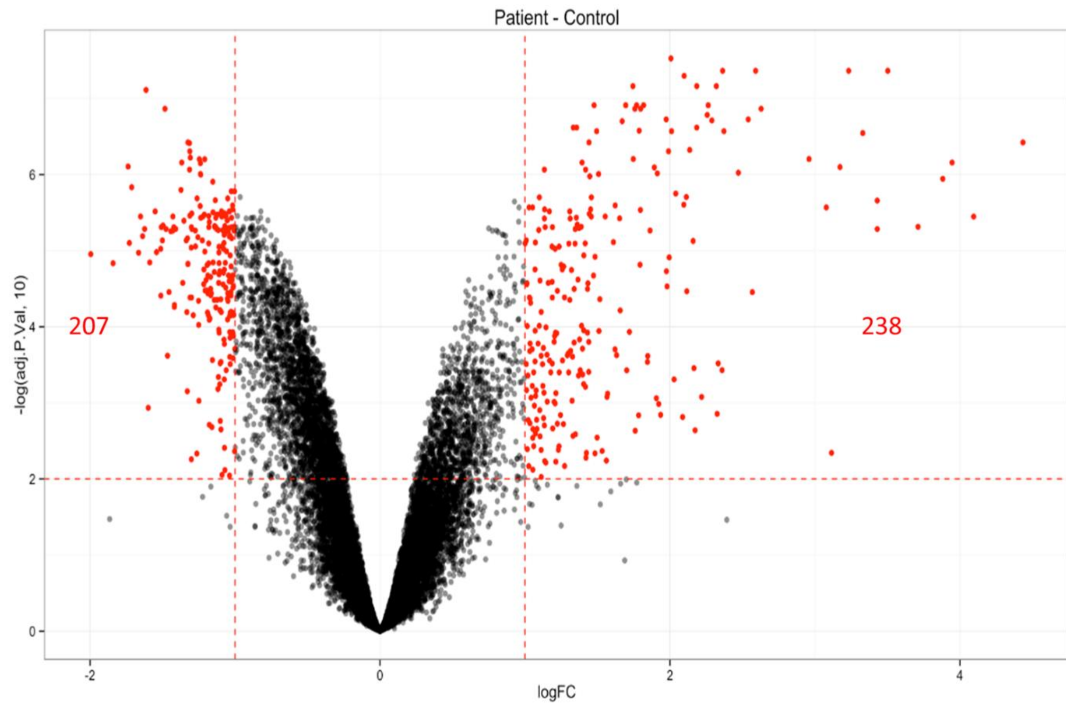


Figure 4.3.4 : Volcano plot of differentially expressed genes in patient vs control. Red dots represent the differentially expressed genes that are≥ 2-fold up- (238 genes) or downregulated (207 genes), adjusted P < 0.01.

A volcano plot was generated to visualise an overview of the differentially expressed genes (Figure 4.3.4). 445 genes were identified as differentially expressed in patient compared to controls (red dots in Figure 4.3.4); 238 genes were upregulated and 207 gene were downregulated. Gene Ontology (GO) Terms (http://geneontology.org/) were assigned to differentially expressed genes to identify those that are involved in apoptosis/ survival pathways.

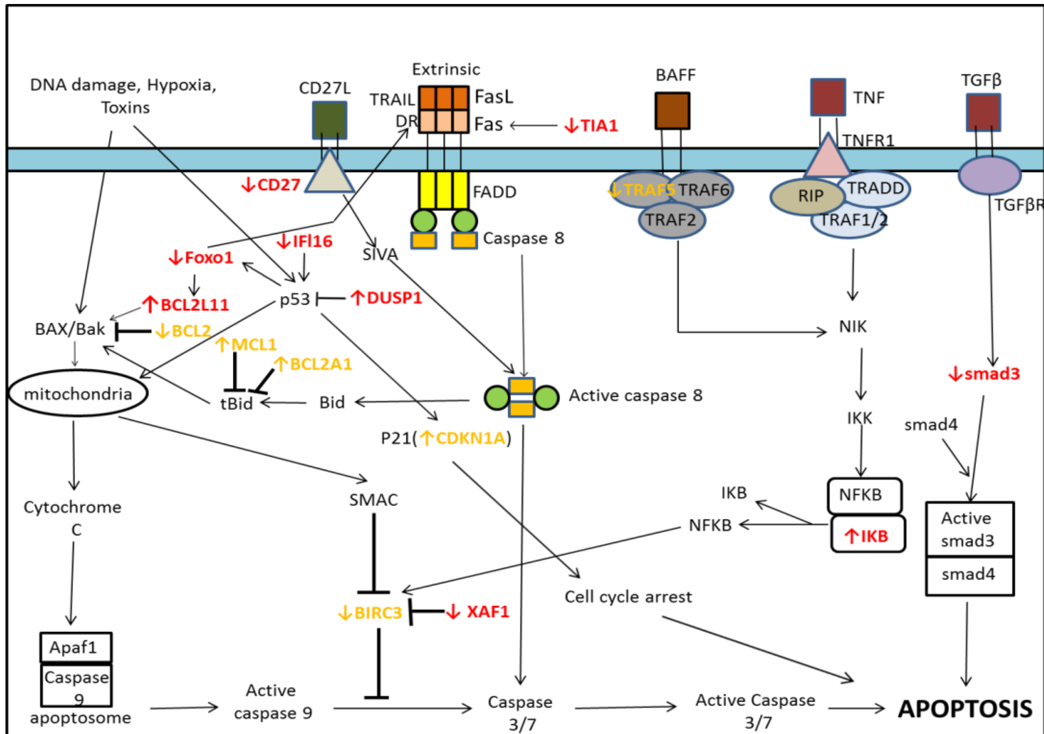


Figure 4.3.5: 15 dysregulated genes from the microarray analysis that regulate apoptosis identified by Gene Ontology (GO) Terms. (-) indicates pro-apoptosis genes and (-) indicates anti-apoptosis genes. (↑) indicates gene upregulation and (↓) indicates gene downregulation.

A total of 34 genes were identified that influence the apoptosis pathway; 16 genes were pro-apoptotic and 18 gene were anti-apoptotic (Table 4.3.1). Some of these are mapped into a diagram illustrating pertinent signalling pathways in Figure 4.3.5.

Proapoptotic genes	Upregulated/downregulated	Fold change
ANXA 1	Upregulate	2.89
NFKBIA	Upregulate	4.8
IL1B	Upregulate	3.74
PMAIP1	Upregulate	2.5
BCL2L11	Upregulate	2.01
DUSP1	Upregulate	11.33
HIPK1	Upregulate	2.2
JUN	Upregulate	4.54
XAF1	Downregulate	2.04
FOXO1	Downregulate	2.1
IFI16	Downregulate	2.3
CD27	Downregulate	2.48
TIA1	Downregulate	2.26
DYRK2	Downregulate	2.27
CD24	Downregulate	3.58
Smad3	Downregulate	2.2

Antiapoptotic genes	Upregulate/Downregulate	Fold Change
MCL1	Upregulate	2.35
BCL2A1	Upregulate	4.11
SOD2	Upregulate	2.6
CDK5R1	Upregulate	2.28
NR4A2	upregulate	3.59
NR4A1	Upregulate	2.58
CDKN1A	Upregulate	5.14
IER3	Upregulate	3.82
BIRC3	Downregulate	2.13
MEF2C	Downregulate	2.2
TMX1	Downregulate	2.1
SYVN1	Downregulate	2.03
SON	Downregulate	2.1
HDAC1	Downregulate	2.5
BCL2	Downregulate	2.2
FAIM3	Downregulate	2.46
TRAF5	Downregulate	2.58
CREB1	Downregulate	2.01

Table 4.3.1 : Pro- and anti-apoptotic genes that were identified to be differentially expressed in patient B cells compared to controls. The genes expressed with above 2-fold change were considered to be either upregulate or downregulated in patient compared to controls.

These results suggested that TET2 mutation leads to dysregulation of certain pro-and anti-apoptotic genes. However, before deriving any conclusion, qRT-PCR needs to be performed to validate these data.

4.3.4.2 qRT-PCR confirmation of the microarray data

To confirm the microarray data, a qRT-PCR using the universal probe library was performed to measure the mRNA expression of the differentially expressed genes. 5 genes were chosen from the differentially expressed apoptosis genes which are CDKN1A, DUSP1, Smad3, TIA1 and TRAF5. cDNA was derived from an aliquot of patient and controls B cell RNA. Two patient replicates and four healthy control RNAs were subjected to qRT-PCR. The primers and probes for each target transcript were designed using the Universal Probe Library Assay Design Centre software (http://lifesciences.roche.com).

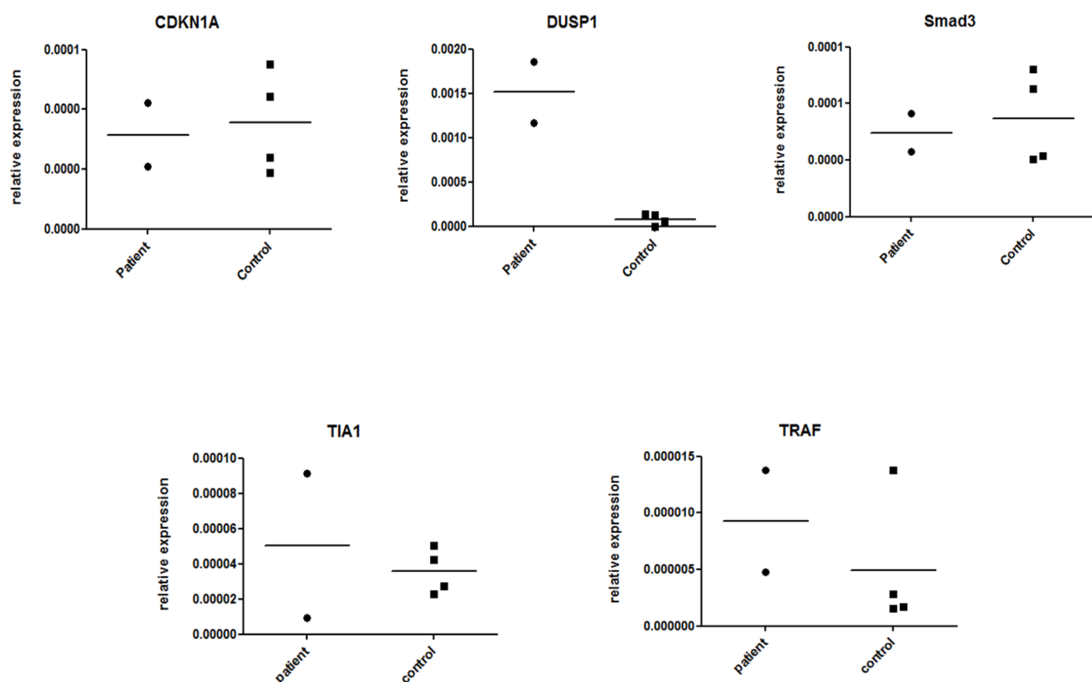


Figure 4.3.6 : Validation of the microarray data by qRT-PCR. The mRNA expression of each gene was expressed as normalised fold change relative to 18S. The data and graph were plotted using Prism 4.0 software and the centre line represents the mean value for patient and controls replicates. Patient replicates (n =2), controls replicates (n=4). The data is representative of one experiment and this experiment was performed twice.

Figure 4.3.6 demonstrated that only one gene, DUSP1, was upregulated in patient compared to control, which corresponded to the microarray data. Expression of genes such as CDKN1A and SMAD3 was shown to be similar to controls. Although patient replicates were shown to be separated far away and not clustered together for TIA1 and TRAF5 genes, the results were still

considered to be in contrast to the microarray data where both genes were shown to be upregulated in the patient. Based on these results, frustratingly, I concluded that the microarray data were unreliable and invalid as the dysregulation of the apoptosis genes that were chosen could not be validated by qRT-PCR.

4.3.4.3 Knock out mouse model

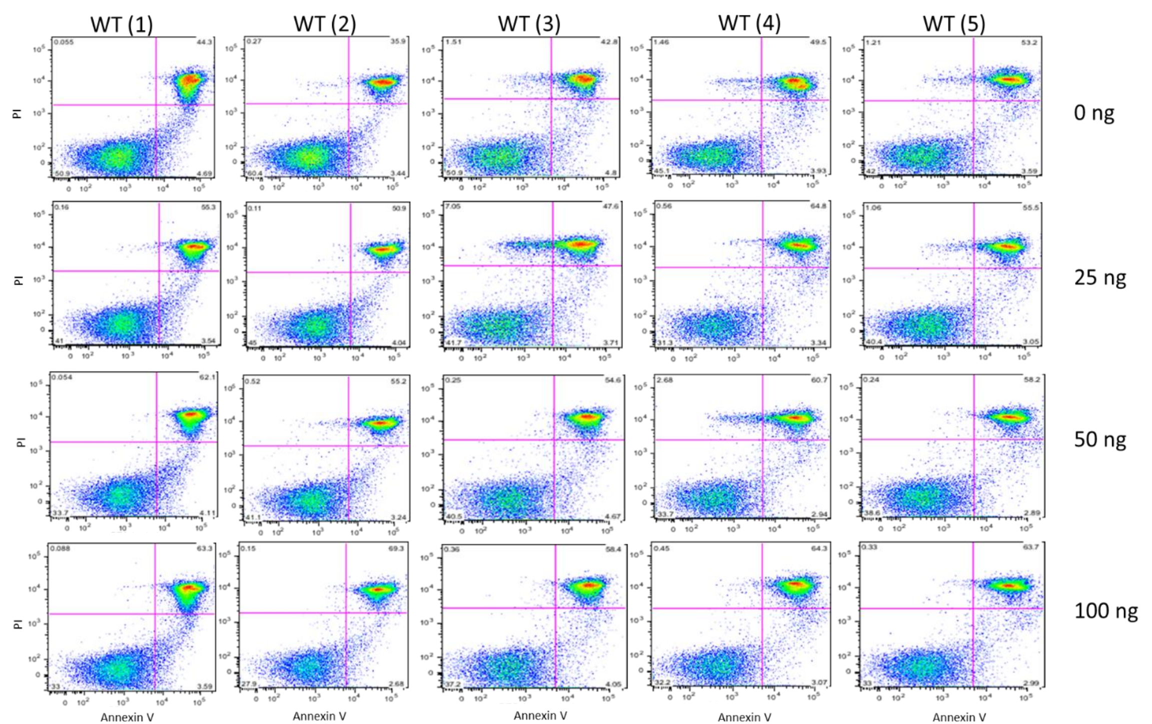
To investigate a possible conserved effect of TET2 deficiency on Fas-mediated apoptosis, Tet2 knock out mice were studied. This work was performed through collaboration with Prof Richard Cornall and Dr Consuelo Anzilotti, Oxford University, where a colony of Tet2 knock out mice was established and I performed the Fas-mediated apoptosis assay.

Briefly, Tet2 floxed allele mice (which possess loxP sites flanking exon 3 of the Tet2 gene) were purchased from Jacksons laboratory, USA. To obtain the full Tet2 knock out mice, the Tet2 floxed allele mice were crossed to mice carrying the Cre-recombinase under the Pgk promoter (Lallemand *et al.*, 1998). To detect the Cre-recombinase alleles carried by Tet2 mice, standard PCR and genotyping were performed using primers similar to the ones used in the study by Quivoron et al. (Quivoron *et al.*, 2011). The mice bearing homozygous null mutation of Tet2 were grown until 10-14 weeks of age and then sacrificed for the Fas-mediated apoptosis experiment.

4.3.4.3.1 Fas-mediated apoptosis assay using mouse B cells

In order to study the Fas-mediated apoptosis pathway in mice, the first experiment that was performed was using B cells and a method adapted from Imtiyaz et al. (Imtiyaz *et al.*, 2006). However, instead of isolating the B cells, I seeded the splenocytes from both wild type and TET2 knock out mice in 48 well-plates at 1 x 10^6 cells /ml. The cells were left untreated or treated with soluble Fas ligand (Enzo Life Sciences, USA) at different concentrations and incubated for 16 hours. An enhancer (Enzo Life Sciences, USA), was added at 0.5 ug/ml to each concentration of soluble Fas ligand, which acts as cross linker to enhance the apoptotic activity. The cells were gated for CD19 (Biolegend, UK), a specific marker of B cells. The apoptosis and cell death were detected with a FITC-Annexin V detection kit (BD Biosciences, UK) which include the

FITC-Annexin V for apoptosis staining and Propidium Iodide for cell death staining and analysed by flow cytometry (Figure 4.3.7). The Annexin V staining utilises the principle that when the cells undergo apoptosis, they expose the phospholipid phosphatidylserine (PS) to the external environment. As Annexin V is a calcium-dependent phospholipid binding protein which has a high affinity to PS, it will identify apoptotic cells by binding to the exposed PS. In dead cells, the membranes are permeable to Propidium Iodide (PI) and allow the PI to stain the cells. Cells that are undergoing early apoptosis will be Annexin V positive and PI negative whereas cells that are either in the late stage of apoptosis or already dead will stain positive for both Annexin V and PI. Cells that are intact and viable will be negative for both Annexin V and PI staining.



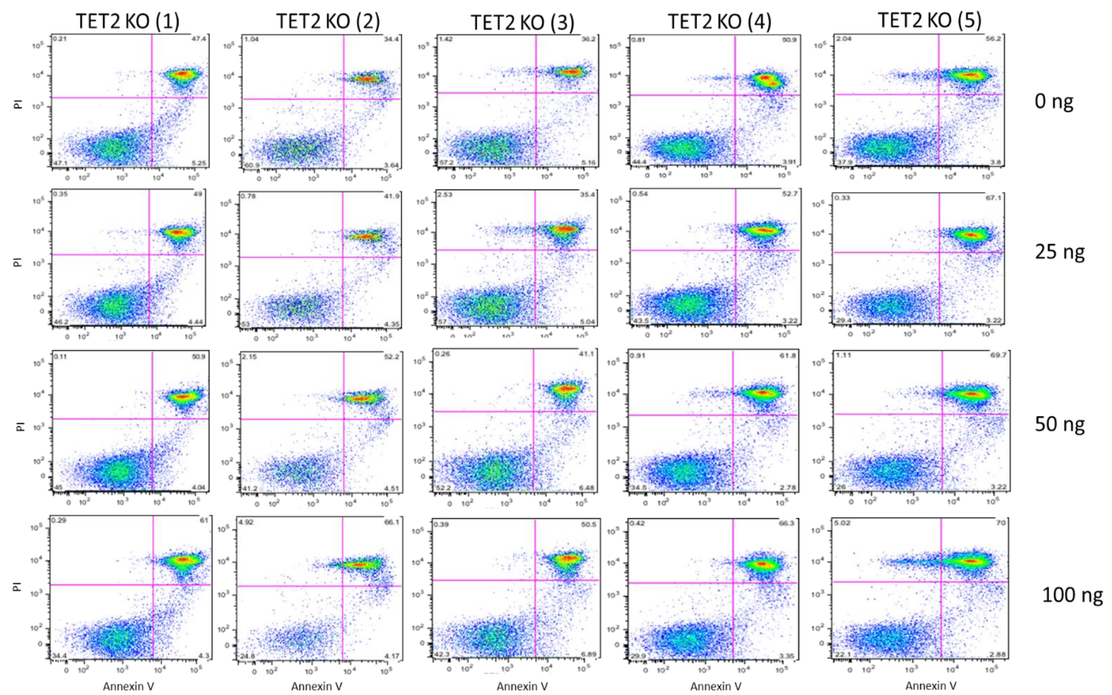


Figure 4.3.7 : Annexin V vs Propidium Iodide (PI) staining analysed by flow cytometry. Splenocytes from five wild type (WT) and five Tet2 Knock out (KO) mice were seeded at 1x10^6 cells/well in a 48-well plate. Cells were left untreated (0 ng) or treated with different concentrations (25 ng/ml, 50 ng/ml, 100 ng/ml) of soluble Fas ligand; enhancer (0.5 ug/ml) was added at each concentration. The cells were incubated for 16 hours. The cells were gated for $CD19^+$ (B cells) and stained with Annexin V and PI and analysed by flow cytometry.

To investigate the Fas mediated apoptosis pathway between the wild type and TET2 knock out mice,the % survival (Annexin V^- PI^-) of cells for each concentration was calculated.

By assuming that the untreated cells were 100 % viable, the percentages of each treated cells were normalised to the untreated cells using the equation below :

$$\text{Normalised cells survival \%} = \frac{\text{Annexin V- PI- (treated cells)}}{\text{Annexin V- PI- (untreated cells)}} \times 100\ \%$$

The normalised percentage of cell survival was calculated as above and plotted in a graph using Prism 4.0 Software (GraphPad Software, USA) (Figure 4.3.8).

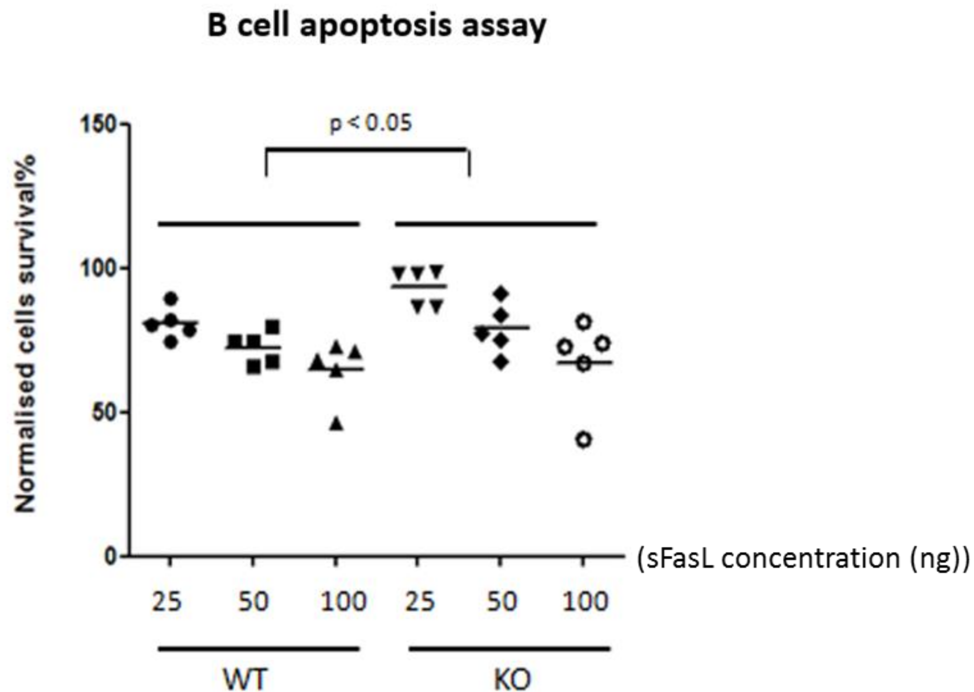


Figure 4.3.8 : B cell apoptosis assay for wild type (WT) and TET2 Knock out (KO) mice . The normalised % of cell survival was plotted for five wild type and five knock out mice at each concentration of added Fas ligand (0 ng, 25 ng, 50 ng, 100 ng). The statistical analysis was performed using Two way ANOVA and $p < 0.05$ is significantly different.

Two way analysis of variance (ANOVA) was performed, testing for differences in apoptosis between WT and Tet2 KO mice at various concentrations of Fas ligand, and this analysis revealed statistically significant differences between WT and KO mice ($p<0.05$). Tet2 KO mice had consistently slightly higher normalised cell survival % across all three concentrations compared to WT mice (Figure 4.3.8).

4.3.4.3.2 Fas-mediated apoptosis assay using mouse T cells

Since patient T cells showed documented abnormalities of Fas-mediated apoptosis, I also performed the Fas-mediated apoptosis assay with mouse T cells. Initially following a similar method to that used for human T cells, the mouse splenocytes were seeded at 1 x 10^6 cells/ml in 48-well plates and stimulated with Phytohaemagglutinin (PHA) at 5ug/ml for 72 hours. Subsequently, the dead cells were removed with Ficoll-paque (Sigma, UK) and viable cells were seeded in 96-well plates and cultured with Interleukin-2 (IL-2)

for 2 days. On the third day, soluble Fas Ligand (Enzo Life Sciences, USA) was added at different concentrations (0ng, 25 ng, 50ng and 100ng) with enhancer at 0.5ug/ml overnight. The cells were gated for $CD3^{+}$ cells (T cells) and apoptosis and cell death were measured as before.

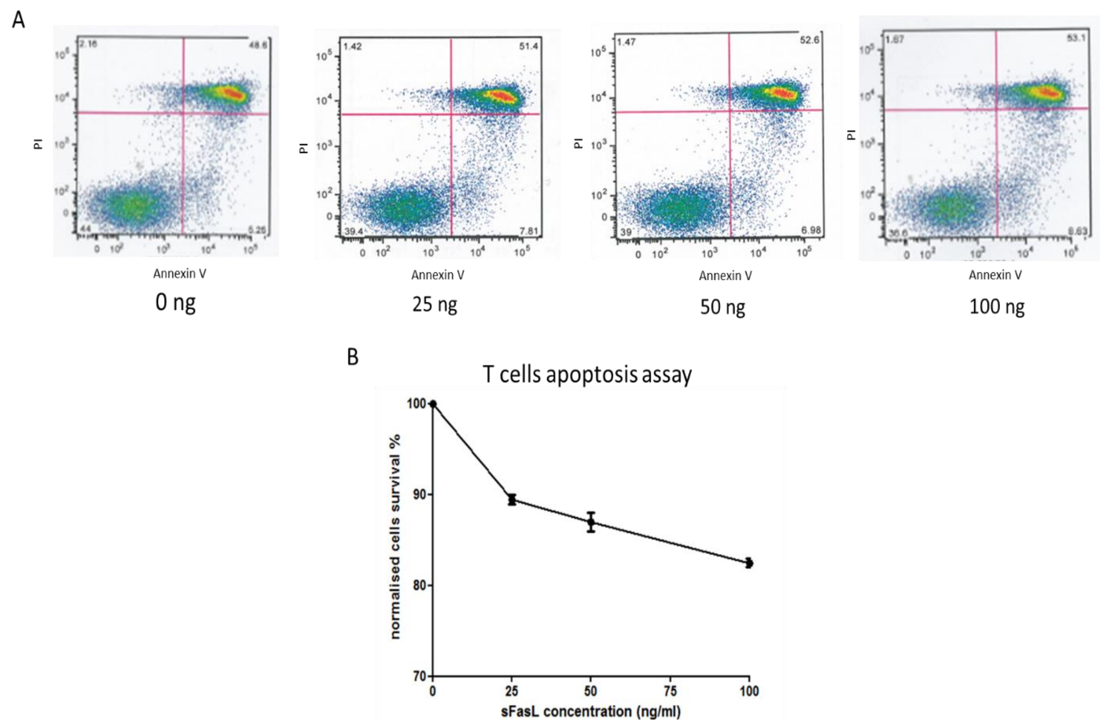


Figure 4.3.9 : T cell apoptosis assay in wild type mice A) Splenocytes were seeded at $1x10^6$ cells/ml in a 48-well plate, stimulated with PHA for 3 days, ficolled and viable cells were treated with IL-2 for 2 days. On day 3, cells were stimulated with different concentration of sFasL (0ng, 25ng, 50ng, 100ng) overnight. The cells were gated for $CD3^{+}$ and stained with Annexin V and PI and analysed by flow cytometry. Data is representative of one of three independent experiments. B) The normalised cell survival % was calculated using the equation described in section 4.3.1.3. The normalised cell survival % was plotted on a graph using Prism 4.0 software. Data is representative of three independent experiments.

To optimise the T cell apoptosis assay, pilot experiments were performed using wild type mice. As expected, all three optimisation experiments demonstrated that the normalised cell survival % was reduced with increased concentration of soluble Fas Ligand (Figure 4.3.9(B)), implying that this method works successfully. Background rates of cell death were however quite high (50-60 %).

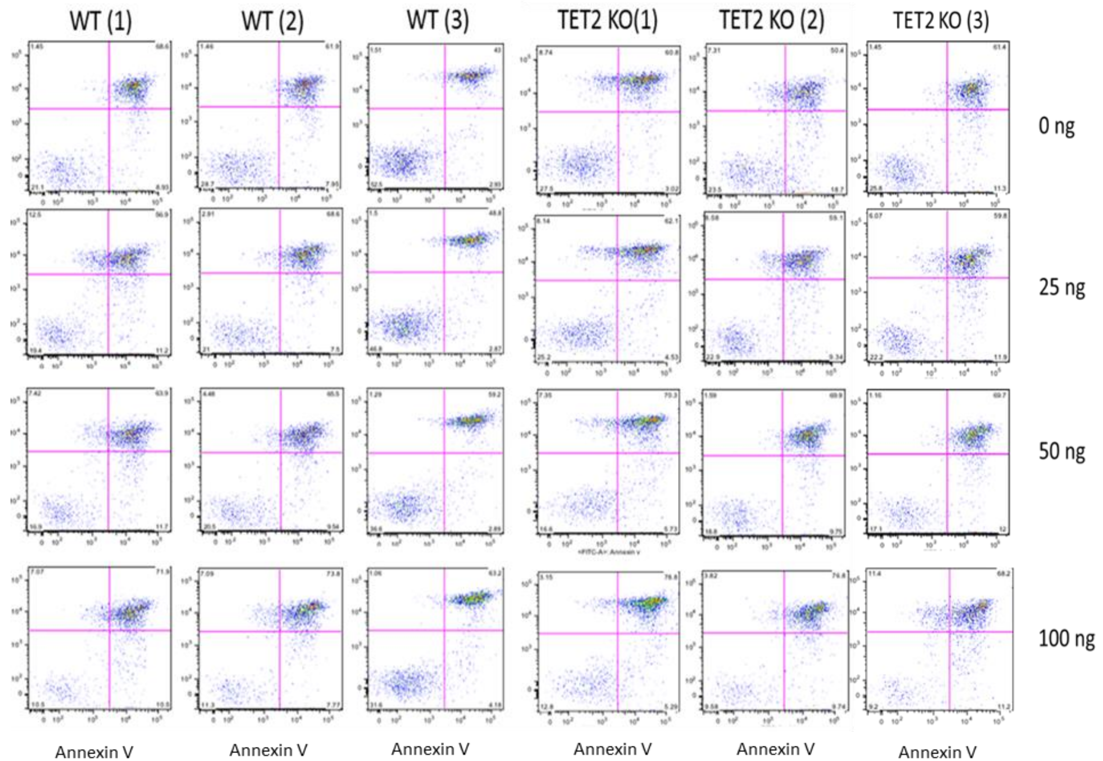


Figure 4.3.10 : T cell apoptosis assay in three wild type (WT) and three Tet2 knock out (KO) mice. Cells were seeded at $1x10^6$ cells/ml in a 48-well plate and stimulated with PHA (5ug/ml) for 3 days. Dead cells were then removed using Ficoll-paque (Sigma,USA). Viable cells were seeded and treated with IL-2 (100IU/ml) for 2 days and stimulated with sFasL at different concentrations (0ng, 25ng, 50ng, 100ng) with enhancer (0.5ug/ml) overnight. The cells were stained with anti-CD3 (T cells), Annexin V and PI and analysed by flow cytometry.

However, unexpectedly, when the same method was used to assay Tet2 knock out mice and local wild type controls, rates of cell death following the PHA stimulation step were very high. As a result, the viability of untreated cells in each assay was poor, ranging between 20-30% (Figure 4.3.10), implying that most of the cells were already dead before stimulation with sFasL. No clear difference was evident between Tet2KO and wild type mice but it was not clear that the experiment would have been capable of revealing such an effect and it was considered noninformative.

On review of the methodology, the choice of phytohaemagglutinin to provide mitogenic stimulation was felt to have been misguided since it is known to produce high rates of activation-induced cell death in mouse T cells. With the guidance of a colleague, Dr Arian Laurence (Institute of Cellular Medicine, Newcastle University), a new method was established. This requires the isolation of a pure homogeneous naïve CD4+ T cell population using the CD4+CD62L+T cell isolation kit (Miltenyi Biotec, UK). The isolated naïve CD4+ T cells were stimulated with plate-coated anti-CD3 (10ug/ml) and anti-CD28 (10ug/ml) antibodies for 3 days. The cells were treated with IL-2 (100IU/ml) on days 1 and 2, providing an additional survival signal. On day 3, the cells were stimulated with soluble Fas Ligand at different concentrations (0 ng/ml, 50ng/ml, 100ng/ml) with enhancer (0.5ug/ml) overnight. As before, the cells were stained with Annexin V and Propidium Iodide (BD Biosciences, UK) and analysed by flow cytometry.

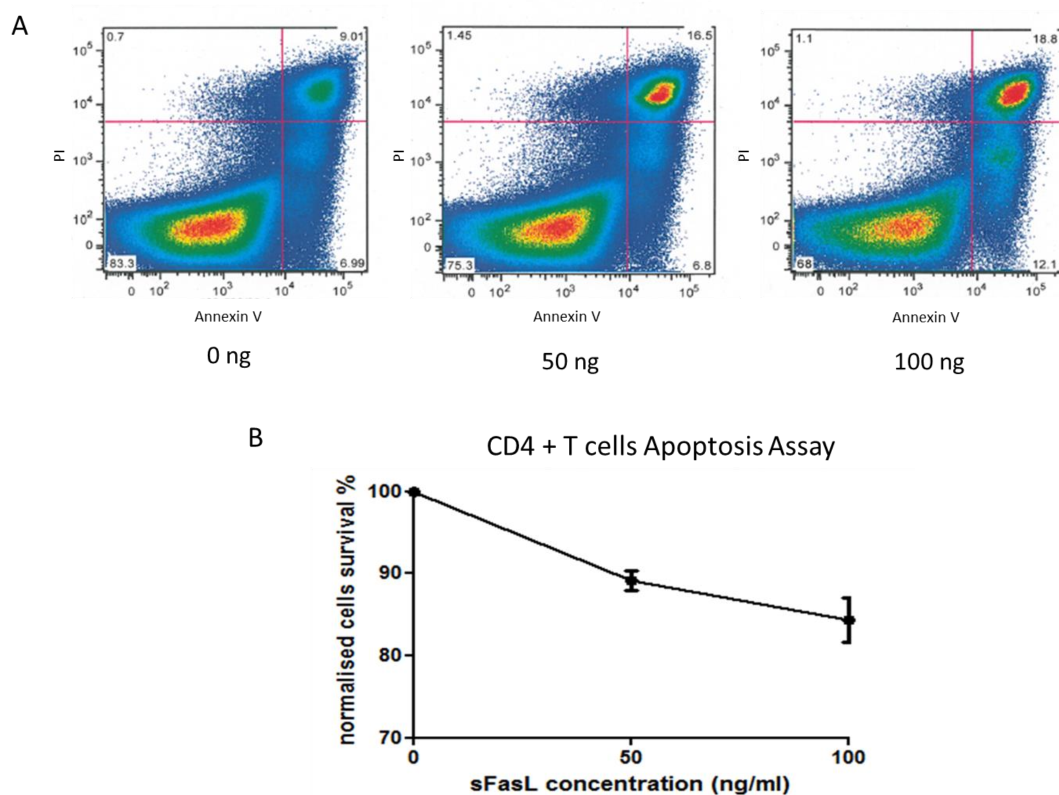


Figure 4.3.11 : CD4+ T cell apoptosis assay with wild type mouse T cells. A) CD4+ T cells were isolated from wild type mouse splenocytes using the CD4+CD62L+ T cell isolation kit (Miltenyi Biotec, UK). Cells were seeded at $1x10^6$ cells/ml in a 24-well plate, pre-coated with anti-CD3 (10ug/ml) and anti-CD28(10ug/ml) antibodies and incubated for 3 days. The cells were treated with

IL-2 (100IU/ml) every day for 2 days. On day 3, the cells were stimulated with different concentrations of sFasL (0ng, 50ng, 100ng) with enhancer (0.5ug/ml) overnight. The cells were stained with Annexin V and Propidium Iodide (PI) and analysed with flow cytometry. Data represents one of the three independent experiments. B) The normalised cell survival % was calculated using the equation described in section 4.3.1.3. The normalised cell survival % was plotted on the graph using Prism 4.0 software. Data is representative of three independent experiments.

To optimise the experiment, the new method was performed using wild type mice. The experiment demonstrated that most of the cells were viable (Annexin V^-PI^-: 83.3%) before stimulation with sFasL (Figure 4.3.11(A)). Once treated with sFasL at 50ng and 100ng, the % viable cells (Annexin V^-PI^-) was reduced as expected. This method is therefore ready to take forward to assay Fas-mediated apoptosis in Tet2 knockout and wild type control mice. Currently, the number of Tet2 knock out mice is very low and the colony is being expanded again. Therefore, the new method has not been performed yet on both wild type and Tet2 knock out mice.

4.3.5 Discussion

Both patients presented with lymphoproliferative disease, lymphoma and autoimmunity. In addition, the laboratory findings demonstrated significantly increased DNT cells and defective Fas-mediated apoptosis. These phenotypes are the main criteria in diagnosing ALPS (Rao and Oliveira, 2011). Both patients were screened for known ALPS mutations, but no mutations in known disease genes were identified. The homozygous TET2 missense mutation p.H1382R was identified by whole exome sequencing and homozygosity mapping. Tet2-deficient mice in a study by Quivoron et al. developed hepatosplenomegaly, an increased number of DNT cells in thymocytes and features of autoimmunity such as thrombocytopenia and anaemia; all these phenotypes are thought to be due to an apoptosis defect (Quivoron *et al.*, 2011). However, so far, to my knowledge, there is no published evidence that links TET2 to apoptosis.

In order to demonstrate that TET2 is involved in regulating apoptosis, the first approach was to perform a microarray analysis. Microarray is used to measure the changes of expression levels of large numbers of genes simultaneously and it allows the description of genome-wide expression changes in health and disease conditions (Tarca *et al.*, 2006). Given the enzymatic function of TET2 as an epigenetic regulator, we postulated that the expression of apoptosis-related genes would be altered in patients compared to healthy controls. An Illumina HT-12 Array, performed by ServiceXS, Netherlands, was chosen. This beadchip targets 47 000 genes derived from the National Center for Biotechnology Information Reference Sequence (NCBI) RefSeq Release 38 (http://www.Illumina.com). For both patient and healthy controls, between 8 000 – 12 000 genes were detected in all samples and the same number of genes were detected in several in house samples which were used as controls.

As mentioned, the microarray analysis data were analysed using Lumi and Limma Bioconductor packages. From the analysis, 16 pro-apoptosis and 18 anti-apoptosis genes were identified to be dysregulated which was either downregulated or upregulated in patient compared to healthy controls B cells. It is widely known that all microarray data should be validated as it can produce false positive results and vary greatly with platform and procedures used (Morey

et al., 2006). The most common method used for validation is qRT-PCR due to its detection sensitivity and low sample requirement. To validate the microarray analysis data, only 5 genes were chosen because of very limited material for patient replicates and controls samples. However, differential expression could not be confirmed as 4 of the 5 genes were similarly expressed in patient and control cells, indicating that the microarray data were invalid. There could be several reasons that give rise to the invalid data.

Before performing the microarray, all the RNA samples were analysed for its quality and integrity by Bioanalyser (Agilent Technologies, USA). The RNA integrity number (RIN) for the two patient's replicate samples were 6.10 and 6.30. The perfect RIN for downstream applications such as microarray analysis and qRT-PCR is above 7 (Fleige and Pfaffl, 2006). An RNA sample with a RIN less than 7 is considered to be a slightly degraded and contaminated and RIN less than 5 is a degraded and low quality sample. It is crucial to have a good quality RNA in order to obtain accurate gene expression results. Although there is evidence supporting that good and reliable microarray data can be obtained from RIN above 5 (Fleige and Pfaffl, 2006), I think that the RIN less than 7 of patient replicate samples could contribute to the invalid data as it could provide a false-positive result on the abundance of the gene expression in the samples. In addition, it has been demonstrated that for a sensitive and reliable quantitative measurement of low abundance mRNA gene expression by qRT-PCR, it requires an intact, good quality and integrity of RNA samples (Vermeulen *et al.*, 2011). Hence, it is critically important to obtain an intact and non-degraded RNA samples so that the microarray analysis and qRT-PCR results provide a meaningful, reliable and valid gene expression data.

Another factor that could influence the validation results was the fold change of the genes. DUSP1 was shown to be upregulated in qRT-PCR and this corresponded to the microarray data. In the microarray data, DUSP1 was 11.3-fold upregulated in the patient, suggesting that maybe a high fold change is needed for the qRT-PCR to get a similar result to the microarray analysis. For other genes such as Smad3, TIA1 and TRAF5, the fold change was small, between 2 to 2.5, therefore the differences in expression could not be detected between patient and controls by qRT-PCR.

It is proposed to have at least three replicates for a reliable and sufficient analysis. It is important to have sufficient replicates 1) so that the variation in the experiment can be measured and statistical tests can be applied to detect the differences, and 2) to enhance the precision of the gene expression measurements to allow smaller changes to be detected (http://www.Illumina.com). However, due to the patients' illness only very limited material was available; only two biological replicates could be obtained from the patient. This could affect the quality of the data and lead to the invalidity of the results.

Another approach to investigate the defective apoptosis was by using a Tet2 knock out mouse model in which to analyse Fas-mediated apoptosis. A mouse model was chosen instead of knocking down TET2 in a cell line because epigenetic effects are cumulative during development and involved in regulating gene transcription. If knocking down the gene in cell lines, most probably TET2-dependent epigenetic changes influencing gene transcription had occurred earlier and are thus maintained. Therefore, we might not be able to see differences between knock down and wild type cells. The statistical analysis demonstrated there was a difference of cell survival in which the overall cell survival was higher in B cells from Tet2 knock out mice compared to B cells from wild type mice, but the differences were very small, making it difficult to derive a definite conclusion from the experiment. However, it should be noted that the Tet2-deficient mouse models, the Tet2 floxed allele mice (which possess loxP sites flanking exon 3 of the Tet2 gene) were crossed to mice carrying the Cre-recombinase under the Pgk promoter, generating mice bearing a homozygous null mutation of Tet2 whereas both patients bear a homozygous missense mutation of TET2, substituting A>G in exon 9. Therefore the effect could still be different in the patient B cells, and could not rule out the possibility the defect may still be in the B cells. However, this could not be confirmed since the Fas mediated apoptosis assay has never been performed using patient B cells.

Next, I wanted to investigate Fas-mediated apoptosis in mouse T cells to see if there is a stronger effect. This is also being suggested by the fact that the Fas-mediated apoptosis assay was performed on both patients' T cells before

transplant, which showed a defect in the Fas-mediated apoptosis. The first experiment performed on wild type and Tet2 knock out mice where the T cells were blasted with PHA was unsuccessful as most of the T cells were dead. Another method was applied where the cells were stimulated with plate-coated anti-CD3/CD28 and treated with IL-2 every day until soluble Fas Ligand was added to the cells. It was demonstrated that mouse T cells grow well when stimulated with either Con A (Mukherjee *et al.*, 2005) or anti-CD3 or anti-CD3/CD28. This method was successful as it was shown in wild type mice that more cells were viable before treatment with soluble Fas Ligand. For future work and once the Tet2 knock out mice are available again, this method can be applied to investigate the effect of Tet2 on Fas-mediated apoptosis in T cells.

So far, both approaches that were performed in order to investigate the effect of TET2 on lymphocyte apoptosis were unsuccessful and no conclusion could be derived yet. More future work especially using T cells is required to enable us to understand the disease phenotypes in the patients that was caused by TET2 mutation.

Chapter 5: Discussion and future work

In this study, I identified novel gene defects that cause two different PIDs. These two newly identified disease genes contribute to the current list of gene defects in the PID classification (Picard *et al.*, 2015). IFNAR2 deficiency, which I identified in a patient with fatal illness after routine MMR vaccination, results in isolated susceptibility to viral infection. Recognition of this phenotype provides better knowledge and understanding of the narrow role of type I IFN in human antiviral immunity, similar to the previously described *STAT2* mutation (Hambleton *et al.*, 2013) . The *TET2* mutation that was identified in patients with immune dysregulation, resulting in ALPS with lymphoma in both patients, provides new insights into immune function and the apparently restricted non redundant role of this important protein. So far, to my knowledge, no genes involved in epigenetic regulation have been described to cause PID.

Clinical impact

Besides adding to knowledge regarding immune function, these discoveries provide a molecular diagnosis for the patients. The ability to group patients according to molecular diagnosis can ultimately lead to better knowledge of the natural history of disease and hence prognosis. In addition, it can also inform better treatment of future patients with the same defect.

For the newborn baby and future patients with IFNAR2 deficiency, HSCT is not likely to represent a curative treatment since IFNAR2 acts ubiquitously and not just within the immune system. Not only would HSCT be unlikely to correct the defect, it would pose an extremely high risk of transplant-associated viral infection during the inevitable period of immune suppression. In contrast, HSCT should still be considered a treatment option for future patients with TET2 deficiency, despite the poor outcome of HSCT for both patients described in this thesis. Patient 1 developed split chimerism and recurrent ALPS, whereas patient 2 died after rejecting a haploidentical T-cell depleted transplant. The precise reasons for this outcome after HSCT are unclear and probably multifactorial, but it seems likely that the underlying defect contributed by favouring graft rejection (as seen in mixed chimeric mouse models). Considering the predominant haematological phenotype of TET2 deficiency, it

seems plausible that HSCT with 100% donor chimerism could offer a cure. As an alternative to HSCT, future TET2 patients might instead be treated with sirolimus, an mTor inhibitor, as recently this medication has become the treatment of choice for ALPS patients (Teachey, 2012).

By identifying the *IFNAR2* mutation in the newborn baby, preventive measures can be taken such as avoidance of the live MMR vaccine that contributed to the demise of the deceased sibling. Currently the infant is also receiving both prophylactic aciclovir and subcutaneous immunoglobulin to bolster antiviral defences. Further useful information for possible treatment options comes from our microarray measuring transcriptional responses to both IFNα/β and IFNγ using patient and control cells. It was shown that both type I and type II IFN induce similar ISGs, emphasising that IFNγ also provides antiviral effects that may partially compensate for the lack of type I IFN. This is helpful in suggesting that exogenous IFNγ might be a useful treatment in the face of uncontrolled viral infection in the patient.

Scientific implications

This work raises further questions that need to be answered by performing more experiments in vitro. It was demonstrated that both type I and II IFNs upregulated a similar set of genes in the control cells at one time point; type I-induced ISGs were even upregulated by IFNγ in the patient cells, showing that it occurs independent of an IFNAR-mediated feedback loop. It would be interesting to look at the functional response i.e. antiviral effect of IFNγ as it might be possible that this IFN upregulates the same ISGs at the transcription level but in terms of the function it may be less compared to IFNα. This can be done by treating patient and control cells with IFNγ and infected them with different viruses and observe the antiviral effect i.e. virus replication in these cells.

Recently, there were several studies demonstrating that type I IFN plays a role in mycobacterial infection (Manca *et al.*, 2001; Berry *et al.*, 2010; Desvignes *et al.*, 2012; McNab *et al.*, 2014). For example, one study produced transcriptomic data which were interpreted as showing that both type I and type II IFN ISGs were upregulated in patients with *Mycobacterium* infection (Berry *et al.*, 2010).

Since my work demonstrated that both types of IFN upregulated a similar set of genes, it brings into question the contribution of type I IFN to this transcriptional signature. Irrespective of that, whether type I IFN protects or predisposes to mycobacterial infection is controversial. McNab et al. demonstrated that type I IFNs have a detrimental effect on mycobacterial infection by inhibiting the release of IL-12, IL-1β and TNFα from infected macrophages, thus preventing the cells from achieving the activated state required for mycobacterial killing (McNab *et al.*, 2014). In contrast, Desvignes et al, tested the role of type I IFN by measuring the additive effect of IFNAR knock-out on IFNGR knock-out background. They observed that upon infecting the mice with *Mycobacterium Tuberculosis*, mice deficient in both type I and II signalling showed more severe mycobacterial infection and died earlier compared to the single knock-out IFNGR-/- mice (Desvignes *et al.*, 2012). They concluded that type I IFN plays a protective role against mycobacterial infection, most probably by aiding the recruitment of T cells (Desvignes *et al.*, 2012).

My recent findings suggest the opportunity for future work to clarify the role of type I IFN in mycobacterial immunity using the patient cells as a model. With current emerging techniques, patient fibroblasts can be reprogrammed into iPSCs and then differentiated into a wide variety of cell types including macrophages. This would provide the opportunity to interrogate the role of type I IFN in mycobacterial infection in the relevant human cell type. Furthermore, the use of genome editing techniques such as CRISPR/Cas9 (see below) provides the opportunity to introduce or repair mutations in a specific manner, thus providing an ideal control for patient iPSCs on the same genetic background.

Recent advances have led to the use of iPSCs in modelling many diseases including PIDs. In the latter field, iPSCs can be differentiated into HSC progenitors which later differentiate into either myeloid progenitor or lymphoid progenitor cells (Weinacht *et al.*, 2012), depending on the relevant cell type. This is a promising method because in theory, iPSCs could provide an unlimited cell source for the study of PIDs (Weinacht *et al.*, 2012). Induced PSCs have been successfully used to generate NK cells (Ni *et al.*, 2011) and B cells (Carpenter *et al.*, 2011) although in general lymphoid differentiation remains

challenging. In terms of providing relevant material for the study of PID, Zou et al. were able to differentiate iPSCs from patients who had been diagnosed with chronic granulomatous disease (CGD) into neutrophils and recapitulate the failure to generate reactive oxygen species upon activation, which is typical for this disease (Zou *et al.*, 2011). The ability to generate alternative cell types from iPSCs could help to overcome the limitations of scarce patient material, which is a major problem in the field of PID research. Several factors contribute including the fact that patients are often acutely ill infants, in whom the abundance of the relevant cell type may be reduced as part of the disease state; it may not be possible to obtain the relevant tissue before death or transplantation.

This is the major limitation that I encountered in performing the functional studies for TET2 using patient material, when availability of fresh immune cells was limiting. Patient 2 is dead and patient 1 had a bone marrow transplant substituting his defective immune cells with healthy ones from a matched donor. However, as he developed mixed chimerism with donor T cells and recipient B cells, there was the potential to obtain TET2-deficient B cells. Yet, as he intermittently received rituximab, a drug that depletes B cells, their availability was very limited. The generation of an EBV B-cell line was attempted using patients cells in order to provide immortalized B cells, however, it was unsuccessful. Therefore, to overcome the very limited material from the patients, and to investigate the effect of TET2 deficiency within the stem cell compartment, we generated iPSCs from patients' fibroblasts. In chapter 4.2, I have briefly mentioned the technique used to generate iPSCs. My colleague, Dr Katarzyna Tilgner, performed a pilot study measuring the 5hmC level of patient iPSCs and control human embryonic stem cells (hESCs). Ongoing work involves differentiating these patient iPSCs into HSCs, followed by further differentiation into T and B lymphocytes.

In parallel, another novel approach that is currently used in PID modelling and gene correction is genome editing using engineered endonucleases which include Zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated 9(Cas9) (Ott de Bruin *et al.*, 2015).

These methods can be used to correct the defective PID gene (Ott de Bruin *et al.*, 2015) and/or to model PIDs by introducing disease-associated mutations, allowing the generation of knock-out cell lines or mouse models (Ott de Bruin *et al.*, 2015). In general, these engineered endonucleases create a double stranded break (DSB) in the specific genomic site that needs to be modified (Sander and Joung, 2014). The nuclease-induced DSB can then be repaired either through the error-prone process of non-homologous end-joining (NHEJ) which can produce insertion and/or deletion mutations of variable length at the site of the DSB or through the homology-directed repair (HDR) that can produce precise point mutations or insert the desired sequences through recombination of the target locus from a single-stranded or double-stranded DNA donor template (Sander and Joung, 2014). Various studies have successfully demonstrated correction of the defective gene in human stem cells using these genome editing tools (Hockemeyer *et al.*, 2011; Zou *et al.*, 2011; Ott de Bruin *et al.*, 2015). For example, Zou et al. successfully used ZFNs to correct CGD patient-derived iPSCs which restored the ability of the cells to generate functional neutrophils (Zou *et al.*, 2011).

Since these genome editing tools have shown to be successful, for future work, CRISPR/Cas9 correction of the *TET2* mutation in patient iPSCs will be performed. The corrected cells will provide an ideal control for the patient iPSCs in further functional work with their differentiated progeny. In addition, the CRISPR/Cas9 technique could also be used to introduce the patients' specific missense mutation to generate a mouse that more faithfully models their disease than the full knock out described in chapter 4.3.

For the TET2 patients, as has been mentioned in the chapter 4.2 discussion section, investigation of the epigenome including mapping of hydroxymethylation will most likely be required to understand disease mechanism. New advanced techniques such as single cell epigenomic analysis (Smallwood *et al.*, 2014) can be considered for the small quantities of cryopreserved material still available from patients. However, with current knowledge it will be difficult to interpret the result in terms of comparing differences of hydroxymethylation in the epigenome with a suitable and appropriate control. Nevertheless with new emerging projects such as

EpiGenome, ENCODE and Blueprint which are funded by organisations such as National Institute of Health (NIH) and International Human Epigenome Consortium (IHEC) for the European societies, this problem could be solved. These projects are involved in mapping the epigenome involving DNA methylation and other epigenetic changes in normal blood cells. Hence, these projects will be able to provide the reference epigenome for normal blood cells.

Finally, to conclude, *IFNAR2* and *TET2* are novel disease genes and have never before been described in PIDs. The suggested future work will hopefully be able to elucidate further the role of each gene in the cellular pathways and mechanisms of host defence and regulation of the human immune system.

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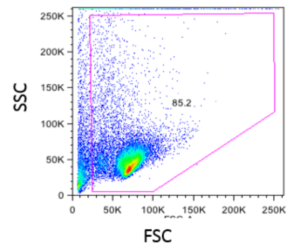
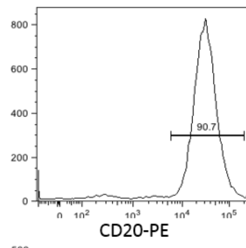
Appendix





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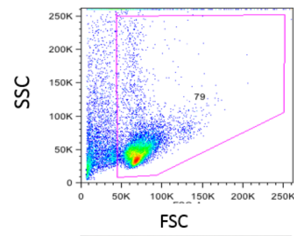

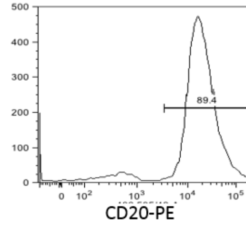

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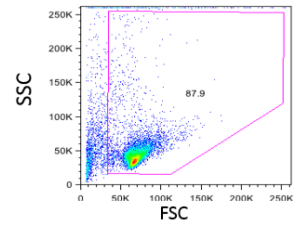

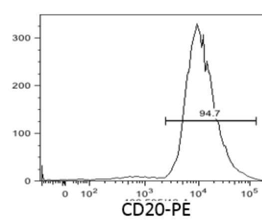

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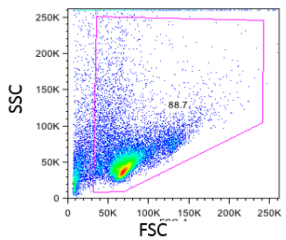

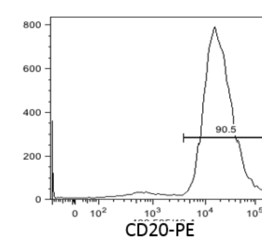

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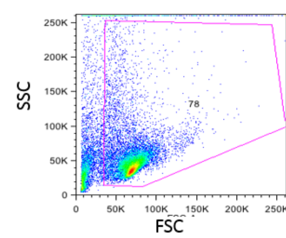

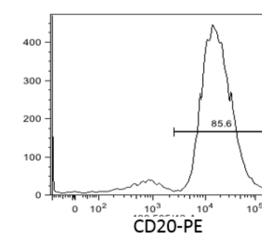

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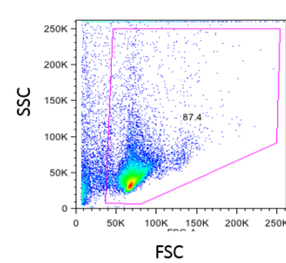

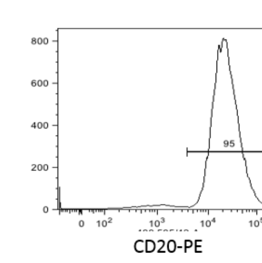

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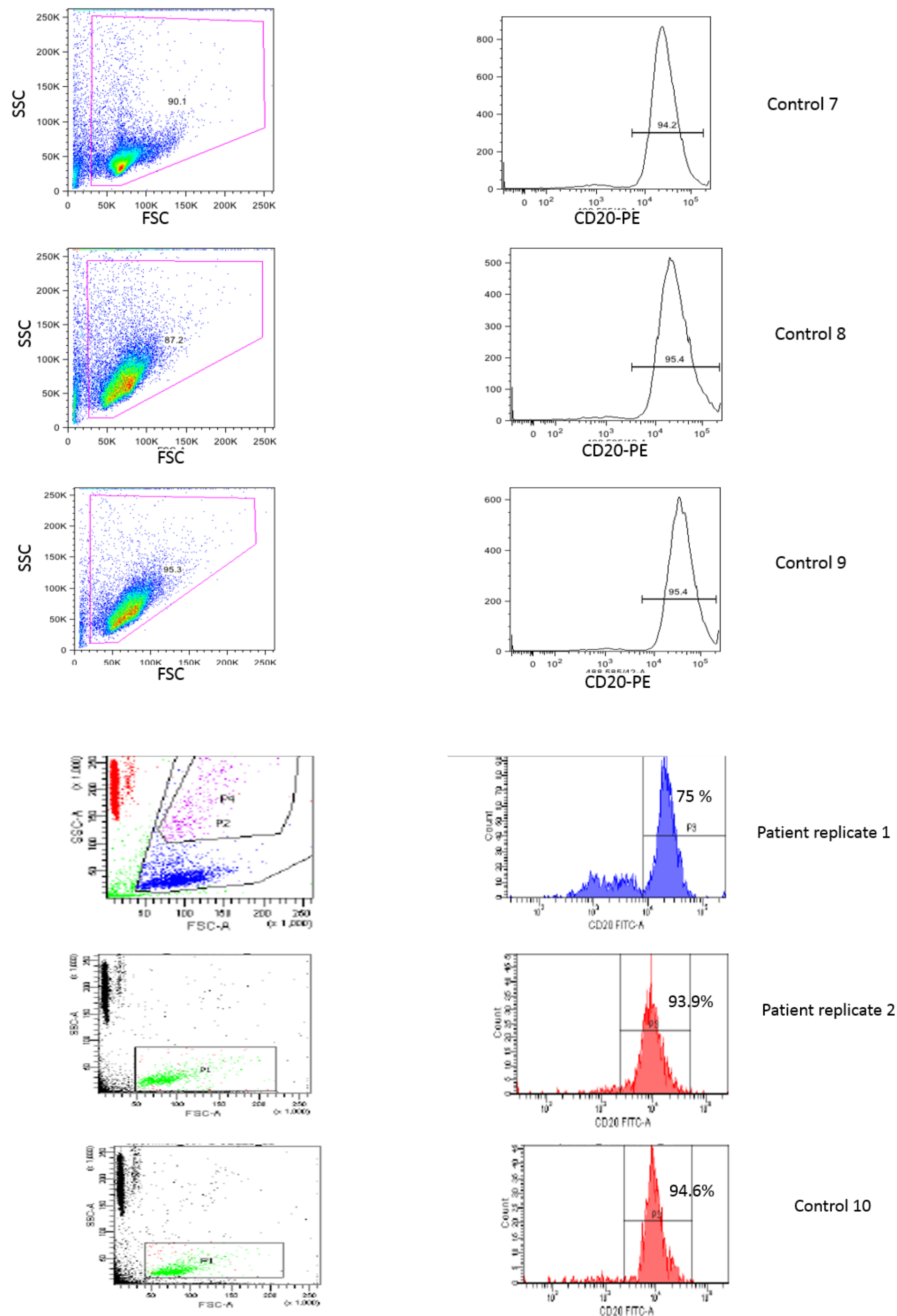
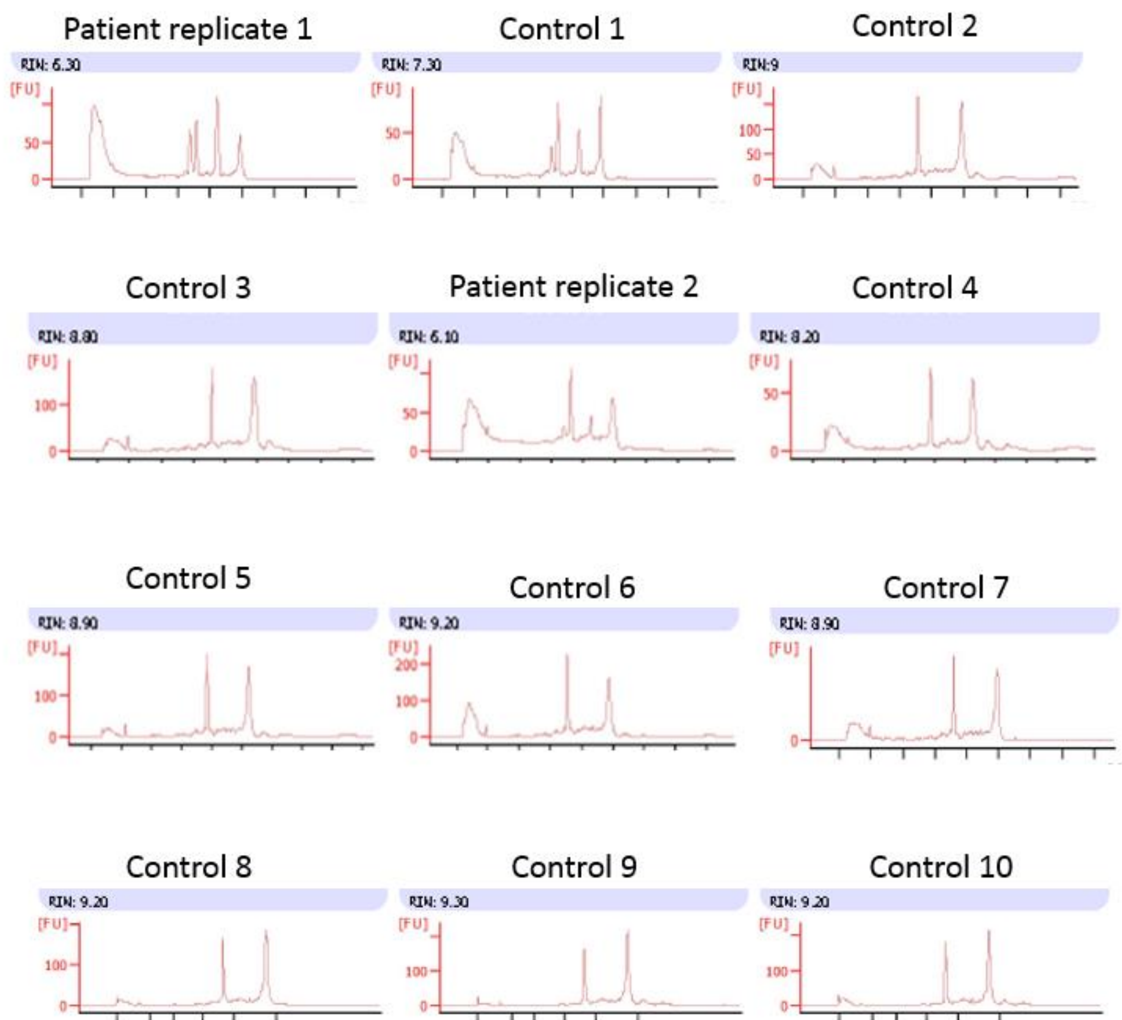
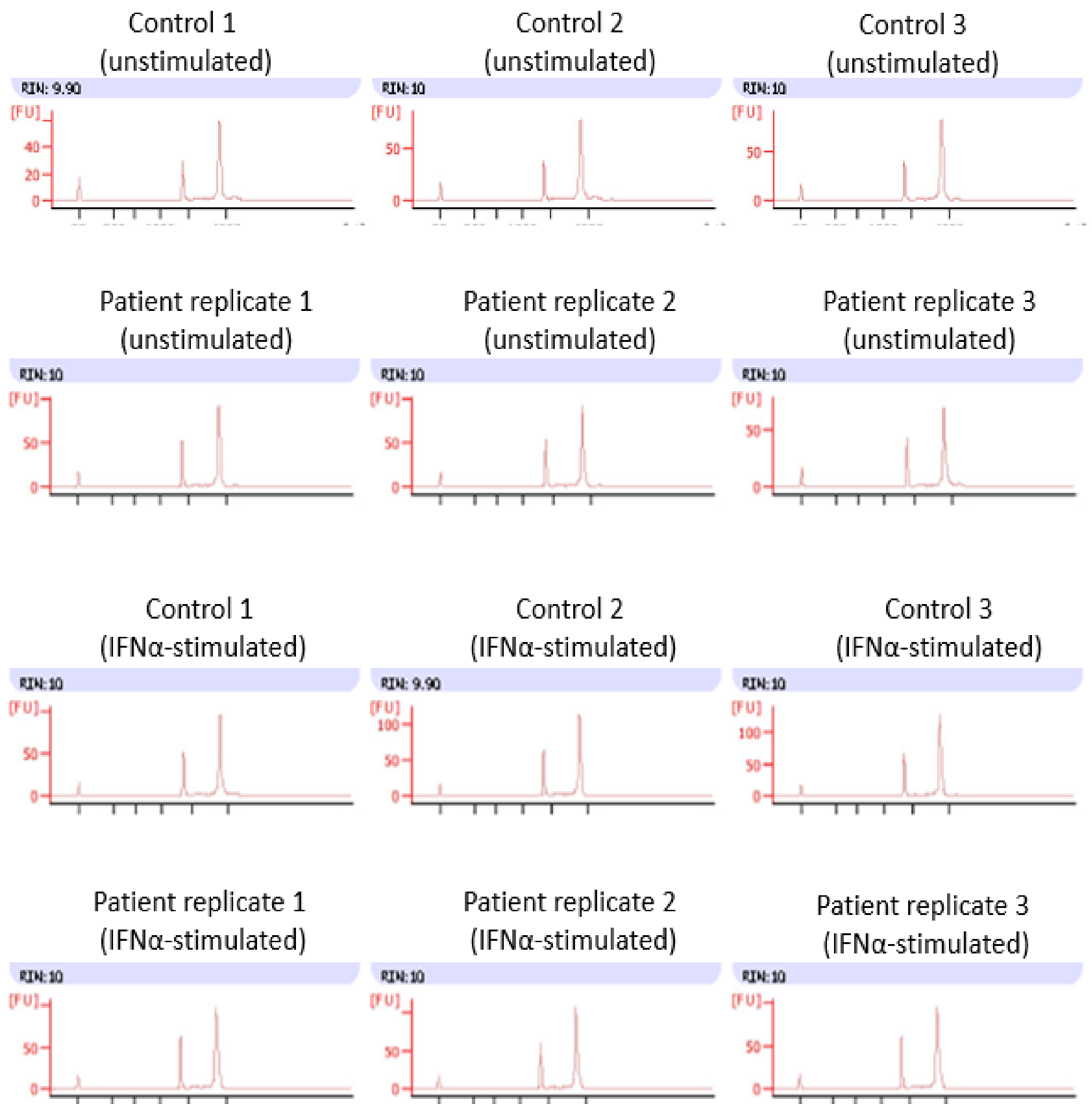


Supplementary Figure 1: B cells isolation purity assessment of patient replicate and controls samples for microarray analysis for the study of the novel defect of autoimmune lymphoproliferative disease,. Left panel : representative of FSC/SSC dot plot and the gated cells were shown in right panel . Right panel : representative of the purity of B cells.



Supplementary Figure 2 : RIN number of each patient replicate and controls B cells RNA . RNA quality and integrity was determined using Lab on-Chip analysis on the Agilent 2100 Bioanalyzer (Agilent Technologies) before performing microarray analysis (Illumina HT-12) for the study of the novel defect of autoimmune lymphoproliferative disease.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(unstimulated)
RIN: 9.90
[FU]
40
20
0
Control 2
(unstimulated)
RIN: 10
[FU]
50
0
Control 3
(unstimulated)
RIN: 10
[FU]
50
0
Patient replicate 1
(unstimulated)
RIN: 10
[FU]
50
0
Patient replicate 2
(unstimulated)
RIN: 10
[FU]
50
0
Patient replicate 3
(unstimulated)
RIN: 10
[FU]
50
0
Control 1
(IFNα-stimulated)
RIN: 10
[FU]
50
0
Control 2
(IFNα-stimulated)
RIN: 9.90
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100
50
0
Control 3
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(IFNα-stimulated)
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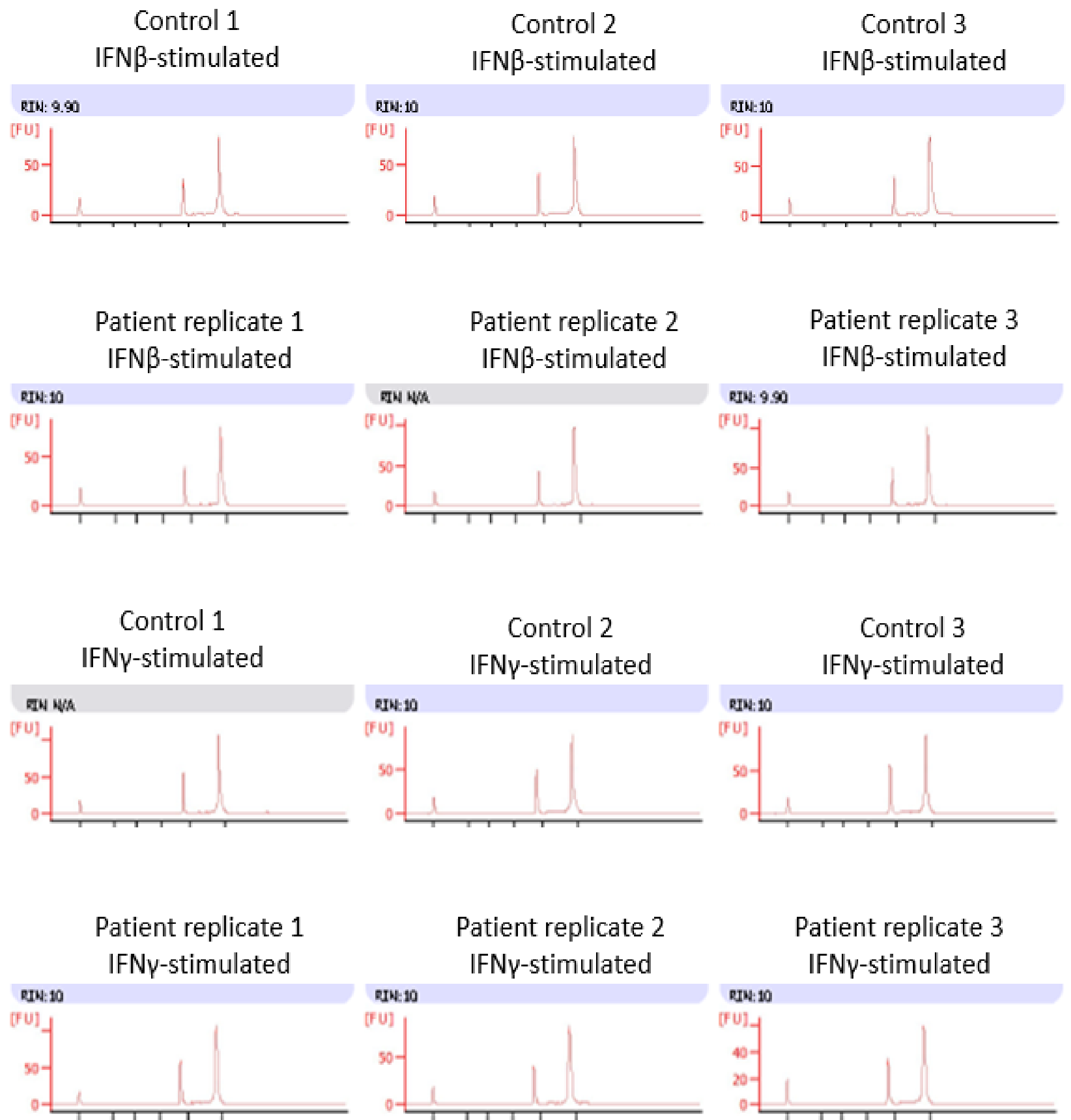

Supplementary Figure 3 : RIN number of unstimulated and IFNα,IFNβ,IFNγ-stimulated controls and patients samples. RNA quality and integrity was determined using Lab on-Chip analysis on the Agilent 2100 Bioanalyzer (Agilent Technologies) before performing microarray analysis (Illumina HT-12 (2x)) for the study of the novel defect of IFN signalling.

Oral presentation

1. A novel defect of Type I Interferon signalling pathway.
   Immunology North East, Annual General Meeting (AGM), June 2015.

Publication arising from this study