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Investigating the role of the B-cell receptor in mantle cell lymphoma

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Thesis for the degree of Doctor of Medicine

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ABSTRACT

Faculty of Medicine

<u>Cancer Sciences</u>

Thesis for the degree Doctor of Medicine

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Mantle cell lymphoma (MCL) is a mature B-cell lymphoma typically characterised by the expression of cluster of differentiation 5 (CD5) and a t(11;14) translocation resulting in overexpression of cyclins. MCL displays significant morphological and clinical heterogeneity. Like chronic lymphocytic leukaemia (CLL), MCL can be divided into two subsets based upon immunoglobulin heavy chain variable (*IGHV*) region status: the more common unmutated (U-) *IGHV* and less common mutated (M-) *IGHV* subset. It has been suggested that, like in CLL, *IGHV* status informs different origin and clinical behaviour, with U-MCL being of pre-germinal centre (pre-GC) origin and aggressive clinical behaviour and M-MCL of post-GC origin and indolent course.

In CLL, B-cell receptor (BCR) levels and signalling capacity have also helped to define subgroups with important clinical and biological significance. This thesis aimed to extend investigations to the BCR of MCLs and understand if BCR expression and signalling variation in MCL could affect variation in clinical behaviour.

A cohort of thirty-six MCL cases were assessed for immunogenetic, phenotypic and functional characteristics including signalling capacity. *IGHV3*-21 and 4-34 were equally identified in U-MCL whilst *IGHV*4-34 was most common in M-MCL. Fifty percent (%) had an U-*IGHV* (>98% homology to germline). Surface immunoglobulin (sIg) M was expressed variably but significantly higher in conventional, nodal MCL (p=0.01) and non-aggressive U-MCL (p=0.03) than in leukaemic non-nodal (LNN) or M-MCL.

Surface IgM (sIgM) expression was higher in MCL than in CLL (p<0.01). Mean signalling capacity was significantly higher in MCL than CLL (p<0.01), and correlated positively with sIgM expression. A subgroup of MCL had low signalling capacity similar to CLL. This 'CLL-like' signalling group was composed predominantly of LNN MCL and *IGHV*3-21 U-MCL with low sIgM expression. In contrast to high signalling MCL cases, this group had a CLL-like BCR engaged functional phenotype: recoverable sIgM expression *in vitro*, high basal proximal signalling kinases and an 'activated' glycosylation profile on the constant Ig region.

MCL tissue and matched lymph node and peripheral blood samples were assessed. The lymph node compartment was identified as the likely site of engagement in non-aggressive MCL; sIgM levels were significantly lower in the lymph node than in the peripheral blood in all tissue and paired samples (p=0.03). Signalling capacity was also significantly lower in lymph node tissue samples (p<0.01). Lower sIgM levels in the lymph node may be indicative of site occurred antigen-induced endocytosis. M-MCL with low sIgM was associated with improved event-free survival (p=0.04).

This study reveals that a proportion of MCL have a BCR engaged signature with low sIgM and low signalling capacity. This likely occurs in the lymph node compartment as a result of the influence of putative (super)antigen, with down-modulation of sIgM. This is particularly prominent in LNN M-MCL and may affect the balance between proliferation and anergy. This mirrors other mature B-cell malignancies, particularly CLL, where this interaction influences clinical outcomes.

Research Thesis: Declaration of Authorship

Print name: Dr David Dutton

Title of thesis: Investigating the role of the B-cell receptor in Mantle Cell lymphoma

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

Acknowledgements

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Abbreviations

°C	degree Celsius
Ab	Antibody
ABC/GCB	Activated B-cell like/Germinal centre B-cell like
BCR	B-cell receptor
BL	Burkitt lymphoma
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
Ca^{2+}	Calcium (ion)
CD	Cluster of differentiation
CDR	Complementarity determining region
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
CSR	Class switch recombination
D (-gene segment)	Diversity (-gene segment)
Da	Dalton
DG	Diacylglycerol
DLBCL	Diffuse large B cell lymphoma
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DZ	Dark zone
EDTA	Ethylenediaminetetraacetic acid
EFS	Event free survival
ELISA	Enzyme linked immunosorbent assay
Fab	Fragment antigen binding
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FCB	Flourescent cell barcoding
F_C	Fragment cristallisable region

FITC	Fluorescein isothiocyanate
FR	Framework region
GC	Germinal centre
HEV	High endothelial venules
Ig	Immunoglobulin
IGHV	Immunoglobulin heavy chain variable (gene)
IGKV	Immunoglobulin κ appa variable (gene)
IGLV	Immunoglobulin λ variable (gene)
IMGT	International ImMunoGeneTics information system
IP_3	Inositol-1, 4, 4-triphosphate
ISMCN	in situ mantle cell neoplasia
ITAM	Immunoreceptor tyrosine based activation motif
J (-gene segment)	Joining (-gene segment)
L	Litre
LNN	Leukaemic Non-nodal
LZ	Light zone
M	Molar
M-	Mutated
MALT	Mucosa-associated lymphoid tissue
MCL	Mantle cell lymphoma
MFI	Mean Fluorescence Intensity
NHL	Non-Hodgkin lymphoma
OD	Optical density
OS	Overall survival
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCNSL	Primary central nervous system lymphoma
PFS	Progression free survival
PCR	Polymerase chain reaction
PE	Phycoerythrin

PLC-γ2	Phospholipase C-γ2
PI3K	Phosphoinositide 3-kinase
PIP$_2$	Inositol-4, 5-bisphosphate
PRR	Pathogen recognition receptor
RAG	Recombination activating gene
RNA	Ribonucleic acid
rpm	Rounds/revolutions per minute
RT	Room temperature
s	Second(s)
SDS	Sodium dodecyl sulphate
SHM	Somatic hypermutation
sIg	Surface immunoglobulin
SLO	Secondary lymphoid tissues
SOX11	SRY [Sex determining region-Y]-box11
SYK	Spleen tyrosine kinase
TBS(-T)	Tris buffered saline (with Tween-20)
TEMED	Tetramethylethylenediamine
U	Units
U-	Unmutated
V (-gene segment)	Variable (-gene segment)
WB	Western Blot

Chapter 1 The Immune system and B-cell immunology

1.1 Introduction

This thesis aims to explore the role of the B-cell receptor (BCR) in mantle cell lymphoma (MCL). B-cells are important cells of the immune system that are characterised by the presence of a specific receptor, the BCR. The BCR functions through the immunoglobulin protein and is a key component of a normal functioning immune system. This introductory chapter will therefore give an overview of the human immune system. It will then focus on the structure of the immunoglobulin gene and protein and subsequent B-cell development. This introductory chapter will then discuss the BCR's role in antigen processing in conjunction with T-cells in the germinal centre. Finally, BCR signalling will be discussed, highlighting its modulation and an induced state of quiescence known as anergy.

1.2 The immune system

The immune system has evolved over millennia to protect cellular organisms from other organisms. In humans, the immune system is an organisation of specialised cells adapted for the detection, prevention and removal of non-self, typically infectious agents known as pathogens.[1] Pathogenic microorganisms such as bacteria, fungi, viruses and parasites have co-evolved during this time and are a constant and changing threat. This is particularly so as their lifecycles, and hence ability to evolve, are so much shorter than humans'. There are three substantive parts to the human immune system that help to protect against pathogens.[2] The first is the barrier of skin and mucosa. The epithelia layer is made of dead cells that physically prevent pathogens entering the body. The mucosal layer secretes mucus and enzymes to prevent and degrade pathogenic invasion. It is aided by physiological properties of different systems; the cilia of the respiratory system move mucus out of the lungs whilst the highly acidic environment of the stomach prevents most organisms from passing through to the gut. Pathogens that pass these barriers then encounter further immunological mechanisms known as the innate or non-adaptive immunity and acquired or adaptive immunity.[3][4]

Innate immunity is a germline encoded primitive defence mechanism. It relies on a limited number pathogen recognition receptors (PRR), such as Toll-like receptors, to conserved pathogenic motifs called pathogen-associated molecular patterns (PAMPS).[3] These receptors are found in both plant and animal kingdoms and hence arose from a distant common ancestor.[5] Congenital disease associated with the innate immune system is rare and usually fatal. Innate immunity tends to immediate, rapid responses and utilises all white blood cells other than T- and B-cells. Macrophages, dendritic cells (DC), mast cells, neutrophils, eosinophils and Natural Killer (NK) cells make up the innate immune system. Macrophages and neutrophils engulf and internalise pathogens utilising lysosomal destruction. Once a pathogen is detected by these cells, a rapid inflammatory reaction ensues inducing intracellular signalling and the eventual release of co-stimulatory molecules, cytokine sand chemokines e.g. interleukin (IL)-6.[3]

DCs are, like macrophages, able to phagocytose but can also degrade pathogenic peptides and then present small peptide fragments at the cell surface. These peptides are known as antigens and the DCs are thus known as antigen presenting cells. Presentation of antigen is associated with complex glycoproteins known as major histocompatibility complex (MHC) molecules. This process links the innate immune system to the adaptive immune system meaning that any persisting infection can be further responded to by the adaptive, and hence more specific, immune system.[6]

A further plasma based innate response known as the complement system is based upon a series of hepatic synthesised proteins that bind to the external surface of cells. This powerful cascade of proteins can enhance neutrophil phagocytosis, leading to cell perforation and activation of the adaptive immune system.[1]

1.2.1 Adaptive immune system

The adaptive immune system evolved after the innate system approximately 450 million years ago.[4] Despite the innate immune systems powerful and rapid response to pathogens, it can fail to eliminate some microbes. Hence a more adaptive and specific approach has evolved allowing it to recognise, respond to, and remember any possible antigen that it

may encounter.[4] It does this through clonally adaptive membrane bound receptors found on T (thymus-derived) and B (bursa or bone-marrow-derived) cells respectively known as T-cell receptors (TCR) and B-cell receptors (BCR).[7] Both BCRs and TCRs undergo a process of gene rearrangement from their germline configuration permitting them to encode a functional antigen recognition structure.[8] The functional molecule in this structure is the immunoglobulin protein discussed later in this chapter. This gene rearrangement process is followed by antigen engagement resulting in clonal amplification, cellular maturation and, in B-cells, somatic hypermutation (SMH) and affinity maturation, isotype switch and finally antibody production. [9] The adaptive immune system is characterised by a slower response to pathogens but it is more specific than the innate system (**Table 1.1**).

Table 1.1: Characteristics of innate and adaptive immune systems

Characteristics	Innate	Adaptive
Receptors	Primitive, non-specific to conserved pathogenic regions	Highly specific – discriminates minor molecular differences
Speed of onset	Rapid: Hours to days	Slow: Days to weeks
Diversity of response	Low	High
Amplification response	Minor	Major
Duration	Short (days)	Long (months/years)
Cells involved	Myeloid: Phagocytes (neutrophils, macrophages, dendritic cells), inflammatory mediators (mast cells, eosinophils and basophils)	Predominantly lymphoid T and B-cells plus antigen presenting cells (APCs)
Plasma components	Complement	Immunoglobulins

T-cells are generated in the bone marrow as pro-thymocytes that then migrate to the thymus for selection and maturation. Single positive $CD4^+$ or $CD8^+$ populations emerge as naïve precursors that can persist for many years. The TCR is a cell surface molecule involved in a diverse range of functions including signalling between B- and T-lymphocytes, inducing cell death in virally infected cells, enhancing cellular cytotoxicity through macrophage activation and immune response regulation.[2][7]

B-cells, identified through the presence of the BCR, are produced in the bone marrow. The 'B' in their name arises from the bursa of Fabricius; this structure is found in birds where the avian B-cell equivalents mature. Broadly, these cells enter the lymphoid circulation as naïve cells that encounter antigen, mature to memory or plasma cells that can secrete immunoglobulin and can persist for many years in the bone marrow.[7] They will be discussed further in the following sections.

1.1 Immunoglobulin Gene

There are three pairs of genes encoding the antigen receptor immunoglobulin; the immunoglobulin heavy chain (*IGH*) gene located at 14q32.3 and the immunoglobulin light chain *κ* (*IGK*) and *λ* (*IGL*) genes found on chromosome 2p12 and 22q11 respectively. There are four parts: variable (*V*), diversity (*D*), joining (*J*) followed finally by constant (*C*) each pertaining to a region of the immunoglobulin heavy chain **(Figure 1.1)**.[10]   In contrast, light chains are made up of only *V* and *J* gene types. This nomenclature identifies a group of genes to a multigene family e.g. *IGHV*. Furthermore, immunoglobulin 'subgroups' identify subsets of genes, that, within a given species, share at least 75% identity e.g. *Homo sapiens IGHV1* subgroup. Mutations at the nucleotide level that identify polymorph variants, also known as alleles, are designated further numbers e.g. *IGHV*1-1*01. The constant region of the immunoglobulin H locus is different as it is designated by a letter and number e.g. *IGHCH*1.[10]

Chromosome 14q32.2
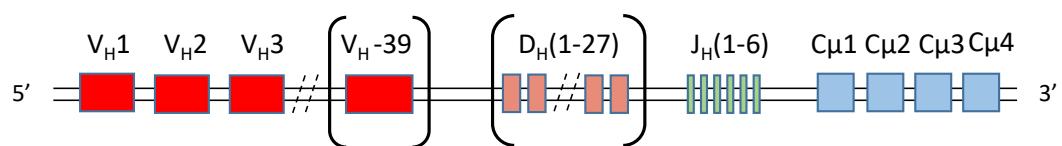
Figure 1.1: Germline configuration of Immunoglobulin Heavy Chain locus.

The heavy-chain (H) locus has approximately 39 functional Variable (V) Heavy (*H*) gene segments and a cluster of approximately 27 diversity (D) segments. These are found between the V_H gene segments and six J_H gene segments. The heavy-chain locus joining (J) genes are followed by a cluster of 4 Constant $_H$ genes. Pseudogenes are omitted. All V gene segments are illustrated in the same orientation. This figure is not to scale: the H-chain locus is over 2 million bases long whilst in contrast the D segments are less than 10 bases long. Adapted from *Immunobiology: The immune system in health and disease, 2001*.[11]

IGHV usage is biased in healthy B-cells. *IGHV*3, 4 and 1 are encoded in 39%, 41% and 10% in naïve B cells with enrichment for *IGHV*3-21 (5%), 1-18 (6%) and 5-51 (3%). IgM positive memory cells also encode *IGHV*3 and 4 but less frequently use *IGHV*1 (~60%, 20% and 5% respectively).[12]

1.2 Gene rearrangement and variability

Gene rearrangement occurs in the bone marrow independent of antigen.[13] The process involves two 'looping out' processes: splicing *J* to *D* segments then *D* to *V* deleting any intervening segments.[2] This appears to be an imprecise process with the addition of nucleotides resulting in huge variability. *V* gene rearrangement is catalysed by the lymphoid specific proteins recombinase activating enzymes RAG1 and RAG2.[14] Terminal deoxynucleotidyl transferase (TdT) is another key enzyme expressed during variable gene rearrangement, which inserts non-encoded sequences (N) between the *V- D-* and *J-* junctions.[15] Interestingly, TdT expression is much higher in the bone marrow than in foetal liver where early haematopoiesis occurs. Accordingly, there is less BCR diversity in neonatal B-cells. TdT expression ceases in late pre-B-cells and thus N sequences are not found in the light chain *V-J* junctions.[1] Following successful gene rearrangement on one allele, the other allele becomes silenced in a process known as allelic exclusion. [16] In this way, each B-cell is uniquely committed to only one BCR.

Once a successful *V-D-J* sequence has been re-arranged the sequence is transcribed along with the downstream µ constant region (**Figure 1.2**). Following translation, cytoplasmic µ heavy chain resides intracellularly defining a pre-B-cell. Heavy chain rearrangement usually precedes light chain rearrangement as further differentiation and maturation requires co-secretion of the µ heavy chain with peptide V_{preB} and λ5. Failure of this process leads to apoptosis. If co-secretion is successful then light chain rearrangement occurs with subsequent transcription translation and the expression of full surface IgM marks the transition from pre-B to naïve B-cell.[13]
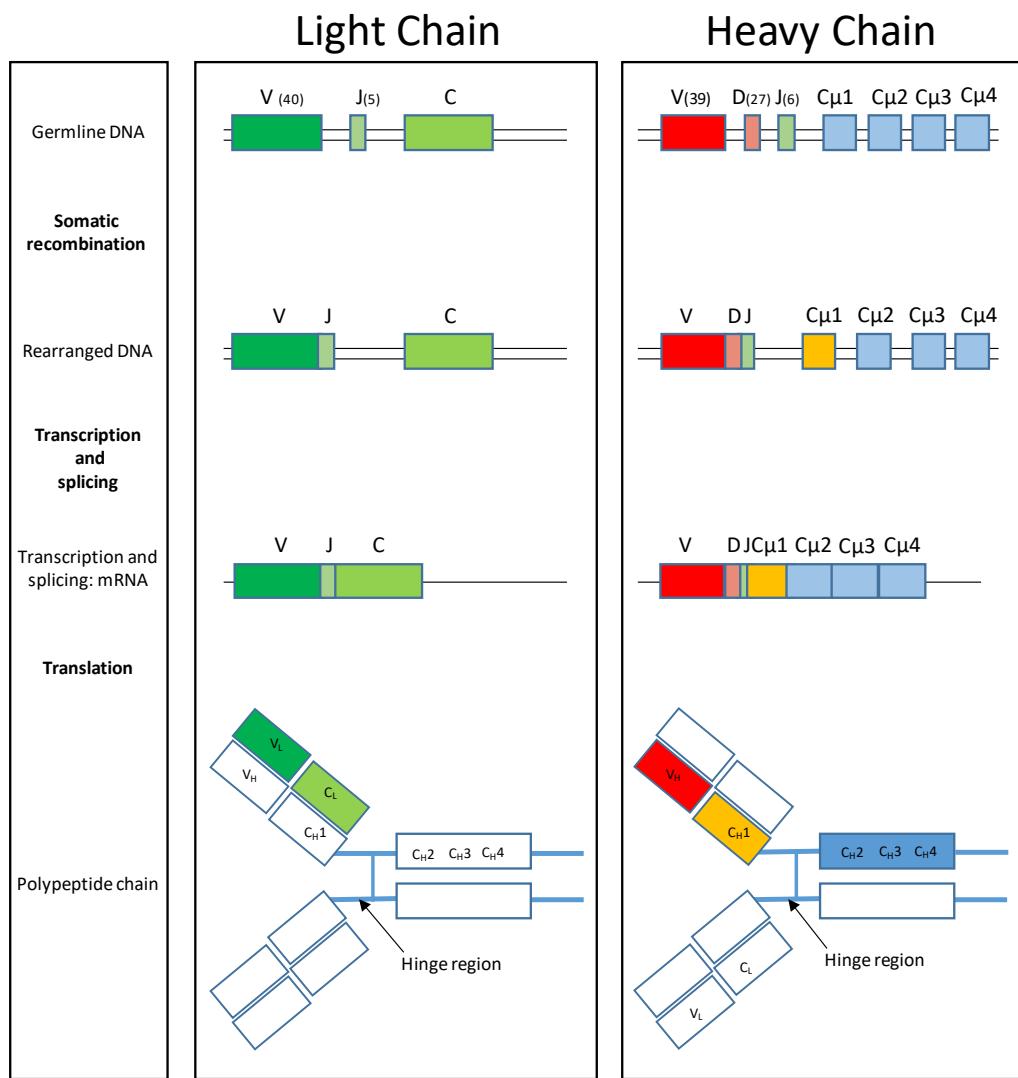
Figure 1.2: Immunoglobulin gene rearrangement

The immunoglobulin (Ig) molecule is made up of two light chains and two heavy chains. Ig heavy chains regions are somatically recombined first rearranging *IGHD* to *IGHJ* at the progenitor (Pro) B stage followed by *IGHV* to *IGHDJ* at the pre-B stage. This forms a complete but somatically unmutated *IGHVDJ* rearrangement and *C*, *μ*, chain. At the pre-B stage, recombination at the *IGK* locus occurs between the *V* and *J* regions. If functional, the light and heavy chains combine and are expressed on the cell surface. See text for full explanation. Adapted from *The Immune system, 3rd Edition*. [17]

1.3 Immunoglobulins:

The immunoglobulin molecules comprise two identical heavy chains cross-linked to two identical light chains forming a Y shaped heterodimeric glycoprotein molecule (**Figure 1.3).** Disulphide bonds bridge the heavy chains together and each heavy chain binds to its light chain. The chains are further divided into domains; five for the heavy chain and two for the light chains. They are functionally split into variable regions, that bind antigen, and constant domains that specify other functions such as Fc receptor binding or complement activation. [13]
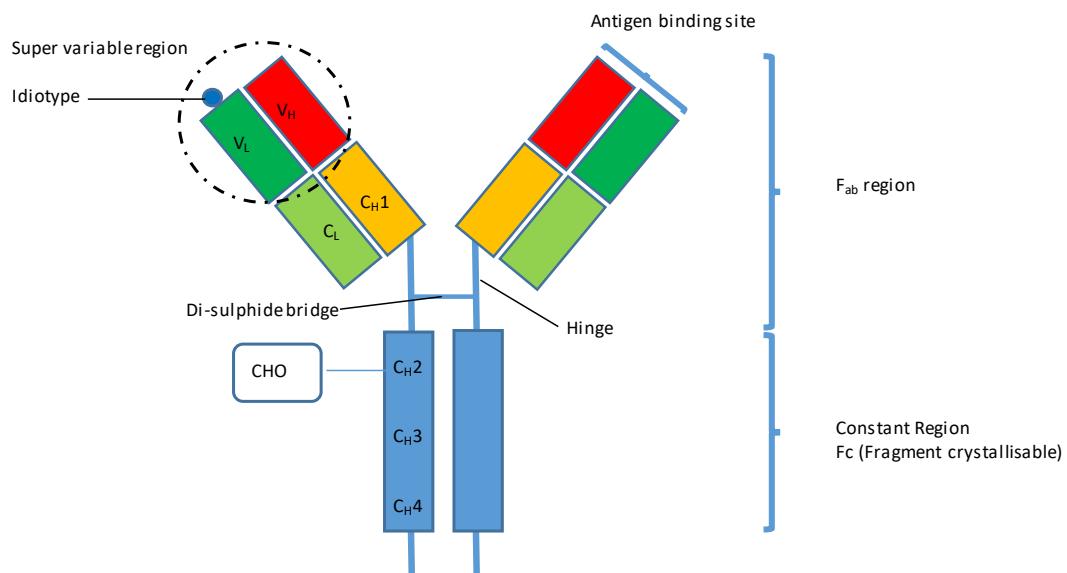
Figure 1.3: The human immunoglobulin molecule

Two heavy chains and two light chains form the F_{ab} region. The variable domain exhibits broad difference between immunoglobulins. The variable region of the heavy and light chain come together to form an antigen-binding site. Antigen binding sites binds to the 'epitope' of the antigen. The variable domain of the immunoglobulin themselves are known as idiotypes; they can be recognised as antigens themselves. Adapted from *Lafranc ML: The immunoglobulin. London, UK:Academic Press, 2001*. [18]

Variability is key to the immunoglobulin's adaptability and the V regions are longer than D and J. However, variability is not spread evenly, but concentrated in shorter hypervariable regions known as complimentary determining regions (CDR) 1, 2 and 3.[19] CDR3 is located at the junction of the J and D segments to benefit from increased variability in order to

recognise a vast number of antigens.[2] These three regions are the antigen-binding site of the immunoglobulin and are known as the antigen determinant. The rest of the V region essentially holds the CDRs in place like a scaffold and are thus known as framework regions (FWR), from one through to four. Antigen determinants can also be identified through their V domains and are called idiotypes. These can act as antigen themselves and be used to identify immunoglobulins.[20]

Immunoglobulins can be split into two regions by the enzymatic action of papain; the 'fragment antigen binding' Fab containing the two arms and the 'fragment crystallisable' Fc portion with no antigen-binding site. Pepsin can separate the two Fab portions further, split by their disulphide bonds, into F(ab')1 and F(ab')2.

The Fab contains C_H1 whilst the Fc portion contains the C_H2-4 chain domains. These C chains define effector function, in contrast to the V segment, which confers antigen affinity.[13] There are five classes of immunoglobulin: M, D, G, A and E. These are based upon variation in heavy chain C region amino acid sequence respectively named mu (μ), delta (δ), gamma (γ), alpha (α) and epsilon (ε). Antibodies function through a variety of mechanisms to enhance the removal of pathogens from the body. These include neutralisation (direct binding inhibit pathogenic function), opsonisation (antibody binding leads to Fc receptor binding on phagocytic cells and pathogen removal), complement activity (the recruitment of complement protein cascades that directly destroy pathogens) and antibody dependent cell-mediated cytotoxicity (ADCC – pathogen bound antibodies bind to cytotoxic cells via the Fc receptor [e.g. Natural Killer cells] resulting in pathogen clearance).[21]

The M subclass is the first Ig expressed in B-cell development and is found in its membrane bound and secreted forms. Membrane bound IgM is a monomeric structure involved in BCR signalling. The larger, pentameric, secreted form, is held together by disulphide bridges between the CH4 chains. IgM is associated with the primary immune response to infection and as such has low antigen affinity but is 'polyreactive' permitting a fast response to a variety of antigens.[13] IgD production results from a splicing variation of the

Cμ chain. Circulating IgD is found at extremely low levels and its effector function is unclear.[22]

IgG makes up the majority of Ig in human blood having the longest half-life. There are 4 subtypes (IgG1-4) based upon structural and functional differences predominantly in the C_H3 chain. IgG functions through activation of the complement cascade and form part of the secondary immune response with high affinity antigen interactions resulting in neutralisation of toxins and viruses.[13]

IgA is an immunoglobulin found at mucosal surfaces and in secretions and is not, therefore, found at high levels within blood. Secretory IgA is a dimeric protein that prevents viral or bacterial invasion of mucosal surfaces through neutralisation and has less complement fixing capabilities. The IgA receptor is, however, found on neutrophil surfaces and may activate ADCC locally.[13] IgE, present at the lowest levels with the shortest half-life, is associated with allergy, hypersensitivity and response to parasitic infections. The IgE Fc receptor is found on eosinophils, basophils, mast cells and Langerhans cells.[13]

1.3.1 Immunoglobulin constant region glycosylation

Carbohydrate (CHO) sugar chains are attached to the constant region of immunoglobulins and affect the biological and functional ability of the proteins.[23] The glycosylated residues are modified throughout BCR maturation and through receptor engagement.[24] Normal B-cells express an N-glycosylation pattern on the heavy chain μ-constant region.[25] The biosynthetic pathway begins in the endoplasmic reticulum where highly mannosylated oligosaccharides are added to asparagine residues.[26] Complex glycans are then added in the Golgi apparatus before the now 'mature' glycosylated sIgM is secreted on the cell membrane surface.[25] Ligation of the BCR by α-IgM results in receptor-antigen endocytosis and modulation of the N-glycosylation pattern revealing immature mannosylated forms. Thus ligation change results in a pattern containing both mature and immature, highly mannosylated, glycans.

1.4 The B-cell receptor

The BCR is the defining functional component of B-cells and is required for mature B-cell survival. [27] The BCR is a complex of membrane IgM, G or D that is non-covalently bound to CD79a (Igα) and CD79b (Igβ). CD79a and CD79b each contain distinct intracellular regions of tyrosine residues, known as immunoreceptor tyrosine based activation motives (ITAM), within their cytoplasmic tail.[2]

Naïve human B-cells express sIgD homogenously but demonstrate variable expression of sIgM. Surface IgM ranges from undetectable (background staining) to the 4th or 5th log. [28] Generally, BCRs are found in large numbers equating to 120,000 to 200,000 copies per cell.[29] The BCRs, including both isotypes M and D, are not randomly associated throughout the membrane but are instead compartmentalised.[30] Super resolution microscopy has revealed distinct, but separate, clusters of IgM-BCRs and IgD-BCRs. These are known as protein islands.[29] Further imaging techniques, including two colour direct stochastic optical reconstruction microscopy, has identified an alteration in protein island composition upon B-cell activation. The BCR protein islands reduce in size and disperse resulting in IgM and IgD-BCRs coming closer together.[31]

The BCR isotypes sIgM and sIgD are expressed variably through B-cell development. The specific role of sIgD has been unclear. Single isotype deficient B-cells can mature, survive and mount normal immune responses to antigen. Furthermore, one isotype will compensate for the other by upregulation.[32] [33] To explore the role of sIgD further, a deficient *dmit* mouse mutant has been investigated. This mutant has a heavy chain constant region amino acid substitution resulting in impaired light chain pairing. Therefore, although transmembrane binding to CD79a/b is not impaired, 'normal competition' with sIgM for the CD79a and b dimers prevents a compensatory sIgM increase seen in previous studies. In this scenario, lack of functional sIgD caused significant survival disadvantage when the *dmit* B-cells competed with wild type B-cells implying a B-cell survival role for sIgD. [34]

1.5 B-cell Lymphopoiesis

At the end of the first trimester, B-cells are first produced in foetal liver before production is switched to the bone marrow. This continues through life and B-cell progenitors continue to make up approximately 2% of bone marrow mononuclear cells.[1] Each stage of B-lymphopoiesis is defined by *IGHV* rearrangement and BCR location (**Figure 1.4**). Differentiation of the pluripotent stem cell to progenitor (Pro) -B-cells, in the form a proliferating lymphoblast, is associated with the expression of recombinase activating genes *RAG1* and *RAG2* and initiation of immunoglobulin gene rearrangement. There is no surface immunoglobulin at this point but the first identifiable phenotypic changes with the expression of surface CD19, CD24, MHC class II and intracellular CD22. CD19 is a surface antigen that is present on early bone marrow progenitor cells and then persists through all stage of maturation regulating BCR signalling.[35 36] This maturation process is cytokine driven and relies upon interleukin-7-receptor signalling (IL-7R).[37]

Pro B-cells develop further into pre–B-cells characterised by the expression of the pre B-cell receptor (pre-BCR). The pre-BCR is composed of polymorphic 'surrogate' light chains, a heterodimer of λ5 and VpreB, that pair with a functionally rearranged μ Heavy chain forming the pre-BCR. Signalling through the pre-BCR results in an expanded pool of precursors that can undergo light chain rearrangement. Ligand independent pre-BCR auto aggregation has been identified on the cell surface; it appears to be driven by interactions between pre-BCR charged, glycosylation residues, a form of cell autonomous signalling.[38] As the pre-BCR molecules aggregate, CD79a and b are phosphorylated by SRC kinases resulting in proximal signal propagation. Spleen tyrosine kinase (SYK) and ζ-chain-associated protein kinase of 70kDa (ZAP70) are recruited and act upon the B-cell linker protein BLNK. This, in turn, recruits phospholipase Cγ2 (PLC-γ2) and Bruton's tyrosine kinase (BTK). PLC-γ2 phosphorylation causes calcium mobilisation and Ras activation leading to downstream extracellular signal-regulated kinase (ERK) activation, proliferation, and growth.[39] Large pre-BCR B-cells undergoing active cell signalling and cell cycling have high nuclear to cytoplasmic ratios. BLNK deficient pre-B-cells express high levels of the pre-BCR and outlive their normal counterparts resulting in leukaemic transformation. Accordingly, a significant proportion of pre-B acute lymphoblastic leukaemia patients have impaired *BLNK* transcription. [40]

Developing B-cells then move through to a period of slower cell division. The smaller, less active cells are now termed "small pre-B-cells". Light chain rearrangement of the *V* and *J* genes occur allowing the developing B-cells to acquire a functionally rearranged but still immature BCR. Immature B-cells are distinguished from their pre-B-cell precursors by this combination and B-cells are characterised overall by the presence of the BCR and other co-receptors such as CD19.

1.6 Central Immune Tolerance

B-cell development is, until this point, an antigen independent process within the bone marrow. However, the B-cell populations now undergo a significant check point to assess the BCR against reactivity to self, i.e. autoantigens. This determines whether the specific immunoglobulin recombination will be admitted into the peripheral B-cell repertoire. This is termed 'central tolerance' and refers specifically to the negative selection of self-reacting B-cells in the bone marrow.[41] This process is fundamental to producing an immune repertoire that is reactive to foreign, non-self, antigen and mostly devoid of self-reacting BCRs. Assessment of the bone marrow early immature B-cell repertoire reveals 50%–75% of BCRs reactive to self-antigen.[42] Removal of these self-reactive BCRs occurs by apoptosis, while rescue of self-reactive BCR employs a process of 'receptor editing', by which the self-reactive BCR undergoes secondary recombination.[43] Transgenic mouse models experiments revealed that autoreactive immature B-cells go through a secondary rearrangement of their Ig light chain locus. This results in new heavy-light chain pairings with altered avidity forming a non-autoreactive BCR.[44] Although an efficient process, persisting self-avidity can be removed by a secondary process of clonal deletion where self-reactivity leads to apoptosis.[9] Following this 'checkpoint' the non-self-reactive B-cells leave the marrow compartment as immature B-cells. However, a minor population of autoreactive B-cells can escape central tolerance and are seen in patients with auto-immune conditions such as rheumatoid arthritis and systemic lupus erythematosus.[42] Patients with congenital BTK deficiency have a peripheral B cell repertoire enriched for autoreactive B cells highlighting the importance of BCR signalling in central tolerance.[45] Following this successful process, immature B-cells move out from the bone marrow in to the peripheral circulation to complete their maturation.

1.7 Transitional B-cells

On exiting the marrow environment the morphologically homogenous immature cells acquire sIgD, whilst transitioning to and from the spleen.[46] The immature cells, known as transitional cells (T1, T2 and T3) based upon their phenotypes, are well characterised in mice.[47] The T1-3 cells represent mature pre-naïve B-cells that have undergone the selection process of central tolerance and egressed from the marrow. They express sIgM, CD93 and CD24 but T1 cells have higher sIgD and have gained CD21 and CD23 (low).[48] The T2 subset express B-cell survival activation factor receptor (BAFF-R) whose expression, in conjunction with the BCR, sustains lymphocyte survival.[49] Transitional cells are said to be developmentally mature when they cease to express CD24 and 93 and are then identified as mature naïve B-cells. These sIgM/D expressing cells migrate to the secondary lymphoid tissues (SLO) to become follicular or marginal zone B-cells. The T3 subset (CD93 positive, sIgM low, CD23 positive) is not thought to give rise to mature B-cells. It appears likely they represent a potentially autoimmune subset that is held in a hypo-responsive, anergic state.[50]
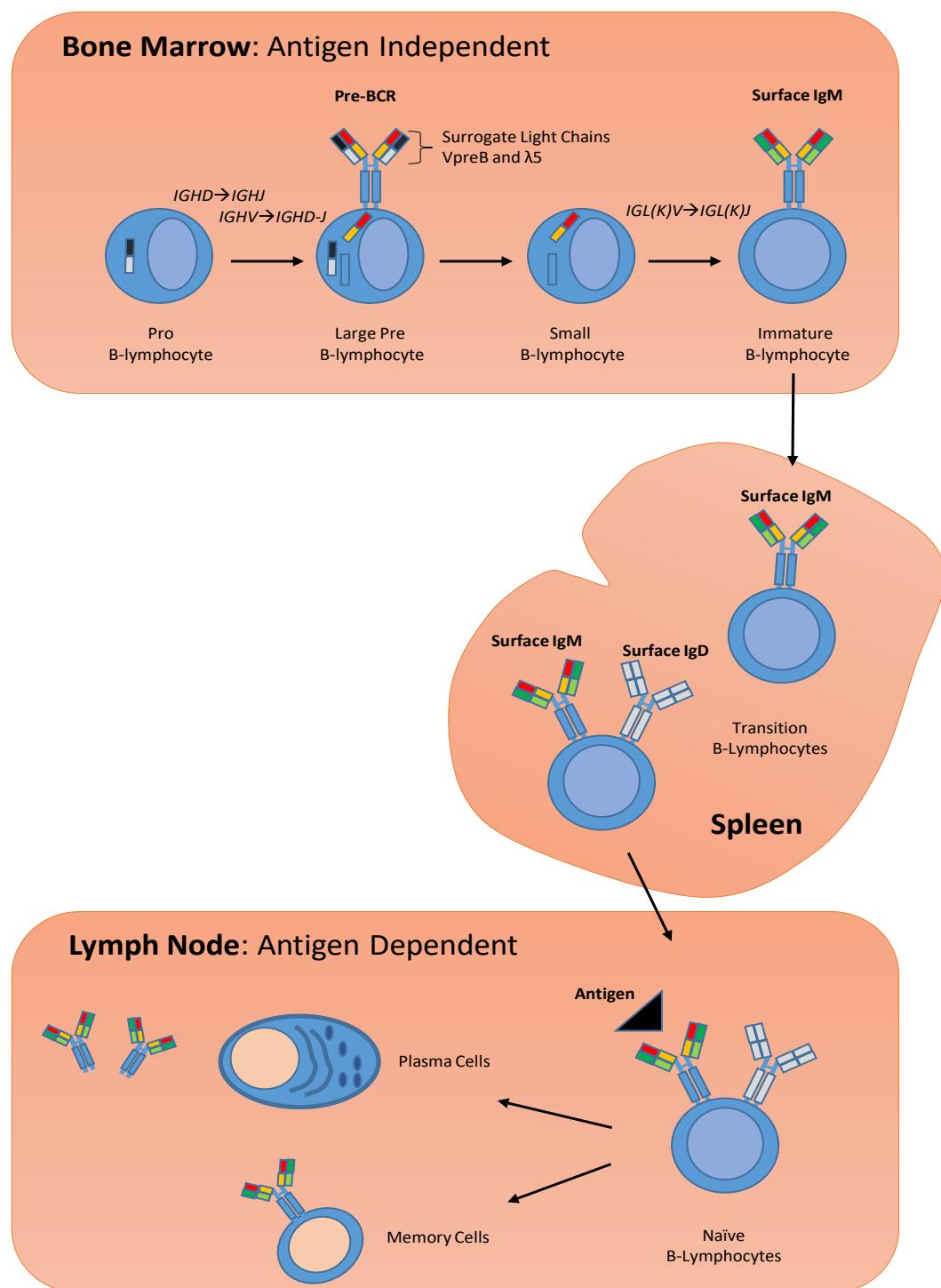
Figure 1.4: B-cell development

B-cell development begins in the bone marrow in the absence of antigen stimulation. *IGHV* rearrangement marks the first step to a progenitor (Pro) B lymphocyte differentiation. Expression of the pre BCR receptor, through assembly of a rearranged heavy chain with surrogate light chains (of λ5 and VpreB), heralds the next stage of development before the resting smaller pre B cell stage where light chain rearrangement occurs. The functionally rearranged light chain pairs with a heavy chain and co-factors resulting in an immature surface IgM molecule. The immature B-cell leaves the marrow environment circulating to and from the spleen where it matures further into transition B-cells acquiring surface IgD. Naïve B-cells migrate to sites of antigen presentation, principally in the lymph nodes, where they encounter antigen and terminally differentiate into antibody secreting plasma cells or long lived memory cells. Adapted *from Rho family GTPases and their regulators in lymphocytes by Tybulewicz.*[51]

1.8 Primary and secondary Lymphoid organs

Given the key role in B- and T-cell production, the bone marrow and thymus are known as primary lymphoid organs.   However, the cells developing in these structures have not yet experienced antigen and are thus termed naïve.[2] To mature further and initiate immune responses, lymphocytes must encounter antigen.[52] This occurs primarily in the SLO and is driven by chemokine co-ordinated movements of lymphocytes (**Figure 1.5 A**).[53 54] These are the lymph nodes, spleen and extra-nodal mucosa associated lymphoid tissue (MALT) that includes the skin, nasal tissue, gut and lung. Secondary lymph organs anatomy vary by their location but generally have T and B regions. Antigen that enters the blood stream are generally processed by the spleen whilst mucosal antigen is delivered to the mucosa associated tissues. Antigen is generally presented to lymph nodes by antigen presenting cells within the SLOs.[52]

The immune response and adaptive development of B-cells thus occurs primarily in the SLO (**Figure 1.5 A**). Lymph nodes have highly specialised functions that are intrinsically linked to their anatomy. The lymphatic system is present throughout the body forming a network that allows antigen presentation, immune system response and redelivery of more active lymphocytes via cytokine and cellular rich liquid, the lymphatic fluid. This fluid enters the lymph node via the afferent lymphatics leading into the sub-capsular space. Dendritic cells within this compartment process begin to process antigen as the sub-capsular sinus bathes the outer cortex.[1] The lymphatic fluid is channelled down through the intra-nodal lymphatic system that surrounds the cone shaped segments of the solid node towards the efferent lymphatic drainage system and the venous blood supply. The endothelial surfaces and tissues are composed of macrophages and other antigen processing cells collectively termed as sinus histiocytes.[55]
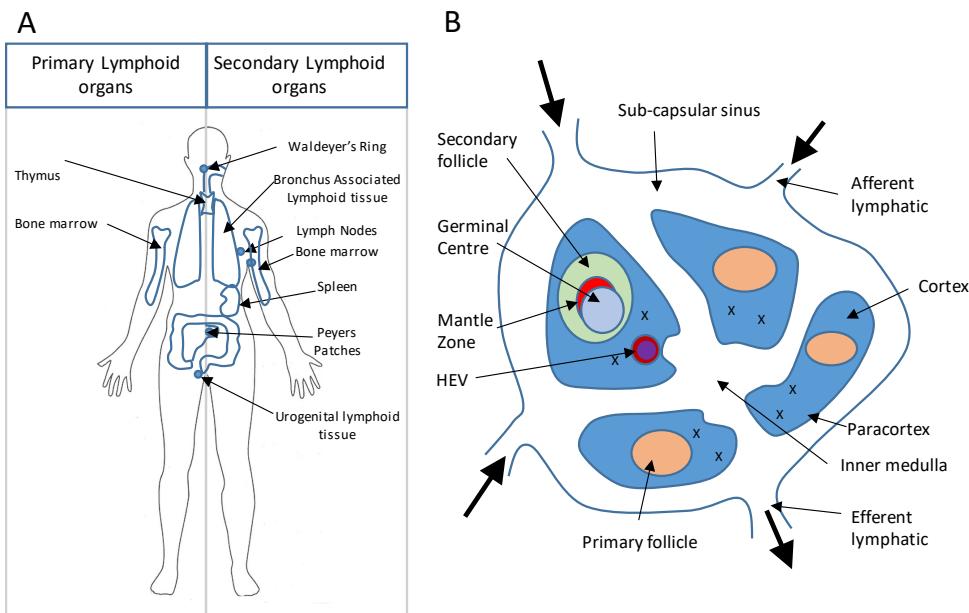
Figure 1.5: The anatomical organisation of lymphoid organs and lymph node structure.

The bone marrow and thymus make up the primary lymphoid organs and act as the site of cellular maturation. Secondary lymphoid organs are the principle site of antigen exposure and thus lymphocyte activation. They are situated at points of pathogen entry: (A) the nose and mouth (Waldeyers ring), bronchus, gut (Peyers patch) skin and the urogenital tract. Antigen flows through the lymphatic system carried within the lymphatic fluid. It arrives in the lymphoid organs via the afferent system and exits through the efferent. The lymph node is a highly organised tissue (B) comprised of three discrete regions: medulla, paracortex (containing T-cells, X) and the follicle containing cortex (B-cell zone). Afferent lymphatics pierce the capsule and lymphatic fluid flows through the sub-capsular sinus. Antigen presenting cells (dendritic cells (DC) and macrophages) process antigen and present them to T cells in the paracortex (x) and B-cells in the cortex within the follicles and germinal centres. Antibody secreting plasma cells reside within the medulla. Naïve B lymphocytes enter the lymph node via medullary piercing high endothelial venules (HEV). Adapted from *Williams Hematology, 8e Marshall A. Lichtman, Thomas J. Kipps, Uri Seligsohn, Kenneth Kaushansky, Josef T. Prchal.*[2]

The solid tissue of the lymph node is comprised of multiple cone-like segments (**Figure 1.5 B**); the base of which borders the sub-capsular sinus and the apex formed by the medullary cords. These cones come together to form a kidney like structure of the lymph node. The cones are comprised of three main areas; the follicular region composed of B-cell aggregates at the base, the middle par-cortical T cell zone and the apical medullary cords.[2] The B-cell follicles are also rich in follicular DCs derived from mesenchymal cells. Blood is supplied through the medullary cones allowing naive B-cells and recirculating B- and T-cells to enter the node in the T-zone via specialised vessels known as high endothelial venules. DCs that processed antigen have now migrated via the afferent lymphatics through to the T-zone and are known as interdigitating cells that are specialised antigen presenting cells. The activation of T-cells occurs in these sites is known as T-cell priming.[1] Further B-cell maturation occurs in T-cell dependent and independent process occurring in specialised follicular regions known as germinal centres (GC) resulting in the production of plasma cells

with increased BCR-antigen.[56] These processes are key to the adaptive nature of the immune system and the biology of mature B-cell lymphoma ontogeny.

1.9 Pre-germinal centre B-cells

In the absence of antigen, naïve B-cells have a lifespan of approximately 48 days.[1] In contrast, the presence of cognate antigen leads to BCR signalling and cellular proliferation. The first step involves the activation of follicle residing IgM[+] IgD[+] naïve B-cells by exogenous antigen. Following this initial event the B-cells move towards the paracortical T-cell zone where they receive co-stimulatory signals by CD4^{+} helper T-cells (**Figure 1.5B**) Once engaged, the sIg-antigen complex is internalised by endocytosis, digested and peptide presented on the B-cell surface in the grooves of MHC class II molecules.[57] BCR signalling upregulates CD40 and the chemokine receptor CCR7. C-X-C Motif Chemokine Receptor 4 (CXCR4) is a key chemokine receptor and remains unchanged at this point preventing egress from the lymph node. The CXCR4: CCR7 gradient permits further movement of activated B-cells towards the follicular boundary where primed T-cells are present. Stromal cells express Chemokine (C-C motif) ligand 12 (CCL12) acting to chemically attract the B-cells.[58] B-cells interact with their cognate TCRs resulting in increased stimulation, activation and proliferation. T-cell priming leads to further T- and B-cell activity causing an early shift of low affinity antibody producing B-cells to local sites. This is the medullary cord in the lymph node and the red pulp in the spleen.[1] Conversely, some of the B-cells activated in the para-cortical T zones will move towards the follicles. These highly active B-cells migrate to the follicles forming GCs.

1.10 Germinal Centre reaction

The GC is a highly specialised region within the lymph node secondary follicle where antigen-activated B-cells undergo two processes of secondary Ig diversification: somatic hypermutation (SHM) and class-switch recombination (CSR). These recombination mechanisms generate antibodies with matured affinity to cognate antigens and with specialised function. Following an immune stimulus, GCs develop and persist for approximately 3 weeks.[59] The GC has classically included two distinct regions; the dark

zone (DZ) and light zone (LZ) (**Figure 1.6**).[60] The DZ contains CXCR4 expressing activated lymphocytes that are large rapidly proliferating undergoing SHM named centroblasts. The DZ stroma is made up of CXCL12 (the ligand for CXCR4) expressing reticular cells that inhibit movement to the LZ.[61] The centroblasts give rise to smaller, non-proliferating, cells (centrocytes) forming the LZ region where centrocytes compete for antigen in the process of affinity maturation. The LZ is less dense than the DZ and also contains infiltrating naïve B cells. The LZ is composed of follicular dendritic cells (FDCs) and T follicular helper (Tfh) cells that bind antigen containing immune-complexes. Cells are selected on the basis of their affinity for antigen through their interaction with FDCs and, crucially, Tfh. Positively selected cells activate anabolic processes, upregulate myc and recycle back to the dark zone for further SMH and proliferation. Cells with low affinity for antigen or that are damaged by SMH undergo apoptosis.[62] The network of FDCs help B-cells organise into well-demarcated areas and hold intact antigen to support affinity dependent testing.[63] The classical model of affinity maturation proposes that B-cells undergo SHM and clonal expansion in the DZ before proceeding to the LZ for clonal selection based upon competitive antigen binding. [64] High affinity B-cells develop through multiple rounds of mutation and proliferation, cycling back and forth between the dark and light zones. [65] Real time imaging studies have shown that B-cells move in both directions within the GC and up to 30% migrate back to the dark zone for further SHM.[66] Once completed, the GC produces memory B-cells and long lived plasma cells.
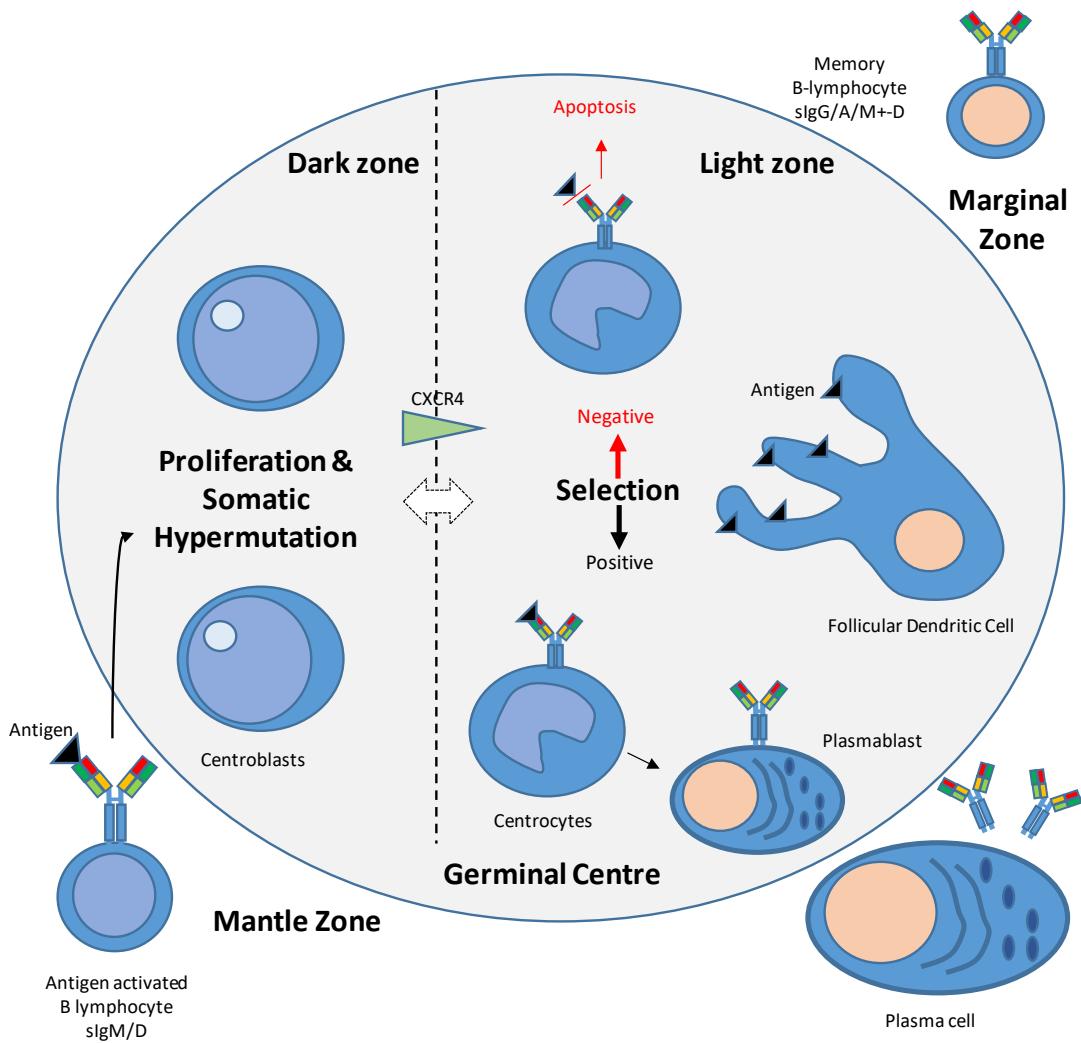
Figure 1.6: Overview of the germinal centre reaction.

Antigen activated B-cell migrates towards the secondary follicle forming the anatomically specialised region of the germinal centre. This is divided into two areas, the dark and light zones. The dark zone contains large rapidly proliferating centroblasts that have active activation induced cytidine deaminase (AID) resulting in somatic hypermutation (SHM) where nucleotide base alterations are induced to increase the affinity to cognate antigen. B-cells cycle between the dark and light zone for rounds of affinity maturation followed by clonal selection in the light zone. Competitive antigen immune complex binding occurs in the T-cell rich light zone with follicular dendritic cells. B-cells are selected for high antigen binding. B-cells that cannot competitively bind antigen undergo programmed cell death. B-cells are either re-circulated to the dark zone, undergo apoptosis or form antibody secreting plasma cells and memory cells. [34]

1.10.1 Somatic Hypermutation

Affinity maturation requires the introduction of multiple single base substitutions increasing the BCR's affinity to cognate antigen. This process requires deoxyribonucleic acid (DNA) modifying processes utilising the enzyme activation-induced cytidine deaminase (AID) which is highly active in the DZ.[67] Nucleotide base insertions occur in hot spot motifs that introduce diversity in both FWRs and CDRs (**Immunoglobulin gene**). CDR mutations accumulate to alter antigen affinity.[68] Following introduction of nucleotide changes the centroblasts slow their rate of proliferation and move to the light zone via the CXCR5 ligand, CXCL13 (**Figure 1.6**).[69][70] During the proliferation process, many B-cells undergo cell death and are hence cleared through macrophages. The GC B-cells then undergo competitive antigen-mediated and T cell mediated positive selection in the LZ.

1.10.2 Class Switch recombination

CSR is a process that affects the function of the Ig as opposed to the affinity of the Ig for Ag as in SHM. However, like SHM, it requires AID to produce base mismatches in the *IGH* locus. The default constant coding region in IgM and D, *Cμ/Cδ*, is switched to an alternative coding exons *Cγ*, *Cε*, or *Cα*.[71] This results in the formation of non-M and D Igs such as IgG, IgE, or IgA (**1.3 Immunoglobulins**). The molecular process requires a deletional-recombination reaction at switch regions (S) that precede all constant coding exons except IgDs, which are formed by a splicing reaction.[71][72] AID induces double strand breaks converting deoxycytidine (dC) to deoxyuridine (dU) in S regions generating dC:dU mismatched activating DNA repair mechanisms which then drive CSR.[73] CSR is not random but cytokine driven. Isotype switching can be induced by a combination of CD40 expression together with IL (e.g. IL-4) or transforming growth factor (TGF)-β.[74] TGF-β induces CSR to IgA , IL -4 causes IgM/D to class switch to IgE and the other ILs result in a switch to IgG1-4.[71][73][75]

1.11 T-cell independent B-cell response

Adult peripheral blood is composed of approximately 10-20% of B-cells.[1] Conventional B-cells account for the majority of adult peripheral B-lymphocytes and are designated as follicular or marginal zone (MZ) cells whilst the remaining comprise B1 peripheral blood lymphocytes. [76] Follicular B-cells circulate between the follicles of secondary lymphoid tissue and MZ B-cells reside in the splenic marginal zone, acting as the first line of defence against blood borne pathogens. Conventional B-cells respond to antigen in a T-cell dependent manner whilst small subsets, the B1 cells and B2 marginal zone cells, can respond in the absence of T cell support producing an innate like response.[77]

B1 cells are well characterised in mice where the cells predominate in the peritoneal and pleural cavity following maturation in the foetal liver. They produce IgM antibodies to carbohydrate and polysaccharides present on commensal bacteria.[78] They have the ability to self-renew in the absence of antigen and analysis of the *V-D-J* genes show the BCRs to be highly conserved with little or no somatic hypermutation. Moreover, their antibodies are poly- and often auto-reactive with broad reactivity. It is likely they are 'innate like' B-cells. In mice, they have a characteristic Immunophenotype CD5$^+$, IgM^{High}, IgD^{Low}, CD23$^{Low/neg}$.[79] There is more controversy in humans about the existence of B1 cells but it appears likely that a CD20$^+$, CD27$^+$, CD43$^+$ population supports a human B-1 equivalent.[80]

MZ cells are composed of a variable mixture of naïve and memory B-cells that persist within the marginal zone. Some have undergone SHM and class switching to surface IgG or A. Unlike the other B2 cells, MZ cells are able to respond to bacterial capsular polysaccharides by rapid differentiation into short lived plasma cells.[81] These are classed as T-cell independent responses and can be elicited by microbial ligands or extensive BCR crosslinking.[81]

1.12 Super antigens

The BCR is adept at responding to its cognate antigen with high affinity to the exclusion of other antigens. However, some pathogenic microbial antigens are able to bind outside of the antigen binding site in a T-cell independent manner and are known as superantigens.[82] They bind outside the CDR activating the BCR through their FWRs. Moreover, these

antigens are able to stimulate a high number of B-cells and target a restricted subset of *VH* genes (*IGHV*3) resulting in potent activity.[83 84] The best studied example is the membrane bound protein of *Staphylococcus aureus*, Staphylococcal protein A (SpA). Binding to SpA occurs through conserved FWR regions, not through CDR3 or the immunoglobulin light chains. It is estimated that up to 46% of circulating B-cells are able to be stimulated by SpA.[83] Super-antigenic dose affects response; naïve non-autoimmune mice respond with proliferation to small concentrations but limited proliferation and death to larger doses.[83] MZ B-cells are particularly susceptible to super-antigenic stimulation and can lead to apoptotic deletion.[83]

1.13 B-cell receptor signalling

BCR signalling describes the process from BCR-antigen engagement that results in a cascade of intracellular reactions ultimately leading to changes in proliferation and cellular function. Signalling is initiated through the immunoglobulin structure as it senses its environment for molecules with sufficient avidity to trigger activation.[2 85] BCR signalling strength varies and is modulated by co-receptors.[86] This ranges from an antigen independent signal essential for survival, tonic signalling, through to stronger, antigen activated, signals that can drive proliferation, anergy or even apoptosis dependent on co-stimulatory effects (**Figure 1.7**).[87]

1.13.1 Tonic B-cell receptor Signalling

BCR engagement with cognate antigen results in robust signalling pathway activation in both normal and malignant B-cells.[88 89] In the absence of ligand, it is continually available for micro environmental stimuli and responds variably dependent on binding avidity of the molecule and co-receptor modulation. This unstimulated, or resting BCR state, is essential for B-cell survival. Iinducible ablation of the BCR surface immunoglobulin in mice models leads to mature B-cell programmed death.[27 90] In these transgenic mice, cell death occurred over 3-6 days but could be delayed by anti-apoptotic bcl-2 expression suggesting this was programmed cell death.[27] They also observed that the ITAM heterodimers CD79a and b were essential for B-cell survival, independent of sIgM.[90] Thus, even in the absence

of cognate antigen trigger, BCRs exhibit some form of low level, constitutive signalling to maintain cell survival. This is dependent upon the BCR and more specifically, the BCR subunit ITAMs. Basal state signalling in the absence of antigen stimulation is known as 'tonic signalling' (**Figure 1.7**).

The downstream nature of these tonic signals has been defined through further selective ablation of BCR intermediate pathways in *cre* mediated transgenic mouse models. Ablation of the BCR was only rescued by concomitant activation of PI3K or deletion of the PI3K negative regulator PTEN. [91] Interestingly, in this mouse model *bcl-2* overexpression, mitogen-activated protein kinase (MAPK)/ERK activation and canonical NF-κB rescue were not sufficient to rescue the BCR negative cells. This data identifies the PI3-kinase pathway to be of central importance downstream to the BCR.

Akt is a serine /threonine protein kinase that is known to play a significant role in cell survival, cell-cycle progression and angiogenesis.[92] It is activated through PI3-K generated PIP3, a membrane lipid that recruits Akt to the plasma membrane (**Figure 1.7**). Akt activation promotes cell survival through the phosphorylation of apoptosis regulators such as BAD and FoxO.[93][92] It has also been shown to play a role in telomere activity, via direct phosphorylation of the hTERT protein.[94] Its central role is further demonstrated by four further targets; the cyclin dependent kinase inhibitor p27[kip1], mTOR, glycogen synthase kinase-3-β (GSK-3 β) and the IκB kinases.[95-99] It is inhibited by the serine phosphatase lipid PTEN - which acts by converting PIP3 back to PIP2 and is lost in different types of cancer.[93][100]

A significant downstream target of Akt is mammalian target of rapamycin (mTOR). It is a serine-threonine protein kinase that regulates cell survival, proliferation and cell cycle progression including expression of cyclin D1.[101] mTOR is found in two protein complexes; TORC1 and TORC2.[102] These highly conserved proteins are involved in cell cycle regulation with respect to growth factors, nutrient status and energy. Once activated, mTOR assists cell cycle progression (G_1 to S phase) by phosphorylation of two proteins: p70S6 kinase (p70S6K) and 4E-binding protein 1 (4E-BP1).[103] p70S6K phosphorylates the ribosomal subunit S6, involved in initiating translation in the nucleolus.[104][105] mRNA translation is

tightly regulated by a complex of proteins (4A to 4F). 4E is the rate-limiting step in CAP dependent translation. In its hypo-phosphorylated state 4E-BP1 interacts strongly with 4E and prevents complex formation and translation.[103]

1.13.2 Antigen-mediated BCR Signalling

BCR engagement with cognate antigen results in conformational changes at the cell membrane with the formation of micro clustered surface immunoglobulins termed lipid rafts.[87] The BCRs lacks intrinsic tyrosine activity, thus closely coupled ITAMs are phosphorylated by the Src family kinases (SFK) Lyn and SYK.[106] SYK is a positive allosteric kinase; phosphorylation results in further recruitment of kinases and lipases which are collectively termed the 'signalosome'. This includes SYK itself, PLC-γ2, BTK and Lyn kinase bound to an adaptor molecule BLNK.   The ligand activated BCR molecules then segregate into lipid rich domains within the plasma membrane. This potentiates further ITAM phosphorylation and amplification of downstream effects (**Figure 1.7**).

Mouse models have been extensively used in this complex area, one such study demonstrated that naïve follicular B-cells are able to recognise a range of endogenous antigens.[107] Zikherman and colleagues used a reporter mouse model with BCR signalling linked to expression of green fluorescent protein (GFP). BCR signalling was identified in the spleen supporting splenic antigen engagement. This resulted in down-modulation of sIgM but not sIgD analogous to the anergic state (**B-cell anergy in normal cells 1.14**). Furthermore, these BCR active mature B-cells, identified through high GFP expression, had lower pS6 and anti IgM induced calcium mobilisation representing reduced signalling capacity.

Further signalling is complex but occurs by two predominant pathways; via PLC-γ2 activity and via phosphoinositide 3-kinase (PI3K)/protein Kinase B(Akt).[88] The B-cell linker protein BLNK is an adaptor molecule which, after phosphorylation by SYK, binds to PLC-γ2 and BTK allowing the phospholipase to produce the secondary messengers diacylglycerol (DAG), inositol-1,4,5trisphosphate (IP3) from phosphatidylinositol-4,5-bisphosphate (PIP2).[88] A

case report of a BLNK deficient human with homozygous splice defect had pro-B-cells but no pre B-cells, absence of immunoglobulin and profound immunodeficiency. [108]

Once produced IP3 binds to its cognate receptor (IP3R) found on the endoplasmic reticulum (ER) membrane. This results in intracellular calcium channel release of stored calcium. Due to its transient nature this, in turn, activates extracellular calcium-release activated calcium (CRAC) leading to sustained calcium signalling.[88] The potent transcription factors NF-κB and N-FAT are activated by calcium dependent atypical phosphokinases and calcium-calmodulin. [109] [110] The generation of Ca^{2+} and DAG enable protein kinase C beta (PKCβ) translocation to the membrane where conformational change and full activation occurs. This enables PKC-mediated destruction of NF-κB inhibitors and hence activity of NF-κB transcription factors.[111] Persistent transcription factor activation results in the activation of *c-myc* and *cyclin D1* and the upregulation of the chemokine CXCR4.[112] [113]
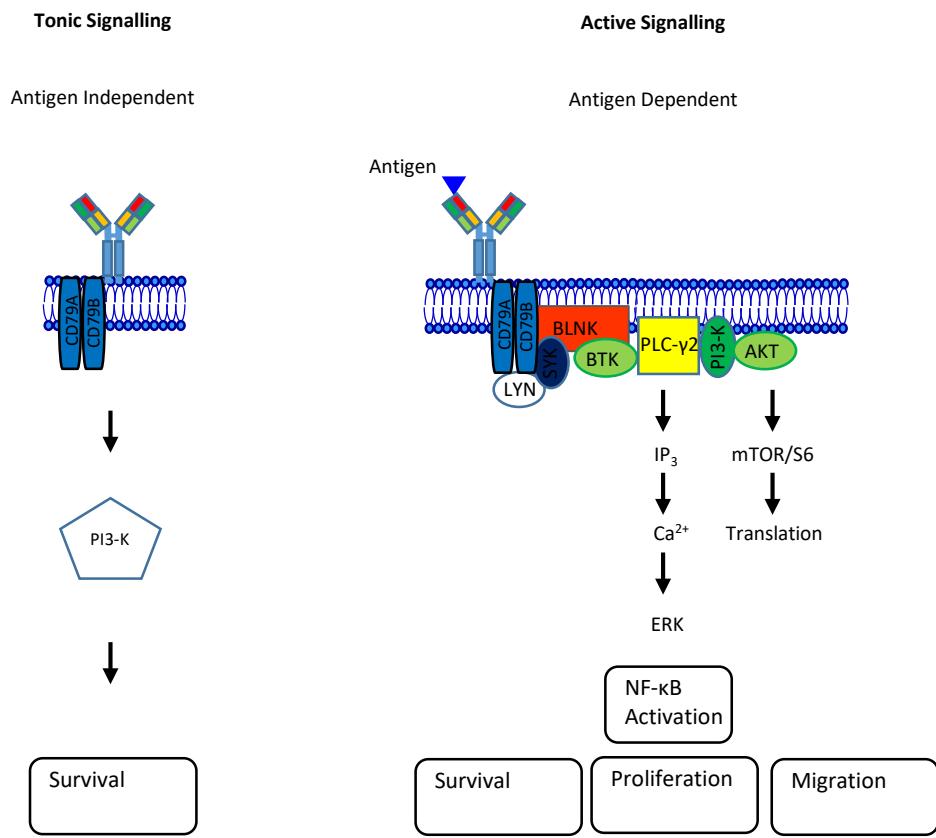
Figure 1.7: B-cell receptor signalling.

Tonic signalling occurs in the absence of cognate antigen and is driven through the PI3-K pathway resulting in lymphocyte survival. Active BCR signalling occurs in the presence of cognate BCR ligation. This results in the production of the signalosome through LYN, SYK, BLNK, BTK, PLC-γ2, and PI3K. Calcium flux secondary to PLC-γ2 phosphorylation and IP3 production results in ERK and NF-KB upregulation. Co-receptor stimulation (e.g.CD19) aids stimulation of the PI3K /AKT /mTOR pathway. The overall result is cellular survival, proliferation and migration. This illustration is heavily simplified and adapted from *Staudt et al.*[114] and *Stevenson et al.*[115]

1.13.3 Downstream Effects

A final downstream pathway of the BCR is the NF-κB family. This family encodes a set of transcription factors involved in regulating broad processes ranging from lymphocyte development and survival through to inflammatory and immune responses.[116] This family shares a Rel homology domain (RHD) which promotes DNA binding and dimerization. It consists of five members termed RelA, RelB, c-Rel, NF-κB1, and NF-κB 2.[117][118] These proteins are held in their inactive form by a group of inhibitory proteins consisting of IκBα, IκBβ, IκBε plus the precursors of P50 and p52, p105 and p100.

Canonical, or classical, signalling of NF-κB refers to transient but rapid activation of transcription factors, such as p50, secondary to a diverse range of stimuli such as proinflammatory cytokines, T-cell receptor and BCR signalling and lymphocyte co-receptors.[119] Conversely non canonical, or alternative, signalling is tightly controlled and based on the metabolism of precursors e.g. p100; signalling is thus slow but persistent.[120]

1.13.4 Positive regulators of BCR signalling

BCR signalling does not occur in isolation, receiving positive and negative stimuli to maintain appropriate signalling response. CD21 is a positive (enhancing) regulator of BCR function. It forms an association with CD19 and CD18 (CD21:CD19:CD81 complex) and binds to antigen bound complement (C3d) forming a link with the innate immune system. On BCR-C3d-antigen binding, CD21 is engaged and co-localises the CD21:CD19:CD81 complex. CD19 harbours an intracellular ITAM region which, once brought into proximity with LYN kinase, becomes phosphorylated and provides a PI3K attachment site enhancing signalling strength.[121]

1.13.5 Negative regulators

Uncontrolled signalling cascades could lead to uncontrolled stimulation and growth. The BCR transduces signalling through their ITAM containing molecules in CD79a and b. Src kinases phosphorylate this reaction and contain a negative regulatory tyrosine in their carboxyl ending. Protein tyrosine kinases (Csk) and protein phosphatases (PTPs e.g. CD45, PTPα, SHP-1 and -2) are able to phosphorylate the tyrosine motif resulting in the formation of an ITAM intramolecular complex with its own SH2 domain.   This inhibits further catalytic activity, altering actin cytoskeletal clustering formation, negatively regulating further signalling. [122][123]

The FcμR is a transmembrane receptor for IgM.   Mice with global FcμR deficiency have increased titres of serum IgG autoantibodies and increased natural serum IgM.[124][125] Confocal microscopy has revealed its importance in the regulation of IgM-BCR isotype transport between the Golgi apparatus and surface. Interestingly it plays no role in sIgD transport. Cell surface expression of sIgM is this regulated, in part, by the FcμR, and its absence leads to increased tonic signalling.[126]

FcγRIIB (CD32) binds IgG and is the only inhibitory Fcγ receptor that modulates immune response of the lymphocyte.[127][128] FcγRIIB possesses an immunoreceptor tyrosine-based inhibitory motif (ITIM) within the cytoplasmic domain which can crosslink to other activating FcγRs or the BCR itself. The action of BCR crosslinking increases the activation threshold reducing antibody production. This occurs through the action of SH2-domain-containing inositol phosphatases (SHIPs) e.g. SHIP1. This molecule results in downstream dephosphorylation and the functional inhibition of calcium influx.[129]

Using knockout mice experiments, Liu and colleagues have recently shown that CD23 also acts as a negative regulator of BCR signalling. It acts by inhibiting actin-mediated BCR micro-clustering preventing BCR-antigen contact.[130]

1.14 B-cell anergy in normal cells

Anergy is a state of B lymphocyte insensitivity to BCR mediated stimuli - 'functionally limited upon stimulation'.[131] It is a normal consequence of B-cell interaction and is classically defined as a two stage process: (1) antigen binding to the BCR and (2) absence of CD4$^+$T-cell co-stimulation.[132]

Clonal anergy of B-cell precursors was identified in the early 1980s – it was thought to be an early developmental mechanism to inactivate B-cells driven by autoantigen .[133] Auto-reactivity and hence autoimmunity would thus be prevented. To understand this process, a transgenic mouse model possessing a BCR specific to hen-egg lysozyme (HEL) was crossed with soluble HEL expressing mice [134]. This manufactured phenotype, of a BCR chronically exposed to its cognate ligand, revealed striking changes. The anti-HEL B-cells stopped secreting anti-HEL antibody and did not undergo clonal deletion. Furthermore, there was a distinct reduction in sIgM expression whilst sIgD was left unaltered. These experiments underpin the understanding of anergy today. Anergy has now been identified in the context of exposure to diverse antigens; DNA[135] (mimicking the auto reactivity that exist in systemic lupus erythematosus), Sm[134] (a small nuclear ribonucleoprotein complex involved in mRNA splicing), insulin[136] and the Ars hapten[137].   These models support a picture where, in the absence of sufficient co-stimulatory help via CD4$^+$ or Toll like receptor engagement, low level, chronic BCR stimulation results in anergy.

These anergic models share common features. A key finding is the selective down-modulation, by endocytosis, of sIgM, but not sIgD, as a consequence of chronic antigen exposure. [134] [137] In keeping with this model, anergy has been shown to be reversible i.e. when antigen exposure ceases sIgM expression increases (as down-modulation ceases). [138] [139] Further common features seen in transgenic anergic cells are increased basal ERK phosphorylation, increased basal calcium levels and poor calcium signalling response to BCR stimulation.[137] [138] [140] In mouse models normal anergic B-cells have a relatively reduced life cycle *in vivo.*[141] BIM, a BH3-only pro-apoptotic protein, is upregulated in anergic cells.[142] BIM deleted self-reactive B cells accumulate *in vivo*.[143]

Anergic B-cells are not confined to transgenic mice and have been observed in healthy humans.[144 145] Mature naïve human B-cells identified via their IgD$^+$, IgM$^-$, CD27$^-$ phenotype (2.5% of B-cells) possessed unmutated *IGHV* genes and had low signalling capacity to BCR ligation.[144] *In vitro* culturing resulted in recovery of sIgM and signalling capacity consistent with the reversible nature of anergy.[146]

Chapter 2 Mature B-cell Lymphomas & Mantle Cell Lymphoma

2.1 Introduction:

The ability of B-cells to somatically rearrange and adapt their immunoglobulins, results in a powerful and evolutionary beneficial immune system. However, its complexity can result in errors; overexpression, uncontrolled proliferation and finally transformation to lymphoma. Mature B-cell neoplasms are much more common than T-cell neoplasms (Annual rate per 100,000 people: 19.21 vs 1.08).[147] This may be due to the three step process required in B-cell maturation (immunoglobulin heavy and light chain rearrangement, SHM and CSR) when compared to the single step of TCR gene rearrangement in T-cell malignancies.[148]

Mantle cell Lymphoma (MCL) is a clinically heterogeneous B-cell lymphoma accounting for 6-8% of all mature B-cell lymphoma cases.[149] It is a disease principally affecting men with a median age at diagnosis of sixty five years old.[150]   Disease usually presents in an advanced stage affecting the bone marrow, peripheral blood, spleen and lymph nodes. It is characterised by cyclin overexpression secondary to the chromosomal translocation t(11;14)(q13;q32) in a CD5^{+}, CD19/20^{+} B-cell lymphoma. Despite clear diagnostic definition, there is striking clinical heterogeneity and overall poor prognosis.[151]

There are three key elements relevant to pathogenesis of MCL which will be explored in this chapter:[152]

- Genetic susceptibility
- Acquired genetic/epigenetic alterations
- Tumour BCR-microenvironment interactions

In younger, fitter, patients MCL is treated with cytarabine containing high dose immuno-chemotherapy followed by stem cell transplantation. Less fit, transplant ineligible, patients

are treated with immuno-chemotherapy. However, the overall outlook is disappointing with a median overall survival (OS) of 4-5 years though this is highly heterogeneous.[153] There is usually an initial response to treatment but this does not persist. Relapse therapy has generally been ineffective and survival post relapse is short with a median OS of 19 months including those undergoing allograft.[154][155] However, novel oral agents that target signalling pathways are transforming therapy. In particular, ibrutinib, an oral small molecule that inhibits Bruton's Tyrosine Kinase (BTK) has prolonged survival.[156] BTK is a kinase found within the B-cell receptor (BCR) signalling cascade.[157]

There are now over fifty WHO classified mature B-cell malignancies; morphological diagnoses are now being overtaken by genetic and molecular diagnostics expanding the known repertoire. [158]   The UK Haematological Malignancy Research Network and the US SEER data identify DLBCL, CLL, follicular lymphoma, marginal zone lymphoma, MCL and Burkitt lymphoma (BL) to be the most common form of B-cell malignancies. In conjunction with less common B-cell malignancies such as splenic marginal zone and mucosa associated lymphomas, they act as context and useful comparators for the understanding of the MCL BCR. [147][159] Furthermore, BCR structure can be used to classify B-cell neoplasm in terms of their likely cell of origin and, more specifically, their germinal centre experience including the role of antigen.

This chapter will briefly review MCL in terms of diagnosis, clinical features of the various subtypes that define MCL and treatment.   It will then discuss the mature B cell malignancies and, the role of the BCR and COO in these malignancies to act as context for understanding the MCL BCR.

2.2 Mantle Cell Lymphoma

2.2.1 In situ mantle cell neoplasia

This is a rare, often incidentally found, Cyclin D1 positive lymphoid neoplasia found in the inner mantle zones of follicles without other features of MCL (**Figure 2.1**).[158] It is sometimes found in association with other lymphomas. They typically have a low proliferation fraction and appear to be indolent.[158]

2.2.2 Classical Mantle Cell Lymphoma

The more common classical, often now referred to as conventional, MCL is a minimally or completely *IGHV* unmutated (U; defined as more than 98% homology to the germline sequence) cyclin dependent small B-cell malignancy (**Figure 2.1**).[152] Through immunohistochemistry it displays positivity for the transcription factor SOX11.[160] This subgroup is typically nodal and has a propensity for the gastro-intestinal (GI) tract due to preferential expression of a gut homing adhesion molecule, integrin alpha 4 beta 7.[161]

Histopathologically, the tumours cells surround preserved germinal centres in an expanded mantle zone in a nodular or diffuse pattern. Progression to more aggressive forms, identified by blastoid or pleomorphic morphology, often occurs with the acquisition of molecular/cytogenetic abnormalities such as *TP53* disruption. They are seen as genetically unstable and will acquire mutations within cell cycle, survival and DNA repair pathways. [158]

As a clinically heterogeneous disease MCL can present in different ways.[162] More than 90% of classical MCL present as advanced disease. Bulky disease is less common but highly aggressive MCL cases can present with systemic symptoms; significant weight loss, night sweats and fevers, commonly termed B symptoms.[163]

Extra-nodal involvement is common with frequent gastrointestinal and liver infiltration.[164] GI involvement is seen in up to 34% of newly diagnosed patients though this has no prognostic impact.[164] 30-50% of patients have at least two extra-nodal sites at diagnosis but central nervous system (CNS) involvement, at diagnosis, is unusual.[165] Extra-nodal infiltration can involve breast tissue, lung, skin, soft tissue, salivary glands and the orbits.[163] [166]

2.2.3 Leukaemic non-nodal leukaemia

Leukaemic non-nodal (LNN)-MCL is a disease defined by patients with "peripheral blood, bone marrow, and sometimes splenic involvement, lacking significant adenopathy" (**Figure 2.1**).[167] This has previously been defined as maximal peripheral nodes of less than 2 cm and, if performed, no or low tumour burden on computed tomography (CT) imaging.[158 168 169] A cut off of 3cm to define clinically relevant lymphadenopathy has been suggested based only on expert opiion.[170] Like all MCL, they harbour the *CCND* disruption but tend to be SOX11 negative cells, avoiding nodal proliferation, and are *IGHV* mutated.[171 168 172 173]

Gene expression profiling reveals down modulation of cell adhesion genes resulting in actin cytoskeletal changes and tumour invasion/progression. [174] A further study identified 8p deletion (tumour necrosis factor–related apoptosis-inducing ligand receptor gene cluster) and gain of 8q (*MYC* locus).[175] Although generally slow growing this neoplasm can also acquire significant molecular alteration, such as to the *TP53* DNA repair mechanism mutations, to become aggressive lymphomas. [171 176]

Clinically, as a more indolent disease, these cases are identified incidentally in primary care as a persistently raised lymphocytosis ($>5\times10^9$/L). However, this subgroup can also present with cytopenias and frank disease progression. LNN-MCL is associated with the CLL markers CD23 and, less commonly, CD200.[177] A cohort of 37 LNN-MCL cases described less frequent CD38 positivity (LNN 48% vs conventional MCL 94%) and a preferential use of *IGHV4-39*.[168]

2.2.4 Blastoid and Pleomorphic Mantle Cell Lymphomas

Blastoid MCL variant (BL-MCL) is a morphological subtype characterised by aggressive clinical course, high proliferation and blastoid cytology.[178] Morphologically, the cells resemble blasts as seen in acute lymphoblastic lymphoma; large with scanty cytoplasm surrounding a larger immature nucleus with open chromatin (**Table 2.1**). Pleomorphic MCL (PL-MCL) is another aggressive variant. It shares much in common with BL-MCL as it has an aggressive course, high proliferation rate and is only distinguished on morphological grounds. Its cells are larger than those seen in conventional MCL and resemble diffuse large cell lymphoma cells. The two aggressive subtypes are commonly referred to as BL-MCL. Classification of BL-MCL can be subjective usually requiring both a high proliferation index and typical morphology. For this reason incidence varies from 10% to 20%.[178][179]

BL-MCL is usually the presenting subtype but cases of co-presentation and transformation are also recognised (**Figure 2.1**).[179][180] BL-MCL demonstrates immuno-phenotypic variation; loss of CD5 is not uncommon. C-myc is commonly overexpressed though c-myc amplifications appear rare. There are no blastoid defining genomic changes to differentiate it from aggressive conventional MCL.[181] However higher levels of chromosomal aberrations and *TP53* disruption are associated, though not exclusively, with BL-MCL.[182]

Clinical management has historically been unsuccessful with reduced progression free and OS compared to other forms of MCL. CNS disease is seen in between 4.1%-7.8% of all MCL cases at relapse. Risk factors for CNS relapse include high Ki67 proliferation index and blastoid morphology. When occurring it usually occurs early (median 15-20 months) and OS is poor (3-8 months).[183-185]
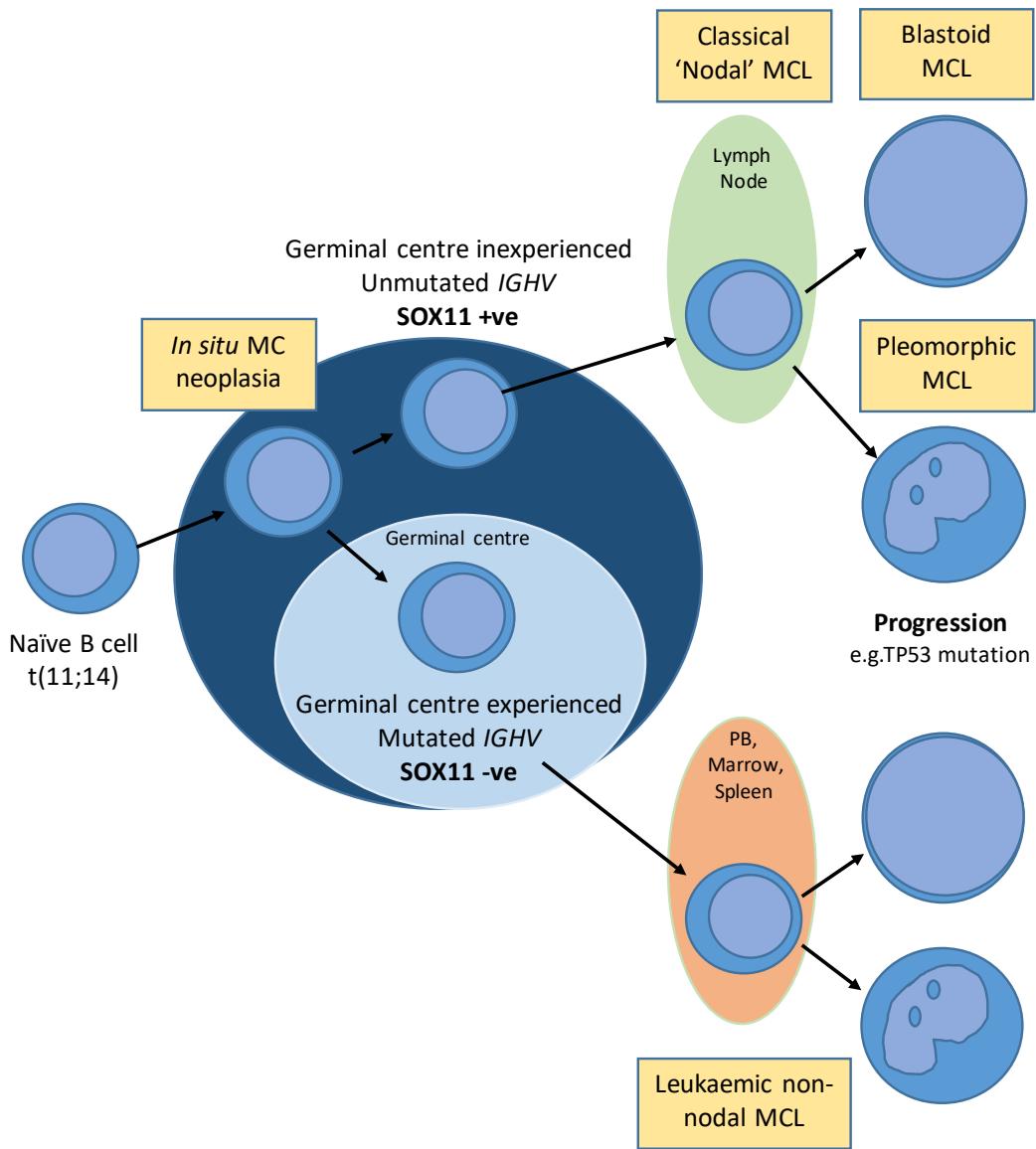
Figure 2.1: Proposed model of MCL pathogenesis.

A pre-cursor cell to the naïve B-cell undergoes somatic mutation and acquires the t(11;14) translocation in the bone marrow. Further mutational events are acquired over time resulting in lymphomagenesis. The t(11;14) harbouring B-cell commonly acquires a SOX11 mutation upregulating BCL6 and avoids the germinal centre reaction. These cells remain in the lymph node and proliferate into nodal disease - conventional MCL. The less common leukaemic non-nodal MCL, generally SOX11 negative, passes through the germinal centre undergoing affinity maturation and somatic hypermutation. They are rarely class switched. This group of MCL is often associated with splenomegaly and have a more indolent course. Either sub-group can acquire further mutations, e.g. *TP53*, and transform to the more aggressive pleomorphic or blastoid MCL. Adapted from *Swerdlow et al*. [158].

2.2.5 Lymphocyte Morphology

Diagnosis of MCL is usually based upon a surgical or core biopsy, as fine-needle aspirates do not reliably evaluate for additional risk factors such as cytology or cell proliferation. In cases of LNN MCL, bone marrow biopsy is sometimes required.[149] Endoscopic biopsy or peripheral blood specimen in GI or leukaemic presentations may also be used to make the diagnosis.[163]

MCL cells are classically monomorphic small to medium-sized lymphoid cells with irregular nuclear contours (**Figure 2.2 A**). The involved lymph node is often completely effaced by a diffuse infiltrate. Less commonly, the infiltrate is nodular. Despite its name, a mantle zone pattern is uncommon.[162] However, a spectrum of morphological variants may be seen (**Table 2.1**) including small round CLL-like MCL (3.6% of cases) or marginal zone-like with larger cells containing more abundant cytoplasm in the less proliferative cases. In contrast to CLL, para-immunoblasts and proliferation centres are not seen.[186]   There is no histological transformation to diffuse large B-cell lymphoma. However, relapse cases can show a loss of the mantle zone pattern, an increase in nucleus:cytoplasm ratio, and an increase in mitotic activity and Ki67 proliferation index in keeping with pleomorphic or blastoid MCL transformation (**Figure 2.2 B**).[158]
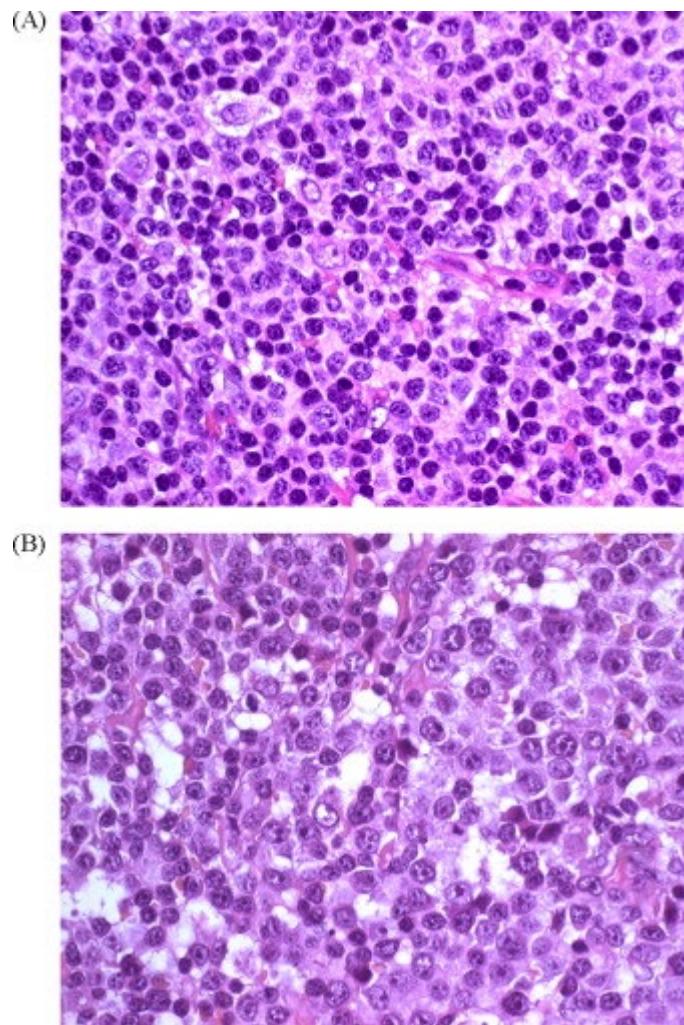
Figure 2.2: Morphology of MCL cells

A) Classic MCL: small to medium sized cells with high nuclear: cytoplasmic ratio, nucleus with irregular contours, clumped chromatin, visible nucleolus. B) Blastoid MCL: medium to large cells with abundant cytoplasm, large nuclei, finely dispersed chromatic and one or more visible nucleolus. Haematoxylin and Eosin stain, 630x from Bertoni et al. [187]

Table 2.1: Morphology of mantle cell lymphoma based on cytological variants.

Mantle cell lymphoma cytological variants			
Classic	**Small cell**	**Blastoid**	**Pleomorphic**
Small to medium size, irregular nuclei, resembling centrocytes	Small round lymphocytes. Resembles chronic lymphocytic leukaemia/small lymphocytic lymphoma cells	Intermediate sized cells, morphology between centrocyte and centroblasts. Resembles lymphoblastoid lymphoma cells	Medium to large size cells, with large cleaved or oval nuclei
Moderately dispersed chromatin	Dense, clumped chromatin	Finely dispersed chromatin	Clumped or pale chromatin, prominent nucleoli
Scant pale cytoplasm		Small cytoplasmic rim	Moderate amount pale cytoplasm
Monomorphic cell populations		High mitotic index, frequent 'apoptotic bodies'	
Infrequent larger neoplastic cells			

Adapted from Bertoni et al (2007)[188].

2.2.6 Immunophenotype

Formalin-fixed paraffin-embedded (FFPE) tissue: MCL are typically CD5$^+$ CD19$^+$ lymphocytes that are CD23$^-$or weakly positive. They express variable but relatively intense surface IgM and D and show a bias towards lambda light chain restriction. MCL cells are usually FMC7 and CD43 positive. Nuclear cyclin D1 is expressed in >95% of cases whilst SOX11 positivity can be seen in >90% of cases using the most sensitive antibodies.   BCL2 is always positive but they are usually negative for BCL6 and CD10. Aberrant phenotypes such as CD5$^-$ or expression of CD10 and BCL6 have been described mostly in blastoid or pleomorphic cases.[189]

Peripheral blood and/or bone marrow: MCL are usually CD5$^+$ CD19$^+$ CD20$^+$, CD79B$^+$, CD22$^+$, FMC7$^+$.   As in the FFPE biopsies, CD10 is seen in a small proportion of cases, typically in blastoid morphology.[190] CLL-like surface antigens have been described very rarely such as LEF1 and CD200 but can usually be used to discriminate between CLL and MCL (**Table 2.2**).[191] Unlike CLL, MCL is less frequently CD23 positive but expresses CD38 and CD49d variably.[192 193]

Table 2.2: Immunophenotype of MCL subtypes and CLL

Immuno-phenotype	cMCL	LNN MCL	Blastoid MCL	U-CLL	M-CLL
CD5/19	++	++	-/++	++	++
CD20	+++	+++	+++	+	+
CD79B	+++	+++	+++	+	+
sIgM	+++	?	+++	+	+
sIgD	+	?	+	+	+
FMC7	+	+	+	-	-
CD200	-	-	-	++	++
CD23	-	+	-	++	++
CD38	+	+	+	+	+
CD49d	+	+	+	+	+

CD – cluster of differentiation, cMCL – conventional nodal mantle cell, LNN – leukaemic non nodal, U – Unmutated, M – Mutated. + weak ++ moderate +++ bright

CD5 has a wide range of functions in the immune system and is predominantly found on T-cells. It is active in T-cell signalling regulation, T-cell anergy and apoptosis acting as an immune checkpoint inhibitor.[194] In healthy B cells, CD5 can associate with the BCR altering downstream signalling but is generally found in aberrant malignant cells.[79] CD5 may play a role in BCR signalling, cytokine signalling and cell survival.[195 196]

CD23 is a transmembrane Fc receptor with low affinity for IgE. It is expressed on B-cells, T-cells, myeloid cells and platelets. [197] Dim to moderate CD23 expression is seen in 13% of MCL cases as opposed to bright expression seen in the majority of CLL.[198] It is associated with LNN, SOX11 negativity and better overall and progression free survival (PFS). [199]

CD38 is a transmembrane glycoprotein with receptor and enzymatic function. It is found at low levels in most haematological tumours but particularly high in multiple myeloma. CD38 facilitates adhesion between circulating lymphocytes and the endothelium.[200] CD31, the ligand that CD38 binds to, is found on endothelial cells, mantle B-cells, plasma cells, alveoli and glomerular cells.[200] The CD38-31 interaction is involved in lymphocyte binding and migration and acts to activate and increase proliferation.[201] It functions as an enzyme (via NAD+) that activates various signalling pathways.[202] CD38 expression has been associated with reduced survival probability in MCL.[203]

CD49D is a surface adhesion molecule that binds to b-integrin CD29 forming very late antigen-4 (VLA-4). It is principally involved in cellular migration. CD49D expression is documented as both low and high in MCL.[192 193] Moreover, it has been suggested that VLA-4 adhesion molecules support drug resistance through VLA-4-dependent marrow stromal cell migration (pseudoemperipolesis), and can be blocked by natalizumab, a VLA-4 antibody.[192]

Lymphoid-enhancing-binding 1 (LEF1) and SRY (sex-determining region Y) box 11 (SOX11) are novel markers to help differentiate MCL from other B-cell lymphomas. LEF1, not found on healthy mature B-cells, is expressed aberrantly on CLL; MCL is usually LEF1 negative.[204]

It is normally expressed on T-cells and immature (pro) B-cells as part of early lymphoid development.[205]

The Bcl-2 protein family, including Bcl-2 and Bcl-X1, primarily antagonise apoptosis. This is in contrast to the bax, bak, bok, bcl-Xs and bim molecules which act as agonists of apoptosis and are expressed widely. [206] Their mechanism of action is through homo and heterodimerisation resulting in mitochondrial dysfunction and caspase activation. Non-neoplastic cells show variable expression of bcl-2 depending on their stage of differentiation.[207] Aberrant Bcl-2 expression results in acquired resistance to apoptosis and a malignant phenotype across many cancers. By immunohistochemistry, normal mantle cells have high levels of BCL2 and it is expressed highly among most indolent mature B-cell malignancies including MCL.[208]

2.2.7 Genetic profile

MCL is derived from mature B-cells that harbour the t(11;14)(q13;q32) chromosomal abnormality. More than 95% of cases have this translocation; the *CCND1* gene locus is juxtaposed with the immunoglobulin heavy chain promoter resulting in cyclin D1 overexpression. Although cyclin D1 expression is expressed in almost 95% of cases of MCL it is not sufficient for malignant transformation.[209] Genome wide next generation sequencing has identified many recurrent mutated genes in MCL. Commonly mutated genes include those affecting DNA repair (*ATM* and *TP53*), Notch signalling and cell cycling (*NOTCH1* and *CCND1*) and the N-end rule pathway (*UBR5*).[210] The epigenetic modifiers *MLL2*, *MLL3* and *SMARCA4* are also frequently mutated.[211] In cases where the alternative NF-κB pathway is activated, *TRAF2* and *BIRC3* mutations are present in 15% of cases.[212]

Cyclins: Cyclin D1

Cyclin D1 belongs to a highly conserved family of cyclins active in the eukaryotic cell cycle. A key characteristic is their periodicity of protein abundance through the cell cycle. They act by activating cyclin-dependent kinases (CDK) which phosphorylates the tumour-

suppressor protein retinoblastoma (Rb). The phosphorylated Rb protein is inactivate allowing transition through the cell cycle, from G1 to S phase, enabling proliferation.[213]

This family of proteins is often upregulated in different forms of cancers. The *Cyclin D1* gene is amplified in breast and respiratory tumours and present as a chromosomal translocation in multiple myeloma and MCL.[214-217] Cyclin D1 also plays a role in oesophageal and endometrial cancers where point mutations disrupt the nuclear export process.[218 219]

In mantle cell lymphoma, the t(11;14) translocation juxtaposes *CCND1* on to the immunoglobulin heavy chain (*IGH*), located on chromosome 14q32, resulting in constitutive overexpression of Cyclin D1.[217] Cyclin D1, not usually present in B-cells, dimerises with CDK4 resulting in persisting Rb phosphorylation. Inactivated Rb allows cell cycle progression and proliferation. [220] Additional mutational events accumulate over time to amplify Cyclin D1 expression increasing its effects.[165]

More recently, cyclin D1 has been shown to dysregulate global transcription.[221] It acts by "widespread binding to the promoters of actively transcribed genes causing increased transcriptional output". Counterintuitively this process is associated with global transcriptional down modulation proportional to Cyclin D1 levels. This is thought to be due to the inhibitory effect that Cyclin D1 plays on RNA Polymerase II (Pol II) causing 'pausing' and transcriptional impairment. The combined effect results in dysregulated cell proliferation with a tendency to genomic instability.

Cyclin D1 negative MCL and cryptic translocations

A small subset of MCL are CyclinD1 negative and do not possess the t(11;14) translocation. [152] Variant CCND1 translocations are described (e.g. associated with Ig light chains) but are uncommon It was initially postulated that these cases were at the limits of Cyclin D1 detection. However, six cases of Cyclin D1 MCL were shown to have the same gene expression and secondary genetic aberrations as conventional MCL.[222] Some cyclin D1

negative cases express high levels of cyclin D2 and D3 and have translocations of these genes.[223] SOX11 positive, CyclinD1 negative cases, frequently harbour *CCND2* translocations using a light chain Ig partner. [224] Less commonly, cryptic insertions of Ig light chain enhancers result in upregulated CyclinD3 and, even more rarely, overexpression of CylcinE1 and 2 (*CCNE1* and *2* genes).[225]

Although diagnostic for MCL in the context of lymphadenopathy or lymphocytosis, healthy individuals may harbour this translocation. This demonstrates that this translocation, in isolation, is insufficient for malignant transformation.[226]

Other Chromosomal aberrations

Non-random secondary chromosomal aberrations are common, greater than 94%, in MCL.[227] Chromosomal gains include 3q26 (31-50% cases), 7p21 (16-34%) and 8q24 (*MYC*, 16-36%). Chromosomal losses include 1p13-31 (29-52%), 6q23-27 (*TNFAIP*, 23-38%), 9p21 (*CDKN2A*, 18-31%), 11q22-23 (*ATM* in 21-59%), 13q11-13 (22-55%), 13q13-34 (43-51%) and 17p13 (seen in 21-45% of cases). Trisomy 12 has been described in up to 25% of cases, but is usually associated with other aberrations.[228]

TP53 mutations were found in 16/82 (20%) of MCL samples in the Leukaemia and Lymphoma Molecular Profiling Project (LLMPP) group and were associated with an unfavourable clinical course.[229]

SOX11

SRY [Sex determining region-Y]-box11 (SOX11) is a neural transcription factor whose function in normal and malignant B-cell development is unclear. In mice, SOX11 plays an important role in organ and neurological development.[230] Within the SOX family, SOX4 has the greatest homology to SOX11 and is crucial to B-cell development.[231] Gene expression profiling of MCL has highlighted variable transcription factor expression; SOX11 has been shown to be a good discriminator between mutated and unmutated MCL subtypes. It is

absent in most but not all cases of indolent non-nodal MCL.[232] It is found in high levels almost universally in conventional aggressive MCL and at low levels in Burkitt's and acute lymphoblastoid lymphomas. Nuclear expression of SOX11 has not been identified in low-grade lymphomas or diffuse large B-cell lymphoma nor is it seen in non-malignant lymphocytes. [233]

The mechanism underlying aberrant SOX11 expression is thought to involve two pathways. Cyclin D1 increases SOX11 transcription, which is associated with increased level of acetylated histones H3K9 and H3K14 whilst increasing STAT3 expression reduces SOX11 expression.[234] It is postulated that indolent SOX11^{-} MCL results from STA3 activity. The oncogenic mechanism of SOX11 is based upon persistent direct positive regulation of PAX5, a B-cell master regulator. This alters terminal B-cell differentiation preventing maturation to plasmacytic lymphocytes.[235] Thus, B-cells with constitutive expression of SOX11 are held in a mature but not fully differentiated state. Impaired terminal differentiation mirrors some t(9;14)(p13;q32) translocated B-cell malignancies and some DLBCL with mutated *PRDM1* which also have forced PAX5 expression.

SOX11 is able to modulate the tumour microenvironment through pro-survival signals. Silencing of SOX11 in a MCL xenograft model reduced tumour growth whilst SOX11^{+} tumour xenografts were larger than their wild type cell lines.[236 237] Further micro-environmental involvement is supported by evidence that SOX11 binds to the regulatory regions of two genes: *chemokine receptor 4* (*CXCR4*), on the C-X-C motif, and *PTK2* which encodes focal adhesion kinase (FAK). Upregulation of these genes leads to activation of PI3K/AKT and ERK1/2 pathways in MCL resulting in increased cell migration, cell proliferation and drug resistance in comparison to SOX11^{-} cells.[238]

2.2.8 Tissue Microenvironment

MCL cells are often found disseminated at diagnosis with lymphadenopathy and bone marrow involvement.[163 239] Circulating mantle cells and splenomegaly are typical of the LNN form of MCL though both can be seen in conventional MCL. The tissue microenvironments is important for MCL pathogenesis and as a site of interaction with the

MCL BCR. BCR activating mutations such as *CD79B* are rare in MCL and thus it is possible that the disease may rely on tumour-microenvironment interactions.[240] Like CLL, ibrutinib treatment releases CD5 positive malignant cells into the peripheral circulation with a concomitant reduction in lymphadenopathy. Recently egressed MCL cells are initially higher in CD38, before reducing, and lower in CXCR4 expression than their lymph node compartment.[241] In CLL, low CXCR4 expression is associated with cells recently egressed from the lymph node unable to return back to their compartment. [242]

Bone Marrow Compartment

The bone marrow acts to support the haematopoietic system allowing growth and differentiation. To facilitate each stage of maturation there are niches within the marrow depend upon the type and quantity of stromal cells. Stromal cells comprise the non-haematopoietic cells including osteoblast, endothelial cells, perivascular cells, mesenchymal cell and nerve cells. Their spatial organisation and biological activity support haematopoiesis.[243]

MCL cells are frequently found in the bone marrow at diagnosis. Stromal interaction enhances survival and drug resistance in MCL.[244] Adhesion molecules drive homing of MCL cells to the bone marrow and require functional intracellular signalling particularly the classic and alternative NF-κB signalling.[245] Stromal mediated activation of FAK, which is highly expressed in marrow infiltrates, leads to increased expression of pAKT and NF-κB increasing survival and proliferation pathways. FAK inhibitors reduced this effect and overcame ibrutinib resistance.[245]

Lymph Node Compartment

Immunophenoytping of fresh lymphoid tissue has revealed variable sIgM expression and low (similar to SLL) sIgD expression.[246] However, Wiestner and colleagues have recently shown that there is greater BCR signalling activity in the lymph nodes, rather than the peripheral blood tumour cells, of patients with MCL.[240] This supports a tumour microenvironment driver in MCL. In another study looking at survival signals, primary MCL

lines demonstrated high basal interleukins e.g. IL-6, TNFa and VEGF. Anti-IgM stimulation of these cell increased autocrine secretion further. Inhibition using ibrutinib and fostamatinib, a SYK inhibitor, led to a reduction of secretion and increased apoptosis.[247]

Gene expression profiling of paired lymph node and peripheral blood CD19+ cells demonstrated at least a 2-fold change in 130 genes; 116 derived from the tumour and 14 overexpressed in the peripheral blood samples. Further analysis by gene set enrichment revealed a BCR gene signature of 27 genes that were expressed more in the lymph node. This data also demonstrated (7 of 17 patient samples) some peripheral blood BCR scores that were comparable to nodal scores. This suggests that some MCL populations may proliferate independent of their microenvironment. Functional analysis using flow cytometry confirmed increased activity of basal pSYK (Y348), pPLC (Y759), pERK(Y204), pAKT(T308) and p-p65(S529). pSYK activity was correlated with BCR score(r=0.64, P<.02).[240]

Splenic compartment

The spleen is a key secondary lymphoid organ where naïve B-cells encounter antigen. Splenomegaly is often identified in leukaemic non-nodal MCL.[158] Here the spleen is enlarged but there is no, or limited (<2cm), lymphadenopathy. Although the spleen is known as a secondary lymphoid region, a recent study by Weiss and colleagues confirmed that B-cells encounter antigen in the spleen. Reporter mice with BCR activation-induced green fluorescence demonstrated sIgM engagement occurred in the spleen resulting in partially down modulated sIgM but not sIgD.[248]

Primary splenic MCL appears to have a more indolent natural history and can be treated, though not curatively, with splenectomy.[249][250] Scientific studies of splenic MCL are rare but a single study identified the biased usage of *IGHV1-8* (5/18) when compared to MCL with nodal or extra-nodal involvement. All splenic samples were sIgM/D positive (8/8). [251]

2.2.9 Prognosis

MCL has a heterogeneous natural history and response to therapy. It was once widely thought to be a disease with uniformly poor prognosis; median OS is widely quoted to be 4 to 5 years.[154] However, this tends to incorporate a group of elderly frail patients and thus prognosis is much more nuanced. Indeed, the outlook for younger patients who are fit enough to receive high dose cytarabine and rituximab containing regimes followed by autologous stem cell transplant have a median OS of 10 years.[252-254] Despite this good outcome, this therapy is not appropriate for all. Ultra-high risk groups, that tend to do badly even with high dose therapy, are characterised by blastoid morphology,[255] high proliferation and high risk MCL International Prognostic Index (MIPI).

A significant minority have indolent disease and do not benefit from initial treatment. The characteristics of this indolent group of MCL include: the LNN sub group, markers of low proliferation (low Ki67 [<30%] on non-marrow tissue, normal lactate dehydrogenase), non blastoid/pleomorphic histology, non-bulky disease (lymph node <3cm), *TP53* wild type, absence of complex cytogenetics and the absence of clinical B symptoms.[256]

2.2.10 Clinical Prognostic Scoring Systems

Attempts at defining risk of relapse and death have used clinical, histological and molecular markers. The MCL International Prognostic Index (MIPI) stratifies patients into risk groups by OS; low (44% of patients, median OS not reached), intermediate (35%, 51 months) and high-risk groups (21%, 29 months). The four independent prognostic factors are age, performance status, lactate dehydrogenase (LDH), and leukocyte count.[257][258] Furthermore, Ki-67%, a nuclear protein involved in proliferation, has been documented as a powerful and independent predictor of survival and has thus been combined into the MIPI score as the MIPI-biological (MIPI-b) and more recently MIPI-combined (MIPI-c). [179] Unlike the CLL IPI score (Age, Clinical Stage, serum ß2 microglobulin, *TP53* status and *IGHV* mutation status) the mIPI does not include characteristics of the BCR.[259]   The mIPI does not determine therapy in current guidelines but does predict outcome.[151][163]

2.2.11 Biological Prognostic Factors

IGHV

The mutation status of the tumour *IGHV* in MCL is thought to be important but does not fully mirror CLL's clear influence on prognosis. However, U-MCL have a reported worse prognosis than patients with M-MCL in two studies.[168] [250] Using an unconventional mutation division between totally or truly unmutated (100% identical to germline), minimally or borderline mutated (97% to 99.99% germline identity) and highly mutated (<97%) U-MCL has a 5 year OS of 40% against M-MCL OS of 59%.[250]

TP53

A critical tumour suppressor gene across all mature B-cell malignancies is *TP53*, located on the short arm of chromosome 17, expressing the p53 protein.[260] It plays a major role in controlling the cell cycle, particularly DNA repair, stress response and apoptosis.[261]   The Nordic MCL2 and 3 trials evaluated 183 samples finding deletions in 16% of cases whilst 11% carried mutations in the *TP53* gene.[262] In this cohort, only mutations of the *TP53* gene impacted OS (HR, 6.2; P< .001).[262] *TP53* mutation is not overcome by intensive upfront chemotherapy even in younger patients.[254] In a smaller study, both chromosomal deletion and *TP53* mutation were associated with improved survival.[263]

The European MCL network also addressed the role of this tumour suppressor gene. It found high p53 expression (>50% lymphoma cells by immunohistochemistry) was associated with a reduced time to treatment failure and OS.[264] This was independent of MIPI and Ki-67. More recently, the SHINE trial, assessed the role of ibrutinib with bendamustine and rituximab in untreated MCL. It identified *TP53* mutaions in 10% of cases and was a poor prognostic risk factor in both treatment arms.{Wang, 2022 #1041}

Cytology

MCL cytology has a clear prognostic impact and is addressed in 2.2.4 Blastoid and Pleomorphic Mantle Cell Lymphomas. It appears linked to proliferation fraction. Blastoid morphological identification can be difficult due to the variability in fixation and the subjectivity of assessing cell size.[163] A recent large prospective trial identified blastoid and pleomorphic disease in 8.6% of patients.{Wang, 2022 #1041}

Ki67%

The Ki67 proliferation index is also prone to subjective bias and inter-user variability but has shown to be a key prognostic factor in routine clinical practice. A Ki67>30% in non-marrow histology is associated with poor outcome.[162 179] Two randomised trials from the German Low-Grade study group (GLSG1996 and GLSG2000) established the utility of a Ki67 threshold of 30% resulting in the MIPI-b.[265 266] However, this scoring system effectively divided patients into two groups and is insufficient to fully discriminate patients. More recently, the European Mantle Cell Lymphoma Network evaluated the European MCL Younger and MCL Elderly trials integrating the Ki67% into the MIPI-c demonistrating the utility of Ki67% expression. The MIPI-c identified four groups with 5 year OS survival 85%, 72%, 43% and 17% (p<0.001) overcoming both cytology and growth pattern.[267]

Growth pattern

Growth pattern has been assessed in several studies with variable prognostic outcome. Mantle zone pattern MCL was thought to have a significantly better prognosis whilst other studies showed no overall difference.[255 268] Overall, growth patern has not been shown to be a powerful prognostic variable.[267]

SOX11

SOX11 expression has been heavily assessed in recent studies in term of its biological and prognostic significance. There is, however, some persisting controversy about the prognostic utility of SOX11. SOX11 negativity is associated with a non-aggressive clinical course in several publications but cohorts have shown discordance; Wang and colleagues demonstrated reduced survival with SOX11 expression. [232 269-271] In a European cohort of 186 MCL cases SOX11 positivity was seen in indolent cases and SOX11⁻ cases had a shorter OS. Nine percent of this registry cohort had clinically indolent disease defined by an absence of treatment for greater than 2 years. Although SOX11⁻ cases made up 7.5% of the total group, only 12% of the indolent group were SOX11⁻ suggesting an absence of association.[272]

Cell markers

CD38 expression is an important prognostic indicator in CLL. [273] However, in MCL CD38 expression and prognosis is less clear with conflicting studies. In a series of 96 cases, CD38 correlated with survival.[203] In two other studies, of 80 and 60 cases respectively, CD38 was not associated with OS.[168 274]

BCR paramteters

Functional analysis using flow cytometry has demonstrated a combination of biochemical parameters that can be used to produce a BCR score reflecting disease activity (including p-AKT, p-ERK, and p-STAT1 and 5). Low, intermediate, and high BCR scores had median survival of 7.4, 5.3, and 1.9 years respectively.[275]

Transcriptome analysis of MCL patient samples has revealed BCR expression status as a marker of disease activity highlighting the BCR's importance. A six gene signature (*AKT*,

BCL2, *BTK*, *CD79B*, *PIK3CD*, and *SYK*), in 83 cases identified BCR High and BCR low activity. This division correlated with progression free and OS.[276]

2.2.12 Treatment

In young, transplant eligible patients, aggressive cytarabine containing chemo-immunotherapy regimens in conjunction with rituximab (R) are first line.[151] This is usually consolidated with autologous stem cell transplantation (ASCT) and followed by maintenance R.[254 277-279] Chemo-immunotherapy is still generally used first line in older transplant ineligible patients.[280] Trials have generally excluded patients for ASCT if aged over 70 but co-morbidity and general fitness is generally a better guide to suitability.[252] A significant minority can be safely monitored without treatment reflecting the great heterogeneity in MCL.[281 282]

Indications for treatment include bulky symptomatic lymphadenopathy, symptomatic organomegaly, B symptoms (fever > 38°C, weight loss > 5% or night sweats), gastrointestinal symptoms and cytopenias without other cause.[151]

The overall prognosis of MCL appears disappointing with a median OS of 4-5 years. [153] However, this quoted median usually includes a majority of elderly patients not fit for treatment. In younger, fitter, patients, MCL treated with cytarabine containing high dose immuno-chemotherapy followed by consolidation with ASCT results in a median OS exceeding 10 years. [252 254 283]

Although the majority of newly diagnosed cases require treatment, a significant minority appear more indolent.[168 269] Active surveillance, or watch and wait (W&W) approach, appears safe in some cases; 3 studies employed W&W revealing no effect on OS but a median times to treatment of 11, 12 and 35 months.[282 284 285] However, identifying which patients are suitable for W&W is less simple. Data from the British Columbia registry, where some patients had a very long observational period, included patient with

traditionally 'high' risk features such as Ki67>30% (14%) and *TP53* disruption (13%), though none were blastoid.Mottok, et al. [282]

Reliable methods to identify these indolent cases have employed clinical, histological and molecular markers. The MIPI and subsequent MIPI-b (plus Ki-67), have been validated to predict OS in those receiving high dose therapy and ASCT. [257] [258] However, it has proven less effective at identifying low risk indolent disease as elevated age and white cell count can increase the MIPI score, overestimating disease severity. Blastoid morphology and *TP53* disruption identify patients who are at very high risk for relapse post treatment and poor prognosis. [181] [264]

Frontline therapy has no recognised standard therapy and none is curative.[151] Current treatment involves careful assessment of patient fitness; younger, fitter patients receive intensive therapies incorporating rituximab and cytarabine with consolidation ASCT whilst generally older, less fit, patients are treated with chemo-immunotherapy with maintenance rituximab.[151]

MCL first line treatment was historically based upon systemic combination chemotherapy (Cyclophosphamide, Doxarubicin, vincristine, prednisolone - CHOP) until the addition of cytarabine demonstrated significantly improved responses (Complete remission (CR) 7% to 84%).[278] Hyper-CVAD (rituximab, hyper-fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone, alternating with cytarabine and methotrexate) dose-intense regimens without ASCT are highly efficacious. Responses included a 30% 15 year failure free survival (FFS), 61% 5 year PFS and 4.8 year median PFS in American centres. The regimen was haematologically toxic with treatment related mortality (TRM) of 2-8% and appeared difficult to replicate outside of trial centres.[286-288]

In Europe intensive R-CHOP/cytarabine regimens with ASCT consolidation demonstrated excellent responses with less TRM. Response range between median 7.3 years PFS, 74% PFS at 3-years, 7.4 years EFS, median 7 year EFS to 71% 4-year PFS with TRM of 1.5 – 5%.[253] [277] [283] [289] [290]   The addition of maintenance rituximab (intravenous 375mg/m^2 2 monthly for 3

years) post ASCT significantly improved PFS, from 64% to 83% at 4 years, and OS, 80% to 89% (P = 0.04). [290] Alternating bendamustine-rituximab with high dose cytarabine followed by ASCT also appears highly effective with 96% PFS and OS at 1 year in a phase II trial. [291]

The majority of individuals diagnosed with MCL are older, less fit, non-transplant ineligible patients.[151] First line treatment is less well established. At 5 year follow up, R-CHOP plus continuing maintenance rituximab has a PFS and OS of 51% and 79% respectively.[292] Bortezomib, a protesome inhibitor, was assessed in a randomised phase 3 trial, replacing vincristine (Velcade-R-CAP) and compared to R-CHOP.[293] Median PFS was significantly higher in the VR-CAP arm (30.7 months vs 16.1 months; p<0.01) at the cost of more haematological toxicity. Bendamustine-rituximab (BR) is efficacious in the less fit group with better median PFS than R-CHOP (69.5 months vs 31.2 months) or equivalent PFS in 2 phase 3 studies. [294] [295] BR was associated with less toxicity in both studies. The role of 2 year maintenance rituximab post BR is less clear with observation having equal PFS and OS at 4.5 years.[296]

Given the efficacy and favourable toxicity profile of BR, it has been used as the backbone in several trials. In conjunction with cytarabine it proved highly efficacious (2 year PFS 81%) but haematologically toxic.[297] BR plus lenalidomide followed by lenalidomide maintenance has impressive efficacy (3 year OS 73%) but significant side effects (grade 3-5 infection - 42%) and secondary primary malignancies (16%).[298]

Relapse therapy has generally been ineffective and survival post relapse is short with a median OS of 19 months post autograft including those undergoing rescue allograft. [154] [155] However, novel oral agents that target BCR signalling pathways are changing therapy.

B-cell receptor Inhibitors

Ibrutinib, an oral small molecule that covalently inhibits BTK has prolonged survival in the relapsed/refractory setting.[299] Pooled data (n=370) of relapsed/refractory patients treated with ibrutinib had an objective response rate (ORR) of 66%. This comprised 20% with

complete response and 46% partial response. The median progression-free survival (PFS) and OS were 12.8 and 25.0 months, with those receiving less lines of therapy performing better.[156] Patients receiving ibrutinib at first relapse had better outcomes (median PFS 25 months, median OS not reached) than if they had received more than one prior line. Moreover, the 37% achieving CR followiign one prior line and confirmed by PET CT, had a remarkale median duration of response of 55 months.[300] In *TP53* mutated subgroup (n=20) including 3 blastoid cases and 12 post second line treatment, PFS was only 4 months.[300]

Ibrutinb is generally well tolerated; the landmark pooled study demonstrated a discontinuation rate of 10% and 5% for adverse events and death respectively. Disease progression was the largest cause of discontinuation in 59% of cases.[300] The most common grade 3 or more treatment emergent adverse events were neutropenia (17%), thrombocytopenia (12%), pneumonia (12%), anaemia (10%), atrial tachycardia (6%) and raised blood pressure (5%). Adverse events decreased over time and were more common in patients treated beyone first line.[300]

 Acalabrutinib, a second generation irreversible BTK inhibitor, shows activity in MCL. In a pretreated population, at a median duration of 15.1 months, acalabrutinib had an ORR of 81% with 40% in complete response (CR).[301][302] In a further study in a heavily pretreated population (median 2 prior lines) with 18 month median follow up, median PFS was 22 months and most grade 3 AEs were neutropenia (19%) and pneumonia (9%). 9% of patients disconsinued zanabrutinib for AEs.[303]

Current trials incorporate BTK inhibitors and frontline therapy (The Triangle Study (EudraCT: 2014-001363-12); ENRICH (EudraCT: 2015-000832-13)). Jain et al, have recently erported a Phase II study incoprrproating rituximab with ibrutinib in treatment naïve MCL aged 65 and older with low risk features (non blastoid and Ki67<50%). Median PFS and OS have not yet ben reached with median follow up of 45 months. Overall response rate was 96%.[304]   Similarly, zanubrutinb-rituximab is being trialed against Bendamustine-rituximab in a prospective phase III study for patients ineligible for autologous stem cell tranaplantation with results expected in 2026 (NCT04002297).[305]

In CLL, resistance to Ibrutinib commonly occurs through mutations of *BTK* and *PLC-γ2.* [306] In MCL, however, resistance to Ibrutinib rarely occurs through these molecules. MCL develops BTKi resistance through several pathways principally involving kinome-adaptive remodelling of the PI3K-AKT, CXCR4 and the NF-κ B pathways. [240] [307] [308] This results in increased proliferation and cellular adherence to the microenvironment stroma rendering MCL independent of BTK dependent proximal BCR and CXCR4 pathways. Pirtobrutinib (LOXO-305) is a selective first in class non covalent BTK inhibitor designed to overcome BTK binding site mutations seen in CLL. The phase I/II BRUIN study (NCT03740529) included heavily pretreated patients with MCL, 90% had received prior cBTK inhibitor therapy. The ORR was 51% and the CR rate was 25%.[309]

Non BCRi therapies

Venetoclax, a pro apoptotic BH3 mimetic, induces apoptosis in malignant cells overexpressing BCL2. [310] In a phase 1 trial including relapsed/refractory (R/R) MCL a best ORR of 75% and CR rate of 21% were reported. [311] Real world data in a high risk group post ibrutinib therapy revealed an ORR of 53% but short median OS 9.4 months. [312] Although strongly cytotoxic to unstimulated cells *in vitro*, venetoclax appears less effective *in vivo* when the microenvironment has stimulated the lymphoma cells BCR.[313] This resistance mechanism may occur through Mcl-1 but can be downregulated by SYK, BTK or phosphatidylinositol 3-kinaseδ inhibition suggesting synergy between BCR inhibitors and venetoclax.[314] [315] A phase 2 trial, assessing dual ibrutinib and venetoclax therapy in R/R patients with MCL revealed a CR of 62% as assessed by positron-emission tomography at 16 weeks. Tumour lysis, neutropenia and gastrointestinal side effects were present. Moreover, in those with response 78% were still responding at 15 months.[316]

In the Oasis phase I/II trial, a triplet combination of ibrutinib, venetoclax and anti CD20 (obinutuzumab) shows early effect in with 1 year PFS of 93.3% and 100% OS.[317] A larger Phase 3 trial is ongoing in assessing the efficacy of combination ibrutinib and venetoclax in R/R MCL.[318]

Anti-CD19 chimeric antigen receptor (CAR) T-cell therapies are effective in R/R MCL and may work irrespective fo prior BCR inhibition.[319-321] The KTE-X19 anti-CD19 CAR-T had a CR of 59% at a median follow up of 12.3 months in an intention to treat analysis. Grade 2 or higher cytokine release syndrome was seen in 31% of patients.[322] A 3 year update of the ZUMA-2 trial revealed a 68% CR rate with median DOR, PFS and OS of 28, 25 and 46 months respectively irrespective prior BTK exposure or high-risk characteristics.[323]

Bi-specific antibodies are off the shelf products that bind a T-cell epitope with a tumour site epitope resulting in T-cell mediated death of the tumour cells.

2.3 Other Mature B-cell malignancies

The mature B-cell lymphomas comprise a varied group of malignancies defined by their possession of a mature BCR. Importantly they can be classified by their cell lineage and cell of origin (**Figure 2.3**).[324] Further diagnosis is based upon morphological, immunophenotypic, molecular and genetic data to define distinct entities. Clinically, they are often defined as low, or high, grade depending upon their clinical proliferation.

Bone marrow pro or pre-B-cells and naïve B-cells, defined by their absence of antigen experience in the germinal centre of secondary lymphoid organs, are the cell or origin for pre germinal B-cell malignancies; subsets of CLL and MCL.

CLL and MCL are mature B-cell malignancies, as both derive from B-cells with antigen experienced mature BCRs. Both CLL and MCL pre germinal subsets are characterised by U or minimally mutated IGHV. CLL and MCL subsets that are derived from cells that arise following germinal centre experience have a M-*IGHV*.[325]

The germinal centre is a highly proliferative compartment where antigen naïve or cycling lymphocytes encounter antigen resulting in memory B-cells expressing high affinity BCRs and antibody secreting plasma cells. The majority of high grade mature B-cell neoplasms arise from this active compartment including diffuse large B-cell lymphoma (DLBCL),

follicular lymphoma (ranging from low grade to high grade DLBCL-like) and Burkitt's lymphoma.[326] Post germinal centre B-cell malignancies, arising from memory cells and immunoglobulin secreting cells, include M-CLL, M-MCL, the marginal zone lymphomas and multiple myeloma (**Figure 2.3**).
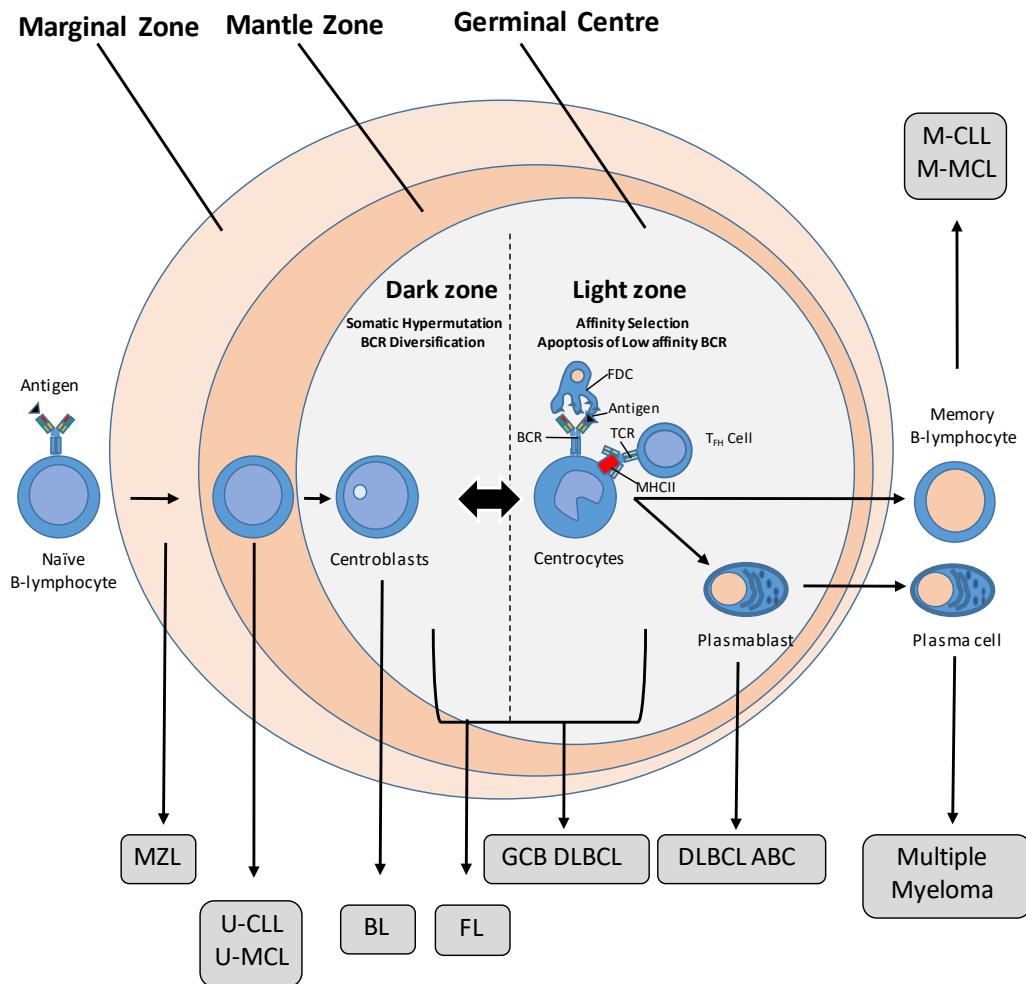
Figure 2.3: Mature B-cell lymphoma cell of origin.

Mature B cell lymphoma arise throughout the stages of B-cell development. Antigen activated B-cell migrates towards the secondary follicle forming the anatomically specialised region of the germinal centre. Marginal zone lymphoma (MZL) arises from the eponymous named region. Unmutated (U) chronic lymphocytic leukaemia (CLL) and U- mantle cell lymphoma (MCL) arise from CD5 positive pre-germinal centre naïve B cell with U-*IGHV*. The aggressive high grade lymphomas Burkitts lymphoma (BL), diffuse large B cell lymphoma (DLBCL) of germinal centre B cell (GCB) origin and activated B-cell subtype (ABC) arise from highly proliferative germinal centre cells. Follicular lymphoma (FL) which is generally more indolent also arises from the germinal centre with variable clinical phenotype. Following somatic hypermutation (SHM) and rounds of affinity maturation followed by clonal selection facilitated by follicular dendritic cells and follicular helper T-cells multiple myeloma arises from generally class switched antibody secreting plasma cells. IGHV mutated (M), antigen experienced, memory cells are the cell of origin for M-CLL and M-MCL. Adapted from *Carbone et al.*[327]

2.4 B-cell malignancies of pre-germinal and post-germinal centre origin

2.4.1 Unmutated and mutated CLL

Chronic lymphocytic leukaemia is a mature B-cell low grade neoplasm characterised by small CD5+, CD19+, CD23+ kappa or lambda restricted B-cells with generally indolent course. Due to relatively common prevalence, indolence and ease of liquid tumour biopsy, CLL is better understood than other mature B-cell malignancies. CLL is divided into two subgroups based upon *IGHV* mutation status with distinct clinico-pathological outcomes.[328] A cut-off of 98% homology to germline *IGHV* defines unmutated (U-) and mutated (M-) CLL. This cut-off has continually demonstrated that U-CLL has a worse clinical prognosis.[329-331] This is underscored by the fact that U-CLL generally possess more genomic aberrations than M-CLL and is more likely to transform to Richter syndrome.[332] [333]

In CLL, the BCR Ig is almost always retained indicating its ongoing influence. In general, the BCR tends to anergy.[85] [115] The normal anergic response renders autoreactive B-cells 'non-responsive' through activation of their surface BCRs.[144] [146] CLL is, although variable, a low signalling disease and its natural history is thus derived from variation within the BCR structure, dependent on the cell of origin, its genetics and the interactions between the BCR and the CLL microenvironment.

U-CLL is derived from naïve CD5+ B-cells whilst the M-CLL clones arise from the less common CD5+ CD27+ post germinal centre memory cells. Mean *IGHV* germline identity, reflecting prior SMH, is low in U-CLL is 99.7% (+-1%) in contrast to M-CLL which is hypermutated with mean *IGHV* mutation of 92.9% (+-3.2%).[334]   A third, much less common subset, contains CLL cells with an isotype switched Ig. These cells usually have a mutated *IGHV* but display *IGHV* gene patterns that are distinct from M-CLL supporting a separate cell of origin.[335]

The role of BCR engagement has long been inferred in CLL pathogenesis. In addition to the presence of *IGHV* mutated CLL, CLL cases also have restricted *IGHV* and IGLV gene usage

and conserved or "stereotypic" IG VDJ sequences.[336] There is over-representation of certain *IGHV*s in CLL; IGHV1-69 and 4-39 in U-CLL and IGHV4-34 and 3-7 in M-CLL cohorts.[334][337]   Moreover, one third of CLL cases have stereotyped BCR sequences where sequence motifs within the variable heavy CDR3 region are 'quasi-identical' between cases.[338] The *IGHV*1-69 fraction of U-CLL shares stereotypic sequences with naïve *IGHV*1-69 B-cells in healthy blood.[337]   These V gene restrictions and stereotypic subsets strongly suggest that CLL ontogeny does not happen 'by chance', but is driven by engagement of antigen in clonotypic lymphocytes.[339]

Complementing the immunogenetic evidence for the role of BCR engagement in CLL, both U- and M- subsets reveal evidence of prior antigen engagement. This principally arises from the observation that sIgM expression is down-modulated in circulating cells and recovers over time *in vitro*. [145] Ligation of the BCR results in sIg-driven signalling and endocytosis of the receptor. Proximal signalling, when assessed by Phosflow cytometry, results in basally elevated p-SFK, p-SYK and p-PLC-γ2 but low levels of p-AKT and p-p65 NF-κB. However, anti IgM induced signalling is low and only slightly greater than normal healthy B lymphocyte controls.[275]

Given the striking clinical variation between U- and M-CLL it is not unsurprising to find variance in CLL BCR signalling capacity. Indeed, signalling is higher in U-CLL whilst lower, but more variable, in M-CLL. U-CLL differs with M-CLL in response to antigen engagement as there is less down modulation of sIgM expression in U-CLL. Retaining more sIgM, U-CLL is thus able to signal more strongly. Thus in U-CLL there is stronger signalling and proliferation whilst in M-CLL, anergy and a more indolent clinical course predominate. Reflecting CLL's tendency to anergy rather than proliferation, sIgM expression is generally low compared to normal naïve and memory B-cells. Crucially, M-CLL has lower sIgM expression than U-CLL. BCR signalling capacity has clinical implications as high surface IgM expressers/high signallers also have shorter time to treatment.[340] In contrast, sIgD expression has no correlation with signalling capacity or prognosis identifying the BCR-M isotype as the most clinically important subgroup.

Some of the putative (super) antigens engaging CLL BCRs have been identified and include both endogenous antigens (auto- or poly-reactive) and exogenous - where the BCR retains more specificity. Given the pre-germinal centre cell of origin it is not surprising that autoantigens with polyreactivity appear to be more common in U-CLL.[341] The repertoire of endogenous antigens that may engage with CLL BCR include myosin heavy chain IIa, vimentin (an intermediate filament protein that is expressed in mesenchymal cells of eukaryotes) and 'neoantigens' - the by-product of protein oxidation.[146 342-344] This process of BCR engagement secondary to endogenous antigen is unlike that seen in some, CD5 negative, marginal zone lymphomas. The BCR in splenic marginal zone lymphoma (SMZL) and mucosa associated lymphoid tissue (MALT) can be driven by exogenous pathogenic antigens from hepatitis C and *Helicobacter pylori* respectively.[345 346]

CLL BCRs react with auto antigens found in the environment and have also been shown to react with themselves, induced by self-recognition of the BCR. This process, known as 'autonomous signalling', causes intracellular calcium mobilisation without standard BCR ligation. CLL cells recognise a highly conserved epitope within a FRW2 region found in the heavy chain V region.[342 347]

It is likely that the site of antigen exposure, whether endogenous or exogenous, is the site of CLL proliferation i.e. the lymphoid tissue. Here, dendritic cells may present unprocessed antigen as the CLL cells coalesce in agglomerates known as proliferation centres and interact with the tissue microenvironment.[348] CLL cells migrate from peripheral blood to tissue throughout their life; surface IgM and the chemokine receptor CXCR4 are upregulated in the absence of cognate ligand (antigen and CXCL12 expressed on stromal cells) forming a chemokine gradient aiding recirculation.[349] This is evidenced by intraclonal heterogeneity where CLL cells, having just left tissue sites, are identified by an 'imprint' of recent receptor engagement.[85] Recently egressed proliferating CLL cells, possessing a distinct stimulated gene expression, are identifiable by a $CD5^{bright}$ $CXCR4^{dim}$ immunophenotype.[242 350]

Evidence for *in vivo* effects of the microenvironment on the CLL BCR came indirectly from proving reversibility of sIgM expression. Surface IgM expression 'recovers' when incubated in 'antigen free' media *in vitro* or *in* vivo following ibrutinib therapy.[145][351] This is accompanied by a rapid rise in CXCR4 reflecting its recent release from receptor down modulation in the tissue microenvironment. Ibrutinib induced lymphocytosis provides further *in vivo* evidence of tissue based receptor down modulation. Ibrutinb functions in two ways; first, by inhibiting Bruton's tyrosine kinase (BTK) in BCR signalling and secondly, by inhibiting CXCR4/5 adhesion and migration pathways.[352-354] Blockade of this second pathway results in the release of malignant cells into the peripheral blood away from microenvironment support and, crucially, antigen. Subsequently, CLL surface IgM, but not sIgD, increases 1 week following ibrutinib therapy.[351] Supporting this hypothesis of chronic antigen engagement, basal pSYK is raised in pre-treatment CLL cells and, following ibrutinib therapy, pSYK levels are found to be reduced, presumably in the absence of (auto)antigen stimuli.[351]

Further evidence of sIgM down-modulation in CLL can be detected through changes in immunoglobulin µ chain N-glycosylation patterns (**Section 1.3**). Both glycoforms (mature and immature) are detectable in CLL but more immature forms are present in U-CLL than M-CLL.[355] Post ibrutinib treated cells also revert to a mature glycosylation pattern.[351] This suggests that, in CLL, BCR engagement is chronic and perhaps is preferentially acting on the poly-reactive unmutated Ig in the tissue compartments.

CLL is a naturally recirculating disease where the tissue microenvironment not only affects proliferation but also trafficking and survival. *In vitro* CLL cells undergo apoptosis but can be rescued by the addition of stromal cells. Stromal cells are able to confer resistance to anti CLL treatment.[356][357] CD49D, which binds to stromal fibronectin, is expressed in CLL/SLL and prevents spontaneous and chemotherapy induced apoptosis. Unsurprisingly increased CD49D expression is associated with reduced OS.[358] CD49D mediates cell-to-cell and cell-to-stroma interactions and is involved in CLL homing to SLOs.[359] Its importance is underlined by the association of its expression with the nodal form SLL.[360] CD49D is expressed at lower levels in CLL than in MCL or marginal zone lymphoma.[361]

2.5 Germinal centre mature B-cell malignancies

2.5.1 Follicular lymphoma

Follicular lymphoma is a common low grade B-cell lymphoma characterised by the overexpression of BCL2 due to a t(14;18) translocation during V(D)J recombination. It is clinically characterised by slow growing lymphadenopathy treated by local radiotherapy in localised stage 1 disease or immune-chemotherapy for more advanced stages with symptoms. Active surveillance is undertaken for some patients until symptomatic.[362]

In spite of the early loss of one Ig allele in the t(14;18) translocation, there is retention if the surface Ig. This translocation is necessary but not sufficient for tumour development. During normal B-cell activation in the germinal centres, t(14;18)[+] follicular B-cells have a likely survival advantage due to the overexpression of BCL2 whilst undergoing selection and proliferation. Thus, follicular B-cells accumulate and recycle through further rounds of T-cell dependent clonal selection. Affinity maturation continues with ongoing SHM changes and crucially, the cells do not undergo apoptosis. Further genetic lesions are slowly acquired before the full malignant phenotype becomes apparent.[362]

One distinctive and unusual phenotypic change is the acquisition of N-glycosylation sites during SHM in the V region of the sIg.[363] Acquired high mannose glycans on the V region are able to bind to dendritic cell–specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), a lectin found in lymphoid tissue (**Figure 2.4**). Lectin-mannose binding induces intracellular calcium mobilisation and BCR downstream activation. In vitro incubation of follicular lymphoma cells does not lead to sIg expression changes.[364] Surface Ig expression starts at relatively high levels in comparison to the chronically engaged CLL tumour cell. Moreover, cross-linked DC-SIGN does not cause sIg endocytosis. These findings support an antigen independent model of follicular lymphoma pathogenesis.

2.5.2 Diffuse Large B-cell Lymphoma

DLBCL is a high-grade mature B-cell lymphoma and the most common lymphoid malignancy in the UK with peak incidence in men aged over 80.[147][159] It has two molecular subtypes with different clinical outcomes: The activated B-cell-like (ABC) subtype is aggressive and generally has inferior outcomes compared to its less aggressive counterpart, germinal centre B-cell like (GCB) DLBCL.[365] GCB DLBCL tumours express many genes found in healthy germinal centre B-cells, such as *BCL6*.[365] In contrast, ABC DLBCL gene expression profiling resembles healthy antigen-activated B-cells with constitutive expression of NF-κB signalling likely derived from a plasmablastic cell.[366][367]

DLBCLs of both subtype retain their BCRs despite chromosomal aberrations that may disrupt the IGH locus.[368] The cell of origin in all subtypes of DLBCL is germinal centre derived (**Figure 2.3**).[62][367] The ABC subtype generally expresses an IgM-BCR whist the GCB subtype, with ongoing SMH and having undergone class-switching recombination, typically expressing the IgG-BCR isotype.[369][370] Although the importance of the BCR is apparent from the link between clinical outcome and BCR isotype, the first evidence to support this came from RNA interference screening. This indicated that BTK was critical to survival in DLBCL cell lines without *CARD11* mutation. Moreover, further knockdown of BCR components (IgM, CD79a and b, SYK, PI3Kδ and PKCβ) revealed a survival disadvantage when compared to wild-type cells. DLBCL lines with a *CARD11* activating mutation were not susceptible to BCR interference but were to knockdown of NF-κB components.[371] The CARD11-BCL10-MALT1 (CBM) complex links BCR signalling to the NF-κB signalling pathway.

However, only 10% of patients with ABC-DLBCL have acquired somatic mutations in *CARD11* suggesting proximal pathogenic aetiologies. The mechanism of chronic BCR activation in ABC DLBCL (**Figure 2.4**) was proven through experiments that altered the *IGHV* region of three ABC cell lines. This identified three mechanisms of possible antigen driven auto reactivity in ABC DLBCL: One V_H 4-34 cell line had V region dependent self-glycoprotein auto-reactivity, the second BCR isotype responded to self-antigens in apoptotic material whilst the third line was driven by its own V region idiotypic epitope.[372]

Furthermore, microscopic examination of ABC and GCB-cell lines revealed BCR clustering, as seen in normal antigen stimulated B-cells, in only the ABC lines. [371]

Genomic sequencing revealed mutated membrane-proximal regions of CD79a and b in 23% of cases. These mutations prevent receptor endocytosis and LYN mediated negative regulation, promoting chronic signalling.[371] More recently, another gain of function mutation, targeting the Toll-like receptor (TLR) signalling adaptor MYD88 in ibrutinib sensitive ABC cell lines, identified a pathogenic super complex formed by MYD88, TLR9 and the BCR (My-T-BCR) linking the two proximal pathways with NF-κB signalling pathway.[373] Thus, both antigen-dependent activation and gentic aberrations contribute to ABC DLBCL BCR signalling activation.[374]

In contrast, cell surface BCR clustering has not been identified in GCB DLBCL supporting a different pathogenic mechanism to its ABC counterpart. Moreover, GCB DLBCL are insensitive to BTK inhibition and are not NF-κB activated.[366] Initially labelled tonic signalling, due to detectable basal phosphorylated (p)SYK and BLNK in the absence of BCR crosslinking, GCB BCR signalling is now named constitutive germinal centre BCR signalling.[373][375] Constitutive signalling is antigen independent; alteration of the antigen binding regions has no effect on BCR signalling.[376] Moreover, induced mutations of CD79A Y188 inhibit GCB constitutive signalling but do not affect calcium flux following BCR cross-linking, further supporting antigen independence in this subgroup.[376] Phelan et al. have revealed CD19 and LYN dependency in this group whilst PTEN mutations are well documented.[373][376]

More recently, a significant subset of GCB DLBCL have been identified with additional glycans inserted in the Ig antigen biding site reflecting extrinisically driven signalling and a follicular lymphoma origin. These cases are associated with more aggressive disease and have enriched BCR, PI3K and MYC signalling pathways.[377]

2.5.3 Burkitt Lymphoma

Burkitt lymphoma (BL) is a rare high-grade mature B-cell lymphoma of germinal centre cell of origin (**Figure 2.3**). It is defined by translocation of the *MYC* gene; Ig heavy chain t(8;14), typical histology and high proliferation factor, Ki67> 95%.[378] This suggests an alternative pathogenesis to other high-grade lymphomas such as DLBCL. There are three forms of BL; sporadic BL (sBL) seen in developed countries, Epstein Barr associated (EBV-BL), endemic to Africa and HIV associated (immunodeficiency-related BL). Although the three forms share a common gene expression profile the sporadic form can be differentiated by its gene expression. BL originates from the GC, sharing gene expression profiles with normal cells, but is distinct from GCB DLBCL.[379]

The BCR is retained in BL as the *MYC* translocation always spares the productive IG heavy chain allele allowing the heavy chain to form a productive BCR.[380] Like GBC DLBCL, BL is NF-κB independent and CD19/LYN dependent suggesting constitutive germinal centre BCR signalling (**Figure 2.4**).[373 381] Tonic signalling engages the PI3K pathway and transgenic mice, engineered to constitutively express MYC and PI3K, produced BL like (by histology and gene expression profile) lymphomas.[382] There is further evidence of the role of constitutive-PI3K BCR signalling in BL. RNA interference screening reveals that PI3 kinase activity is increased by the transcription factor TCF3, which acts by upregulating surface BCR through upregulated expression of the IG heavy and light chains.[383] TCF3 has a further role inhibiting *PTPN6* that encodes SHP-1 phosphatase. This phosphatase slows BCR signalling by dephosphorylating ITAM motifs. Constitutive signalling is thus increased by two mechanisms; upregulated BCR expression and a reduction in negative regulation by loss of SHP-1.

Furthermore, and with interesting parallels in relation to MCL, a second mutation is present in 38% of sBl cases that affects the cell cycle kinase cyclin D3.[384] *CCND3* mutations in BL produce highly stable cyclin D3 isoforms that dysregulate the cell cycle regulation promoting proliferation. Overall, this leads to increased sIgM in BL that is more active resulting in highly aggressive constitutive BCR signalling and a highly aggressive mature B-cell tumour.[383]

Low Grade Lymphomas

CLL SMZL MALT Follicular Lymphoma

Antigen Dependent:
Microbial/Auto (super)antigen)   *VH* CDR3 binds to FR2   Hepatitis C Virus   *Heliobacter pylori*   Glycan - DC-SIGN

Antigen

High Grade Lymphomas

ABC DLBCL GCB DLBCL Burkitt Lymphoma

Antigen Co-Dependence
Chronic Active BCR signalling   Antigen Independence (50% Glycan-DC SIGN)
Constitutive Germinal Centre BCR signalling   Antigen Independence
Constitutive Germinal Centre BCR signalling

Antigen

CD79A/B CD19 LYN TCF3 CD19 LYN

CARD11 PTEN + c-MYC

PI3-K PI3-K

NF-κB
Activation MYD88 TNFAIP3

Figure 2.4: BCR signalling activation in Mature B-cell malignancies

Low Grade lymphomas: BCR signalling occurs through ligand resulting in downstream signalling cascade. The BCR is internalised post stimulation in CLL and negative regulators are activated to prevent prolonged activation. Follicular lymphoma acquires atypical glycans in the CDR3, which signals via DC-SIGN. The BCR does not internalise. (SMZL: Splenic marginal zone lymphoma, MALT: Mucosa associated lymphoid tissue, DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin)

High Grade lymphomas: Chronic active signalling through micro-clustered sIgM results in continued activation of BCR and downstream signalling in ABC DLBCL. BCR and downstream mutations in red e.g. CD79B preventing BCR recycling or CARD11 perpetuating signal effect to NF-κB and Transcription factor-3 (TCF3) overexpression leading to BCR over activity in Burkitt. Constitutive germinal centre B-cell signalling in GBC and Burkitt lymphoma are CD19/PI3-K dependent. 50% of GCB DLBCL signal through the follicular lymphoma associated glycan-DC SIGN pathway. (ABC DLBC: Activated B-cell diffuse large B-cell lymphoma, GCB: Germinal Centre B-cell) See text for further details. Adapted from *Young, R. M., Shaffer, A. L., Phelan, J. D., & Staudt, L. M. (2015). B-cell Receptor Signalling in Diffuse Large B-cell Lymphoma. Seminars in Hematology, 52*(2), 77–85. [114]

2.6 The B-cell receptor in Mantle Cell Lymphoma

The functional unit of all normal B-cells and the majority of B-cell neoplasms is the BCR and BCR signalling is a key determinant of behaviour in CLL and DLBCL.[27 385 386] Like other mature B-cell malignancies MCL retains expression of a functional BCR, predominantly M and D, and knowledge of its structure and function may also explain MCL's behaviour and help improve its management.[387]

2.6.1 IGHV

The MCL repertoire is skewed (in reducing order of prevalence, from *IGHV3-21*, *4-34*, *1-8* to *3-23)* accounting for 46.3% of cases in one large cohort (n=807) but varies from the CLL and normal naive and memory repertoires (**Figure 2.5**). [388] 10.4% cases, principally employing *IGHV*3-21 and *4*-34, possessed stereotyped heavy complementarity determining region 3 (VH CDR3) sequences. Immunoglobulin light chain bias is also present in MCL identified by an inverse λ:κ ratio of 2:1 whilst normal healthy lymphocytes express a λ:κ ratio of 1:2.[188] A subgroup of MCL patients has been identified with abnormal heavy light chain pairings: *VH*3-21 co-express almost uniformly λ light chains of the V$_λ$ 3-19 gene. These patients tend to be younger at diagnosis and have longer median survival when compared to other MCL patients (53 vs 34 months; P = .03).[389]
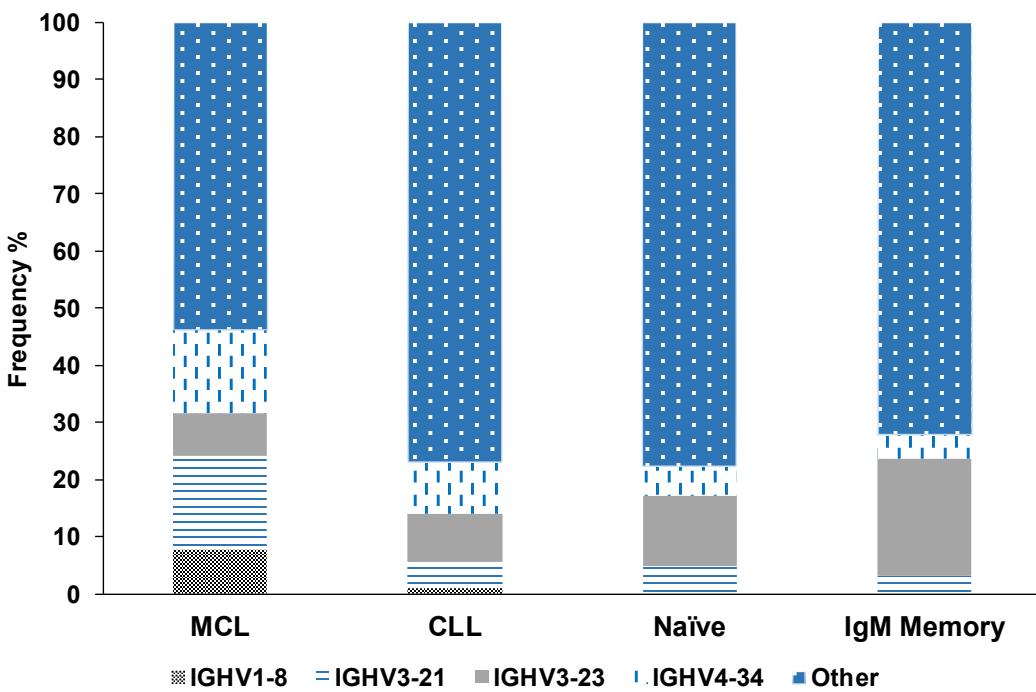
Figure 2.5: *IGHV*-gene repertoire of MCL, CLL and naïve B-cell BCRs

Biased immunoglobulin usage is seen in healthy and malignant B-cells. MCL displays a distinct IGHV usage in comparison to CLL and healthy B-cells. MCL bias in order of decreasing frequency: *IGHV3-21*, *4-34*, *1-8* and *3-23*. Adapted from *Fichtner et al.* [390]

The mutational status of the *IGHV* gene in MCL is relatively low when compared to other mature B-cell malignancies. In the largest study to date, 23% of primary MCL samples were classed as mutated using the accepted 98% cut off.[388] Smaller studies have shown MCL to possess >2% variation in its sequence in 16-29% of cases.[203 391 392] In comparison 3 large CLL cohorts were mutated in 38-61% of cases.[393] The 98% germline variation cut-off has also been questioned in MCL; initially used as a useful marker for prognostication in CLL, larger studies in MCL have found 97% variation to have more clinical utility.[250 388] Using this cut off only 13.8% of cases are classed as mutated whilst 29.5% were completely unmutated.[388] Using this cut off, one study found variable outcomes with a 5 year OS of 59% and 40%, M vs U-MCL (P< 0.01).[250]

2.6.2 *IGHV* & Cell of origin

Historically, MCL had been seen as an aggressive B-cell lymphoma that developed directly from naive B-lymphocytes without germinal centre experience or antigenic stimulation.[158] [167] However, it is now accepted that MCL is comprised of two major molecular subtypes derived from a CD5^{+} positive B-cells that harbour the t(11;14) translocation defined by the status of the tumour *IGHV*.[158] [209] The more common subtype, clinically defined as classical or conventional MCL, has a U- or, minimally M-, *IGHV*. It expresses SOX11 and clinically involves nodal or extra-nodal sites (**Figure 2.1**).[158] Conversely, the less common leukaemic non-nodal MCL, derives from a SOX11 negative B-cell with a mutated tumour *IGHV* and generally has lower genomic complexity with better prognosis.[250] The two subsets retain epigenetic features that reflect their likely cell of origin; U-MCL has DNA methylation and expression signatures that reflect a naïve B-cell and M-MCL retains those of a memory B-cell (**Figure 2.3**).[394]

2.6.3 Surface Immunoglobulin

MCL cells are characterised by relatively high but variable levels of BCR as identified through the transmembrane surface immunoglobulin (sIg) M or D in lymphoid tissue [186] [246]. This is unlike other ibrutinib sensitive B-cell neoplasms such as CLL which have generally much lower sIgM expression.[186] [340] A case series of 103 MCL samples identified 71% as dual sIgM/D positive, 23% were sIgM positive/D negative whilst only 6 (3%) were either sIgM negative/D positive or sIgG positive.[251] In another series looking at tissue samples, reactive hyperplasia tended to much higher sIgD but similar or low levels of sIgM when compared to MCL.[246] Another large study assessed sIgM via CD79B, a BCR subunit; surface IgM levels in MCL were high, variable but significantly higher than in CLL; ranging from low levels, similar to CLL and marginal zone lymphoma, to high, like healthy volunteer lymphocytes, follicular lymphoma and DLBCL.[275] Unfortunately, unlike CLL, there is much less availability of MCL samples due to its relative rarity. There is therefore, less published evidence regarding MCL BCR expression, function and drivers.

2.6.4 BCR Activation

In other mature B-cell malignancies, such as CLL and DLBCL, there is evidence to support a role for (auto)-antigen driven or activated BCR signalling (**Figure 2.4**). In MCL, there is minimal evidence for a candidate auto-antigen. However, in over a third of cases in one study, MCL BCRs do, like CLL, show auto-reactivity to vimentin.[395] This is significantly less than M-CLL (56.7%), U-CLL (89.6%) and appears more similar to mature B cell auto-reactivity (40%).[42 396] Superantigens, which bind to FWRs on the BCR, are candidate causes of BCR engagement and signalling.[397] A small study showed that *Staphylococcus aureus* protein A binds is able to bind to the FWR of the *IGHV*3 immunoglobulins causing calcium flux.[390 397]

2.6.5 Proximal Signalling

Although BCR associated therapies have improved survival, the signalling mechanisms in MCL are only partially understood.[156 299] Due to its rarity most cell signalling understanding is derived from cell lines which, although useful, may not reflect the function and diversity of primary MCL samples.

BCR expression is intrinsically linked to proximal BCR activity. Although not known to associate with basal phosphorylation, surface BCR expression (CD79B and surface IgM) positively correlates with α-IgM-induced signalling. [275] Experimentally increased expression of CD79B subunits established a direct relationship between the surface BCR unit and proximal induction.[275]

Constitutive phosphorylation of SYK, BTK, PLC-γ2, ERK and AKT has been demonstrated in mantle cell lines and primary cells.[247 275 398-400] BTK is more commonly detectable by Western blot in both cell lines and primary cells whilst constitutively phosphorylated SYK, PLC-γ2, ERK and AKT is less commonly identified reflecting MCL's heterogeneous nature. Stimulation with α-IgM results in high and sustained (1 hour) phosphorylation in MCL cell.[247]

Gene amplification and overexpression of SYK and the PI3K catalytic subunit is present in the cell line Jeko-1 and GRANTA respectively and a subset of primary MCL samples.[401][402] In these cases gene alterations did not always correlate with detectable phosphorylated pAKT.[402] Hyper-methylation of SHP-1, an antagonist of growth-promoting and oncogenic protein tyrosine kinases, results in sustained activation of BCR-mediated pathways. [403]

The MCL cell lines, Mino and Jeko, are sensitive to the BTK inhibitor ibrutinib undergoing cell death and a reduction in phosphorylated BTK and anti-apoptotic molecules Bcl-2, Bcl-xL and Mcl-1.[400] SYK is overexpressed in some primary MCL cases and several cell lines including Jeko. SYK inhibitors cause apoptosis *in vitro*, particularly when SYK expression is high.[401][404] The kinase PKCβ, found downstream of BTK, has been shown to be elevated in some MCL cases.[405]

In mice models over expressing SOX11, mass cytometry (CyTOF) assays have demonstrated increased BCR baseline signalling in mice expressing SOX11 and increased response to anti-IgM stimulation. BTK inhibition with ibrutinib reversed elevated basal signalling. [406]

2.6.6 PI3K-Akt pathway

Constitutive pAKT is seen in the more aggressive blastoid variants whilst in only one third of conventional MCL by Western blotting of primary cells.[398] In contrast, some studies have not been able to detect basally phosphorylated pAKT and have had to resort to stimulating the BCR in order to study pAKT.[407] The protein PTEN, which acts by degrading PIP3, a potent activator of AKT activity, is implicated in MCL pathogenesis. The inactive phosphorylated form of PTEN is correlated with Akt activation in MCL lines.[399] There is great variability in pAKT activity but gene expression reveals upregulation of the *PI3K*, *AKT* and mTOR pathways which is supported by more recent Phosflow comparisons.[408][275] These show elevated basal pAKT compared to healthy peripheral blood mononuclear cells but significantly less than is seen in DLBCL.[275]

Mammalian target of rapamycin (mTOR).

Clinical effectiveness of mTOR inhibition has been demonstrated with concomitant de-phosphorylation of GSK-3β and Akt.[409][410] GSK-3β is a signalling kinase regulated by both the PI3K/Akt and canonical Wnt pathway and is constitutively phosphorylated in over two thirds of MCL tumours detected by immuno-histochemistry staining. It has shown to be correlated with cyclin D1 expression and OS.[411] Furthermore, MCL cell lines, such as Granta 519, are held in the G1 phase of the cell cycle with concomitant reduction in Cyclin D3 but not D1 with the use of the mTOR inhibitor rapamycin.[412]

2.6.7 NF-κB Signalling

MCL cell lines have been investigated by pharmacological profiling using the BTK inhibitor ibrutinib and pan-protein kinase C (PKC) inhibitor sotrastaurin. PKC functions by coupling BTK activation with the CBM complex to allow activation and downstream activation of NF-κB.[413] This has demonstrated BCR signalling inhibition sensitivity dependent on NF-κB signalling pathway type. Tumours using CBM- NF-κB, or classical, signalling display sensitivity to BCR inhibitors.[212] Alternatively, tumours with somatic mutations identified in the alternative NF-κB pathway may show BCR signalling inhibitor insensitivity. This pathway is dependent on the protein kinase NIK and may be a target for therapeutic intervention.[212] The cell lines Jeko-1 and Mino were shown to be sensitive to sotrastaurin and demonstrated NF-KB activation by gene expression profiling.[212] CARD11, a scaffold protein linking BCR signalling to NF-KB, is mutated in ABC DLBCL. More recently, whole exome sequencing has also identified these mutations in 5.5% of MCL cases conferring *in vitro* resistance to ibrutinib and lenalidomide.[414]
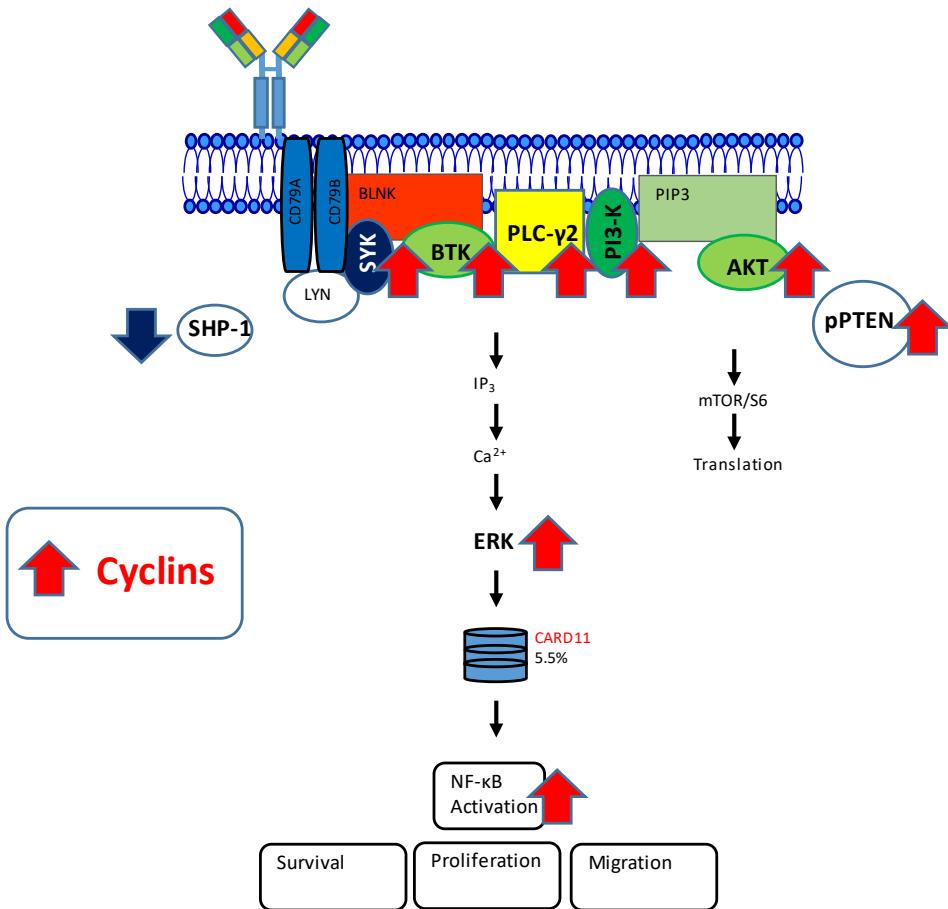
Figure 2.6: Pathogenic BCR signalling pathways in MCL

Schematic representation of the BCR signalling pathway in MCL.   The activation mechanism of the BCR is unknown but immunogenetic data supports a role for chronic BCR engagement in at least a significant minority of MCL.   SYK, BTK, PLC-γ2, PI£-K catalytic subunits, phosphorylated PTEN, ERK, and NF-κB may be upregulated (red arrow) in the pathogenesis of MCL. CARD11 mutations are seen in a small percentage of cases resulting in BCR independent signalling, analogous to ABC DLBCL. The negative regulator SHP-1 is downregulated (blue arrow) in some MCL cases allowing uncontrolled signalling

Table 2.3: Mature B-cell malignancies and the BCR.

Malignancy	Postulated COO	Primary BCR Drive	*IGHV* bias	Anergy	BCR activator	sIgM expression	BCR Mutations	BCR Effects
U-CLL	Pre-GC	Antigen	+	+	Auto antigen Pathogen Autonomous	Reduced		Proliferation/Anergy
M-CLL	Post-GC	Antigen	+	++	Auto antigen Pathogen Autonomous	Reduced		Anergy
DLBCL ABC	GC	Chronic active	-	-	Autoantigen Autonomous	Increased Micro clustered	CD79a/b CARD11 MYD88	Impaired BCR internalisation and enhanced BCR signalling via reduced LYN activity
GCB DLBCL	GC	50% Extrinsic Constitutive	-	-	Lectin (DC SIGN) Constitutive	Reduced Class switch IgG	IG-N-Gly site CD79a	Chronic extrinsic BCR stimulation Constitutive signalling
Burkitt	GC	Constitutive	-	-	Constitutive	Increased	TCF3	Increased BCR expression Constitutive signalling
Follicular lymphoma	GC	Extrinsic	-	-	Auto antigen Lectin (DC-SIGN)	Increased	IG-N-Gly site	Chronic extrinsic BCR stimulation
MCL	Pre-/Post- GC	Unknown	+	Unknown	? Autonomous	Variable	CARD11 (5%)	Unknown

Comparison of mature B-cell malignancies demonstrating the principal different signalling pathways (constitutive, antigen driven, extrinsic and chronic active), presence of anergy, principal BCR activation, surface IgM expression, principal oncogenic mutations and their effects. U- and M-CLL result from antigen drive evidence by low sIgM and anergic B-cells. There is evidence for multiple BCR activators such as autoantigen, pathogen and autonomous signalling. [415]

2.7 Project Hypothesis and Aims

The MCL BCR is sensitive to inhibition identifying the important role that the BCR plays in its natural history. MCL is a heterogeneous and relatively rare disease meaning that little is known about BCR expression and signalling activation. Like other mature B-cell malignancies, above all CLL, its biased immunogenetic repertoire and stereotyped sequences suggest a role for antigen-interaction in its patho-biology, in at least a subset of cases.[388] Therefore the aim of this study is to characterise and functionally phenotype the MCL BCR. To achieve this goal, primary MCL samples, principally blood but including lymph node and spleen where available, will be analysed. BCR structure, expression and function will be assessed, looking for associations by *IGHV* mutation status and WHO clinical subgroup. This will then be compared to the known BCR functional phenotypes in other B-malignancies, chiefly CLL.

2.7.1 Overarching Hypothesis

B-cell receptor structure, expression and function are important determinants of MCL behaviour; a subgroup of MCL possess BCR characteristics, similar to CLL, revealing the role of BCR engagement in MCL behaviour.

2.7.2 Aims:

1. To phenotype MCL BCRs and identify associations between *IGHV* structure, clinical WHO groups, sIg expression, signalling capacity and cell surface adhesion molecules.
2. To look for functional evidence of prior BCR engagement suggesting antigen interaction; sIg down modulation, upregulation of proximal BCR kinases, IGH constant chain glycosylation changes and Ig variation across tissue compartments.
3. To assess for clinically significant associations by *IGHV* status, clinical WHO groups, and sIg expression.

Chapter 3 Material and Methods

3.1 Primary MCL Samples

Cryopreserved samples were taken from patients presenting with lymphocytosis to hospitals recruiting to the Observational Study (formerly Lymphoproliferative disorder study) from January 2001 to August 2019 based at Southampton University Hospital Trust. The Observational Study was approved by the Institutional Review Boards at the University of Southampton (228/02/t). The NHS Health Research Authority approved ethical opinion with REC reference 16/SC/0030, protocol number LLR/12021 and IRAS project ID 186109. LLR renewal application 2015, 971 is the Clinicaltrials.gov identifier. Patient tissue samples from lymph node and spleen were available from the University of Southampton Tissue Bank, Bournemouth hospitals and the University of Karolinska (material transfer agreement RITM0172125). All patients provided written informed consent prior to inclusion in the study.

Diagnosis of MCL was made according to the World Health Organisation classification of haematopoietic and lymphoid tissue. This requires a B-cell clonal population with t(11;14) translocation or tissue cyclinD1 expression [167]. Characterisation of MCL samples (*IGHV* gene, mutational status, sIgM expression, soluble α-IgM antibody induced intracellular calcium flux) was carried out by research technicians in some cases. [340]

CLL samples are included and used for comparative studies in the forthcoming chapters. All CLL samples were recruited to the Observational trial (formerly LPD) at the Department of haematology of the University of Southampton from January 2001 to June 2019. The 2008 International Workshop on Chronic Lymphocytic Leukaemia (iWCLL2008) criteria were used to confirm the diagnosis with a Matutes score>3. B-cell receptor phenotypic characteristics (*IGHV* gene and mutational status, sIgM expression, soluble α-IgM antibody induced intracellular calcium flux). Interphase Fluorescence-in-situ-hybridization (FISH) was performed when available (**Table 8.5**).

3.2 Cell counting and viability determination by Trypan blue exclusion

Counting of cell lines and patient samples were performed in a Class II safety cabinet. An equal volume (1:1) of 10µl cell suspension was added to 10µl 0.4% (w/v) Trypan blue in an Eppendorf tube and mixed well. The cell/dye suspension was pipetted to then edge of the haemocytometer chamber which was then drawn up capillarity. Trypan blue is a negatively charged dye staining cells with damaged cell membranes. Hence, healthy viable cells appear bright and unstained. Viable cells in 25 squares (volume = 1×10^{-4} cm^3) were counted using light microscopy. The average of two counts was calculated using the following equation: Cell concentration = Average cell count x two (dilution factor) x 10^4 (cells/ml). Calculated cell concentration was multiplied by the volume of cell suspension in order to calculate the total cell number.

3.3 Phenotypic analysis of MCL cells using flow cytometry

Cryopreserved PBMCs and tissue samples (Spleen, lymph node and gastric tissue) from MCL patients were thawed, centrifuged (1500rpm (350g) for 5 minutes) and recovered for 1 hour in complete RPMI medium at 37°C/5% CO$_2$. Subsequently the viability and number of cells was determined by trypan blue exclusion. Cells were centrifuged and re-suspended in fluorescence activated cell sorting (FACS) buffer (1% BSA, 4 mmol/L EDTA, and 0.15 mmol/L NaN3 in PBS) to a minimum final cell concentration of 1×10^7 cells/mL. Cells (1×10^6 cells /100µl) were transferred to individually labelled FACS tubes and subjected to immunofluorescence staining. Each sample was stained with up to four monoclonal antibodies labelled with fluorescent dyes. These were CD19, CD5 and two markers of interest or two corresponding isotype controls (**Table 3.1**). Antibody master mixtures were used in order to minimise staining variations. Each tube was gently scraped to mix the cells with the antibodies and incubated on ice in the dark for 30 minutes to allow antibody binding. Following incubation, 2ml of FACS buffer was added to each tube and centrifuged at 1500RPM (350g) for 5 minutes. The supernatant was discarded and each cell pellet re-suspended in 300µl of FACS buffer. The tubes were then kept on ice and in the dark before proceeding to acquisition on the flow cytometer. To maintain the same procedure for all samples, the phenotypic characterisation samples were acquired on BD FACSCaliburs. Using forward and side scatter properties, 10,000 lymphocytes were recorded and the

results were analysed using FlowJo software (version 10). For recovery experiments, samples were acquired on a BD FACSCanto II using the identical procedure at 0, 24, 48 and 72 hours.   FACS CantoII appropriate antibodies were used in the recovery experiments (**Table 3.1**)

Table 3.1: Antibodies used for Immunophenotypic characterisation of MCL cohort.

Flow Cytometer Experiment	Antibody	Supplier	Catalogue Number	Volume
	Mouse CD5 Per CP cy5.5*	Biolegend	302212	2 μl
	Mouse CD19 APC*	Biolegend	302212	2.5μl
	Rabbit anti-IgM PE*	DAKO (Alere)	R511101	10μl
	Rabbit F(ab')2 PE (isotype control) *	DAKO (Alere)	X093001	5μl
	Rabbit anti-IgD FITC*	DAKO (Alere)	F018901	5μl
	Rabbit F(ab') 2 FITC (isotype control) *	DAKO (Alere)	X092901	1μl
FACSCalibur	Mouse anti-CD23 PE*	Biolegend	338508	5μl
Surface Phenotype	Mouse IgG1k PE (isotype control)*	BD Biosciences	555749	1μl
	Mouse anti-CD38 PE*	Biolegend	356604	5μl
	Mouse IgG1k PE (isotype control)*	BD Biosciences	555749	1μl
	Mouse anti-CD49d FITC*	Biolegend	304316	5μl
	Mouse IgG1k FITC (isotype control)*	BD Biosciences	555748	5μl
	Mouse CD5 Per CP cy5.5*	Biolegend	302212	2 μl
	Mouse CD19 Pacific Blue*	Biolegend	302232	1μl
	Mouse anti-IgM Antibody*	Biolegend	314508	5μl
CANTOII	Mouse IgG1, κ Isotype (Isotype control)*	Biolegend	400112	0.6μl
Recovery Phenotype	Mouse anti-human IgD*	Biolegend	348206	5μl
	Mouse IgG2a, κ (Isotype control)*	Biolegend	400210	1μl
	APC anti-human CD184 (CXCR4) *	Biolegend	306510	2.5μl
	APC Mouse IgG2a, κ (Isotype Control)*	Biolegend	400220	2.5μl

*Stored at 4oC; (anti-human unless otherwise specified) Matching Isotype control below test antibodies.

3.4 Intracellular Calcium Signalling

Cryopreserved MCL samples were thawed, allowed to rest for 1 hour in RPMI whilst cell count and viability was performed. Cells were centrifuged (1500rpm (350g), 5 minutes) and re-suspended in complete RPMI medium at 1×10^7/mL. Cell suspension (1ml; 1×10^7 cells) was aliquoted into labelled FACS tubes and 10% Pluronic acid F-127 (4µl) and Fluo3-AM (1.6µl) were added in the dark to prevent deactivation of the Flou3-AM dye (**Table 8.2**). Following incubation at 37°C for 30 minutes, cells were washed once in 2mL complete RPMI at room temperature and re-suspended in RPMI medium (2mL). The sample was divided into 4x500µl aliquots ($2.5\times10^{\wedge}6$ cells per tube) and kept at room temperature in the dark. Prior to acquisition, the cell suspension was transferred to a 37°C water bath for 5 minutes before analysis was performed on a BD FACSCalibur flow cytometer. A ligand (20µl of soluble F(ab')$_2$ α-IgM or α-IgD (20µg/ml)) was added at 34 seconds of acquisition and the response was recorded for a total of 5 minutes. Ionomycin (20µl), a calcium channel ionophore, was added to the final tube for 3 minutes as a positive control. Data were analysed using FlowJo software (version10) on the lymphocyte gate. Calcium flux was calculated using the difference between basal calcium and peak or mean flux. This was divided by the percentage of CD19+CD5+ cells, which had been calculated during the phenotypic analysis of the patient sample, to produce an intracellular calcium flux percentage.

3.5 Phosflow phenotypic analysis

Cryopreserved mononuclear cells from MCL patients were thawed, rested for 1 hour and cell number was determined by trypan blue exclusion. Cells were centrifuged and re-suspended in complete RPMI medium at 10×10^6 cells/mL. Cells (2×10^6 cells in 200µL) were pipetted into a 96 well round bottom plate and treated with 10µM of SYK signalling inhibitors (Entospletinib (GS-9973) or DMSO control) for 1 hour at 37°C. Following treatment, cells were stimulated with α-IgM (20µg/ml) or α-IgD (20µg/ml) for 4 minutes at 37°C. Paraformaldehyde (PFA) was subsequently added to each well at a final concentration of 1.6% for 5 minutes at room temperature. This process stopped the induced signalling by chemically crosslinking adjacent macromolecules. The samples were

transferred into labelled FACS tubes, washed in 2ml of phosphate buffer solution (PBS) and subsequently centrifuged at 1500RPM (350g) for 5 minutes. The supernatant was discarded and 1.5mL of 90% ice-cold methanol was added to permeabilise the cells. The samples were vortexed for 5 seconds and left for 10 minutes on ice. Following methanol treatment, the samples were washed twice in 2ml PBS; on the last wash, the supernatant was removed and the tube blotted on tissue paper to remove as much liquid as possible. The cell pellet was re-suspended in PBS (200μL) ready for fluorescent cell barcoding.

3.5.1 Fluorescent cell barcoding

Fluorescent cell barcoding is a process that allows flow cytometric isolation of cell groups combined in a single sample this allows for efficient use of antibodies and cells. In this study Pacific Blue and Pacific Orange succinimidyl esters were used to barcode the cells.

The optimised (**Barcoding and Phosflow validation using Cell Lines**) concentration of Pacific Blue and Pacific Orange dyes was added to each sample tube before vortexing and incubating at room temperature, in the dark, for 30 minutes. The samples were then washed once in PBS (2ml), then again in 2ml PBS + 1% bovine serum albumin (BSA). The cell pellets were re-suspended in PBS + 1% BSA and the samples pooled. The samples were subsequently added to labelled FACS tubes ensuring equal volumes were added resulting in 100μl/$1x10^6$ cells per tube. The sample tubes were incubated at room temperature in the dark for 60 minutes with appropriate phosflow antibodies (**Table 8.3**). Following incubation, PBS+1% BSA (2ml) was added to the tubes and the sample was centrifuged at 1500RPM (350g) for 5 minutes at room temperature. The supernatant was discarded, cell pellet re-suspended in 100uL of PBS+1% BSA and the samples acquired on the BD CANTOII flow cytometer.

3.6 Barcoding and Phosflow validation using Cell Lines

Human leukaemia-lymphoma cell lines have been used since the establishment of a Burkitt lymphoma derived line in 1963. The advantages of continuous cell lines are their unlimited

supply, infinite storability in liquid nitrogen and thus worldwide availability to the scientific community of a monoclonal population. Cell line establishment is difficult but there are now numerous MCL cell lines that are well characterised. Due to its relative rarity of primary MCL tissue, cell lines are helpful in understanding MCL biology and developing new assays.

3.6.1 Jeko-1

JeKo-1 is an Epstein-Barr virus (EBV) negative large cell mantle cell lymphoma cell line established in 1998 from a seventy eight year old. The sample was taken from peripheral blood in a patient undergoing leukaemic conversion.   Established surface immunophenotypic characterisation displays positivity for CD5, CD19, CD20, CD22, CD45RA and HLA-DR antigens, and negative for CD3, CD4, CD8, CD10, CD14, CD23 and CD56. The cells are cyIgMDκ+ and sIgMDκ+.[416] Jeko-1 demonstrated sensitivity to ibrutinib with an IC_{50} of 0.115µmol/L.[417]

3.6.2 Barcoding Confirmation

Barcoding has been successfully performed and published using DyLight 350, DyLight 800, Pacific Orange and Pacific Blue at various concentrations and combinations [418-421]. Pacific Blue (PB) and Pacific Orange (PO) dyes were chosen due to availability and as per the Myklebust et al[422] (2017) and Irish et al[423], (2010) publications. PB (Irish 0.78, 7.0 and 50ng/mL, Myklebust 31.2, 7.8, 1.56 and 0.195ng/mL) and PO (Myklebust 8.7, 87 and 510ng/mL, Irish 20.89, 3.48 and 0.348ng/mL) were assessed as part of the optimisation process (**Figure 3.1 A**). Optimum dye concentrations were chosen based on clarity of cell separation. The use of 9 conditions was possible but introduced overlap of cell barcodes and hence error.   Outer concentrations typically appeared clearest allowing 6 conditions (including no barcode) as required (**Figure 3.1 B**).

Pacific Blue ng/ml	Pacific Orange ng/ml
0.78	20.89
0.78	87
0.78	520
7.8	20.89
7.8	87
7.8	520
50	20.89
50	87
50	520

Pacific Blue ng/ml	Pacific Orange ng/ml
0.78	520
7.8	520
50	520
50	87
50	20.89
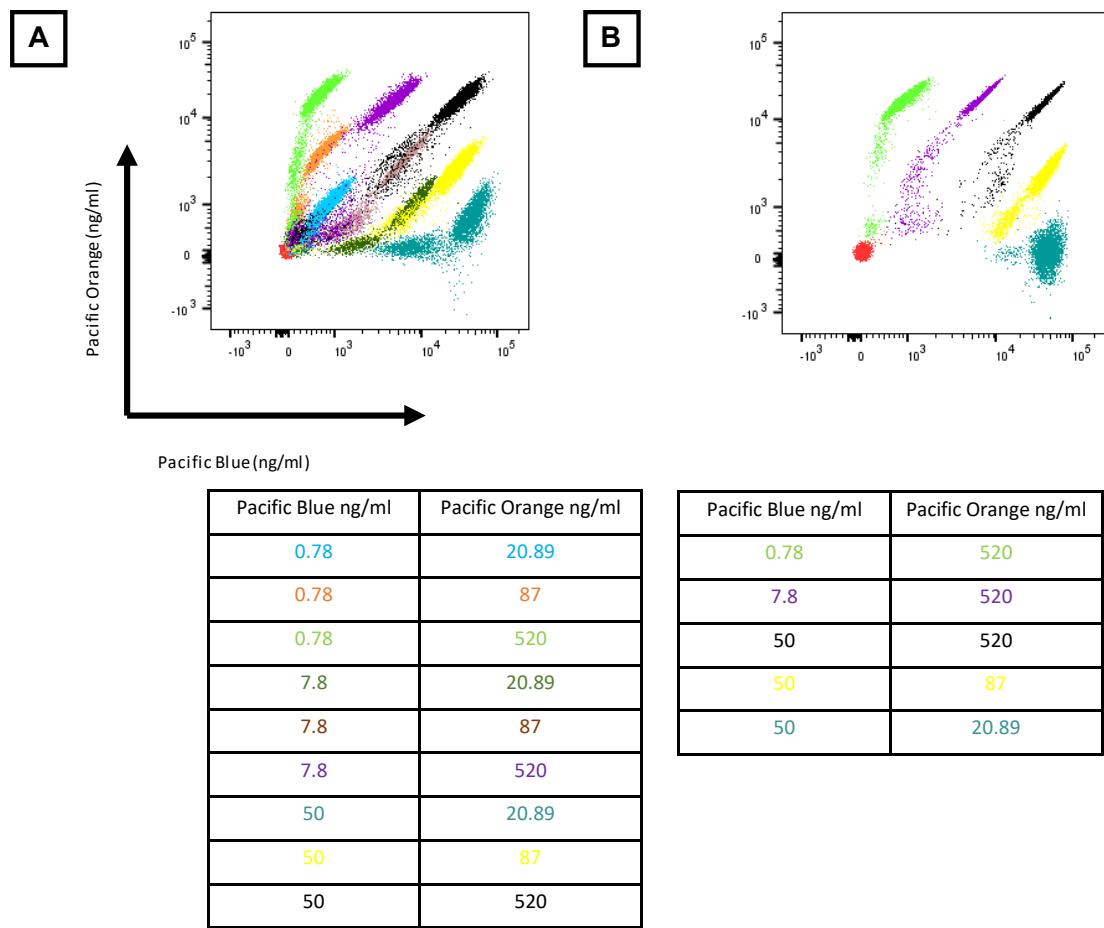
Figure 3.1: Barcoding dye combination development.

Dye combination allowing maximum separation and minimal overlap. Tails represent possible overlap and introduction of error. A) Overlay of all 5 chosen concentrations of PB (0.78, 7.8 and 50ng/mL) and PO (20.89, 87 and 520ng/mL) in one sample resulting in 9 different distinguishable samples. B) Chosen concentration combinations of PB (0.78, 7.8 and 50ng/mL) and PO (20.89, 87 and 520ng/mL) for optimum Phosflow barcoding on Jeko-1. Red is unstained. PB: Pacific Blue, PO: Pacific Orange.

The Jeko-1 cell lines was successfully identified, isolated and analysed using the bar coding and Phosflow assay. Live lymphocytes were identified through their forward and side scatter profile residing in a well-demarcated group, low side scatter, medium forwards scatter. To avoid the assessment of doublets, a gated population removing low forwards scatter –area/height (FSC-A and FSC-H) was formed. The lymphocyte population could then be reassessed before cell populations are isolated by their pre-assigned dye concentrations (**Figure 3.2.A –D**)
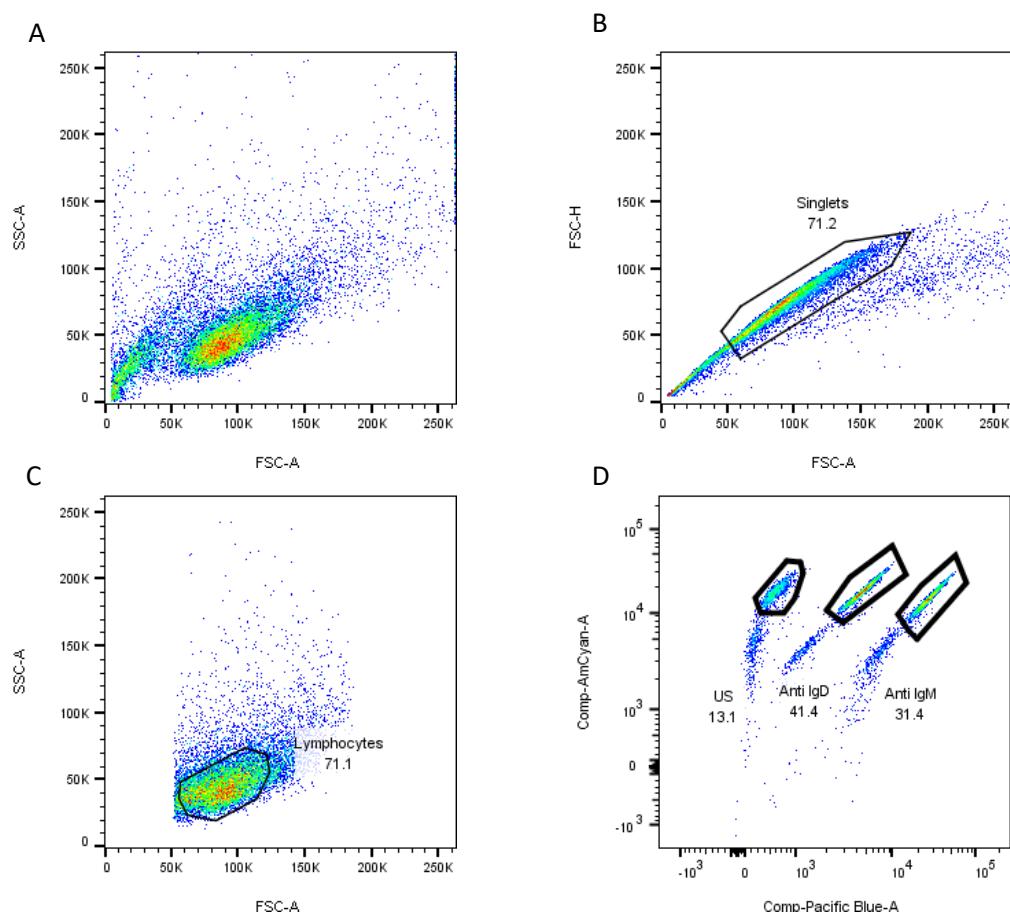
Figure 3.2: Cell line analysis of barcoding

FlowJo analysis of cell line Jeko-1 cell line. A) Forward versus size scatter plot of lymphocytes before analysis. B) Doublets are removed leaving only single lymphocytes. C) Single cell lymphocytes are identified using forward scatter and size scatter revealing typical lymphocyte gate. D) Lymphocyte subsets are identified by their barcoding signatures revealing three populations assessing three conditions, unstimulated (US), soluble anti IgD stimulated (Anti IgD), soluble anti IgM stimulated (Anti IgM) The sample was analysed until 5000 cells were counted in a single condition.(Anti IgM).

3.7 Immunoblotting 'Western Blotting'

3.7.1 Protein Extraction

Cryopreserved PBMCs from MCL patients were thawed, rested for 1 hour in RPMI at 37°C and cell number was determined by trypan blue exclusion. MCL lymphoid cells were centrifuged and re-suspended in RPMI at $1x10^7$/mL before being plated out in 500μl wells. Samples were left unstimulated in RPMI or DMSO and stimulated with α-IgM (20μg/ml) at 37°C. After 4 minutes the samples were immediately placed on ice stopping any further reactions. The cells were collected in 1.5ml Eppendorf tubes and washed with ice-cold PBS (400μl). Samples were then centrifuged at 2000 RPM for 5 minutes at 4°C, and then washed again with ice-cold PBS (900μl). The supernatant was discarded and the cells were mechanically broken up with a pipette tip before cells were lysed using RIPA lysis buffer. Protease and phosphatase inhibitors were added to the lysis buffer to prevent further phosphatase activity and maintain protein stability. The cells were incubated with the lysis buffer on ice for 30 minutes before being vortexed and centrifuged at 13000 RPM (16,100g) for 10 minutes at 4°C. The supernatant was added to new labelled tubes on ice and the pellet discarded. At this point the samples were kept on ice awaiting protein quantification.

3.7.2 Protein quantification.

Sample protein concentration was calculated using Pierce BCA Protein Assay Kit (**Table 8.7**). A standard curve was calculated using a serial dilution of known concentrations of bovine serum albumin (BSA) (0, 0.15, 0.3, 0.6, 1.25, 2.5, 0.5 and 1μg/μl) in duplicate. Protein extract (4μl) was mixed with 46μl of H2O from each experimental sample. According to manufacturer's instructions 200μl working reagent (A+B) was added to each sample and incubated for 30 minutes at 37°C/5% CO_2. A Varioskan Flash plate reader (ThermoScientific) was used to detect the colour change at 595nm which could then be used to calculate the estimated protein concentration. This was determined from the standard curve ensuring an equal amount of protein for each sample was analysed by immunoblotting.

3.7.3 Gel electrophoresis

Protein (15 µg) from each sample cell lysate was taken and made up to 20 µl with distilled water. Dithiothreitol (DTT; 1 µl) and 9 µl of loading dye were then added to each sample before heating at 95°C for 5 minutes. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate and visualise the protein samples (**Table 8.7**). SDS-polyacrylamide gels were made as described in 'Molecular Cloning a Laboratory Manual' [424]. To make the running gel, 10% acrylamide was poured into the space between two glass plates. After 30 minutes, the gel polymerises and a stacking gel is added containing a 10 well comb. Once set, the comb was removed and the gels placed in a running buffer chamber filled with SDS detergent (1:9 Distilled water). The samples and molecular weight marker were loaded into separate wells. An electric current was run across the solution for 90 minutes, 100V for the first 15 minutes and 120 V for the remaining time.

3.7.4 Transfer of proteins from gel to membrane

Following SDS-PAGE, the gel was removed from the glass plates and placed upon a nitrocellulose membrane (**Table 8.7**) together with filter paper forming a 'sandwich'. The 'sandwich' was submerged in transfer buffer in order to allow the migration of proteins from the gel to the nitrocellulose membrane. The filter paper-gel 'sandwich' was then placed into a transfer tank and a voltage of 100V was applied for 90 minutes. A sealed ice unit was submerged in the tank preventing overheating and temperature fluctuations during protein transfer.

3.7.5 Antibody incubation and visualisation

Following transfer, the nitrocellulose membrane was blocked for at least 1 hour at room temperature using a 5% BSA blocking solution in Tris-Buffered Saline – Tween-20 (TBST). The membrane was subsequently incubated with appropriate dilutions of primary antibody (**Table 8.7**) in 5% BSA in TBST overnight at 4°C or for 3 hours at room temperature.

Following incubation, the membrane was washed twice with TBST for 5 minutes.   The membrane was then incubated with the corresponding secondary antibody diluted 1:2000 in 3% milk in TBST at room temperature for 1 hour.   Following the secondary antibody incubation, the membrane was washed three times in TBST for 10 minutes. The secondary antibodies are conjugated to horseradish peroxidase that allows visual detection under ultraviolet light with appropriate substrate. The chemoluminescent substrate, SuperSignal West Pico Chemiluminescent Substrate (400μl Reagent1 + 400μl Reagent2), was added to the membrane as per manufacturer's protocol. The colorimetric signal was imaged using the UVP ChemiDoc – It Imaging System with the BioChemi HR Camera P/N 97-0155-02, 230V ~50Hz (UVP, Cambridge, UK). Densitometry of protein bands was carried out by ImageJ software (1.41v, US National Institutes of Health, USA).

3.8 Biotinylation and glycosylation analysis of cell surface IgM

Cell surface glycan structure was assessed by isolation of cell surface structures using biotinylation. Biotin has high affinity for avidin that can be used to select out biotinylated molecules for further immunoblotting assessment.

3.8.1 Biotinylation of cell-surface proteins

Cell-surface proteins were biotinylated using the Cell Surface Protein Isolation kit (Pierce) according to the manufacturer's protocol. Cryopreserved mononuclear cells from MCL patients were thawed, rested for 1 hour and counted by trypan blue exclusion. Primary cells were centrifuged at 1500RPM for 5 minutes and re-suspended in RPMI medium at 1×10^7/mL. Viable cells were isolated following Lymhoprep (Stemcell technologies) centrifugation for 20 minutes at 1500RPM (350g) with brake applied. Cells (20×10^6) were washed twice in cold PBS and the supernatant removed. The cells were incubated with 1.4ml (0.7ml Biotin per 1×10^7 cells) Sulfo-NHS-SS-Biotin (0.5mg/ml) at 4°C for 30 minutes. The reaction was terminated using Quenching solution (Pierce), then washed twice with 1ml cold PBS. The cells were lysed by sonication in Lysis Buffer (Pierce) with added solution of SIGMAFAST Protease Inhibitor Tablets (Sigma-Aldrich). The lysis mixture was incubated on ice for 30 minutes followed by centrifugation at 12,000g for 10 minutes. The

supernatant was mixed with washed Immobilized NeutrAvidin Gel (Pierce) and incubated at room temperature for 60 minutes on a roller. Following incubation, the NeutrAvidin Gel was centrifuged at 12,000g for 10 seconds before washing (3 times) in Wash Buffer (Pierce). The biotinylated proteins were eluted by heating at 95°C for 5 minutes in SDS-PAGE sample buffer containing 50mM dithiothreitol (DTT). Samples were then processed as per the Western blot procedure 'Gel electrophoresis' onwards. Surface IgM was visualised by immunblotting using the primary anti-rabbit anti-μ antibody (Jackson ImmunoResearch Laboratories).

3.8.2 Glycosidase treatment

BCR engagement results in alteration of mannose sugars on the μ chain of B lymphocytes. Glycosidase digestion by Endoglycosidase H (EndoH) and peptide:N-glycosidase F (PNGase) (New England Biolabs) were used to identify these changes in MCL. Following biotinylation of the cell surface proteins, appropriate denaturing buffers were added to the samples before denaturing (95°C for 5 minutes). Following heating the samples were cooled on ice before the addition of 10μl G3 reaction buffer 10X (New England Biolabs) and 3μl EndoH (Endo H digestion) and 10μl G2 reaction buffer 10X, 10μl NP-40, 3μl PNGase (PNGase digestion) to the appropriate tubes (**Table 8.7**). The samples were incubated at 37°C for 3 hours in a water bath with occasional mixing. After 3 hours, 50μl of loading dye + DTT was added and the samples boiled at 95°C for 5min. The sample was stored at -20°C or analysed as per the immuno blot procedure (**Methods**: **Gel electrophoresis**).

3.8.3 Statistical Methodology

The availability of samples determined the number of samples analysed in this study. Clinical data collected was collected prospectively when possible recording details from presentation. Date of diagnosis was recorded from the histopathology report or immunophenotyping report where available. Following diagnosis date, time to progression to first treatment (TTFT) was recorded by date of first intervention (surgical or medical), event free survival (EFS) by date of next line of therapy or death due to any cause following treatment and OS to date of death or last follow up. EFS was used as a primary endpoint as

most patients have historically been treated at presentation. OS was assessed as a secondary endpoint as it may be confounded by era of diagnosis and therapy availability (pre/post rituximab or kinases). High/low signalling cut offs were compared and inferred from absolute CLL signalling (iCa^{2+} mobilisation, %) and correlation (MFI) data.

The normality of data was assessed by the Shapiro-Wilk test. For independent data sets of continuous variables without a normal distribution, the Mann-Whitney U test was employed. Independent data sets with a normal distribution were assessed by the Student's unpaired T test with an assumption of equal variance (Levene's test). All statistical tests were two sided. A p-value <0.05 was considered significant. Survival analysis was performed by Kaplan-Meier method using log-rank statistics. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software v.22.0 (Chicago, IL) and GraphPad PRISM software version 8 (La Jolla, CA).

Chapter 4 Phenotypic Characterisation of the MCL BCR

4.1 Introduction

MCL is a mature cluster of differentiation CD5+ B-cell tumour characterised by common phenotypic and genetic features causing an overexpression of cyclins (typically cyclin D1) affecting cell cycle progression.[158] MCL is heterogeneous with a spectrum of disease from the indolent to the very aggressive. The variability of BCR structure, expression and function, is fundamental to the pathogenesis and clinical course in CLL.[340] Like CLL, MCL express IgM and IgD. Association of *IGHV* status with clinical outcome and sensitivity to BCR kinase inhibition demonstrate the importance of BCR signalling in MCL.[156 299]

Although originally thought to be an antigen naïve disease, immunogenetic evidence now supports a role for selective pressure on the MCL BCR. Almost half of MCL BCRs use four *IGHV*s (in decreasing order of frequency, 3-21, 4-34, 1-8 and 3-23). [388] Biased usage is seen across all MCL but U-MCL typically uses *IGHV*3-23, 4-34 and 3-21 whilst M-MCL uses *IGHV*1-8 and 4-59.[250 388 391] The light chain isotype distribution in MCL is skewed in the reverse (λ:κ, 2:1) direction. [425] Demonstrating the non-stochastic nature of influences on the BCR, U-MCL utilizing *IGHV*3-21 are almost universally paired with a λ light chain, *IGLV*3-19. [392 426] Unlike CLL, MCL with *IGHV*3-21 tend to have a better prognosis, independent of mutation status and have fewer genomic aberrations compared to non *IGHV*3-21 MCL. [392 426] Furthermore, the presence of MCL BCRs with mutated IGHVs (sequence homology to germline <98%) in up to a quarter of cases, typically seen in the more indolent LNN subtype, confirmed prior germinal centre, and hence antigen, experience.[388]

Moreover, approximately 10% of cases show quasi-identical amino acid sequences across the entire IGHV domain, 'stereotypy', particularly, IGHV3-21, IGHV3-23, and IGHV4-34.[388] As in CLL, this shows a proportion are likely to have had antigenic selection act upon the BCR at some point during MCL development, highlighting the importance of the BCR.[388] However, mean *IGHV* germline homology is lower in 'memory like' M-CLL than 'memory like' M-MCL (92.9%+-3.2% vs 95.1%=-1.5%).[152]

Surface BCR is typically of the sIgM and D isotype in MCL.[186 246] In a series of 103 MCL samples identified: 71% as dual sIgM/D positive, 23% were sIgM positive/D negative whilst only 6 (3%) were either sIgM negative/D positive or sIgG positive.[251] Surface IgM expression in lymph node tissue is high, variable, and overlapping small lymphocytic lymphoma and other large cell lymphoma expression, whilst sIgD is low differentiating it from reactive hyperplasia.[246] Surface IgM is generally much higher than CLL suggesting that reversible endocytosis, mediated by (auto) antigen, as appears in CLL is not occurring in the majority of cases.[275]

MCL has no known activating mutations of the BCR like DLBCL or Burkitt lymphoma, but does have weak evidence for autoantigen sensitivity in *in vitro* experiments, like CLL.[395] MCL has dysregulated BCR signalling pathways with increased phosphorylated SYK, BTK, PLC-γ2, ERK and AKT detected in cell lines and primary cells. [247 275 398-400] MCL sIgM expression positively correlates with α-IgM-induced proximal BCR activity and negatively associated with BCR inhibitor efficacy.[275] High surface Ig that retains signalling capacity resembles follicular lymphoma (FL). FL possess an acquired N-glycosylation and appear to interact with non-endocytosable lectin.[364] MCL do not appear to have acquired this glycosylation change but may have other interacting environmental factors.[427]

The overuse of specific *IGHV* genes and BCR variability suggest selective environmental influences may play a role in MCL ontogeny akin to CLL. Given the relationship between sIgM expression, signalling capacity and clinical outcomes in CLL,[340] this chapter will assess MCL BCR phenotypic and signalling characteristics to further investigate the role of BCR structure, expression and function in MCL behaviour.

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4.2 Phenotypic Characterisation: Hypothesis and Aims

4.2.1 Hypotheses

1. The MCL surface Ig immunophenotype mirrors CLL and associates with *IGHV* mutation status.
2. MCL has variable signalling capacity that associates with *IGHV* status and clinical subgroup.

4.2.2 Aims:

1. To explore MCL BCR *IGHV* structure and mutational status.
2. To explore MCL surface immunoglobulin expression: assess for associations by *IGHV* mutation status, clinical subgroup with comparison to CLL cohort.
3. To explore signalling capacity in response to α-IgM, by calcium and Phosflow, and assess associations by *IGHV* mutation status, clinical subgroup and in comparison to CLL.
4. To explore MCL surface adhesion molecule expression: assess for associations by *IGHV* mutation status, clinical subgroup with comparison to CLL cohort.

4.3 Results

4.3.1 *IGHV* use and mutational status in MCL

4.3.2 Sample Cohort

The clinical background and phenotype of 36 peripheral blood MCL samples were assessed (**Table 4.1**). Diagnostic WHO criteria was used to confirm MCL diagnosis; either confirmed t(11;14) positivity or cyclinD1 expression in a mature B cell phenotype. Samples were generally from older (range 45-93, median 70 years) male patients (n=29) with equal M- and U-MCL (n=17). The median lactate dehydrogenase (LDH) level was above the upper

limit of normal (ULN – 425 Units/Litre; range 249-1274, median 457). The MCL

international prognostic index (MIPI) score was available in 32 (89%) patients. MIPI (range

5.6-9.7, median 6.7) was classed as low risk in two patients, intermediate risk in four and

high risk for the remaining cases. Clinical MCL subgroup classification was available in 36

patients. Nineteen (52%) were nodal MCL, whilst one third (n=13, 36%) were leukaemic

non-nodal, one (3%) was pleomorphic (previously nodal MCL) and three (10%) were

blastoid (all previously leukaemic non-nodal (LNN) MCL).

Table 4.1: Clinical, histological and genetic characterisation of peripheral blood MCL samples.

PB MCL cohort (n=36)	PB MCL (n=36)		U-MCL (n=17)		M-MCL (n=17)		P
	Frequency (%)	Median (Range)	Frequency (%)	Range (median)	Frequency (%)	Range (median)	(U vs M)
Clinical Characteristics							
Diagnostic Age (Years)	27 (90)	70 (45-93)		71 (46-93)		67 (45-88)	0.55
Male	22 (75)		12 (75)		14 (83)		n.s
LDH (Units/Litre)	34 (94)	445 (249-1274)		433 (304-101)		477 (249-1274)	0.25
MIPIb	32 (89) High (72%)		High (45)		High (52)		n.s
Ki67%							
Leukaemic Non-nodal	13 (36)		2 (12)		14 (80)		<0.01[*]
Blastoid or Pleomorphic Histology	4 (11)		1 (6)		3 (18)		-
Performance Status (0-1)	33(92)		15 (94)		15 (88)		0.80
Genetic Characteristics							
***TP53* disrupted (Tested n=14, 39%)**	5 (36)		1 (20)		2 (80)		-

Data compiled up to 18th November 2019. Clinical data extracted from electronic records from University Hospitals Southampton 2018 to November 2019. Statistics generated using GraphPad software.   * denotes significance <0.05. Calculated if sufficient number. (PB, peripheral blood. U-MCL, unmutated mantle cell lymphoma. M-MCL, mutated MCL. LDH, lactate dehydrogenase. MIPI, Mantle cell International Prognostic Index. IGHV, immunoglobulin heavy chain variable. CD, cluster of differentiation. sIg, surface immunoglobulin. MFI, mean fluorescence intensity, n.s. Not significant)

4.3.3 IGHV use and Mutation status

Mutation status was available (n=34 of 36, 94.4%). The remaining two cases had low RNA yields with faint unreliable PCR products and hence excluded from the analysis. There were no statistically significant associations between *IGHV* subgroups and age, sex, LDH, or MIPI. Conventional and leukaemic non-nodal MCL cases were *IGHV* unmutated in 90% and 20% respectively. Three peripheral blood cases were classed as blastoid by histology; all cases had mutated *IGHV*. *TP53* mutation assessment was not available but FISH for del17p was available in eight cases of which three were positive (38%).

The *IGHV* usage in the peripheral blood MCL cohort was fully evaluated in thirty-four cases identifying eighteen different *IGHV*s (**Figure 4.1 A**). In order of abundance, *IGHV*4-34 (21%), *IGHV*3-23 (9%) and *IGHV*3-21 (9%) were the most commonly used *IGHV*s. In this cohort, 79% of MCL cases used *IGHV* 3 and 4 (**Figure 4.1 B**). Half of all cases were mutated, using the 98% cut off to germline; six cases were >95% mutated.

The MCL *IGHV* repertoire varied by mutational status. U-MCL used 11 different *IGHV*; including *IGHV*3-21 (18%), 4-34 (18%), 3-23 (13%) and 4-59 (13%) (**Figure 4.1 C**). M-MCL also used 11 *IGHV* sequences; *IGHV*4-34 (24%), 3-74 and 3-15 and 3-7 (12% each; **Figure 4.1 D**). U-MCL, but not M-MCL, used *IGHV*4-59 (n=2), *IGHV*3-21 (n=3), and 3-53, 3-11, 2-5, 1-2, 6-1 (all n=1). M-MCL, but not U-MCL, used *IGHV*3-74 (n=2), 3-15 (n=2), 3-7 (n=2) and one of *IGHV*3-64, 1-46, and 1-18.

IGHV Useage

- IGHV4-59
- IGHV4-39
- IGHV4-34
- IGHV3-74
- IGHV3-64
- IGHV3-53
- IGHV3-23
- IGHV3-21
- IGHV3-15
- IGHV3-11
- IHGV3-7
- IGHV2-5
- IGHV1-46
- IGHV1-18
- IGHV1-8
- IGHV1-2
- IGHV3-48

IGHV4-34 21%
IGHV3-21 9%
IGHV3-23 9%

IGHV Bias

Non IGHV3/IGHV4; 21%
IGHV3 and IGHV4; 79%

Unmutated MCL

4-34 18%
3-21 18%
4-59 13%
3-23 13%

- IGHV4-34
- IGHV3-21
- IGHV4-59
- IGHV3-23
- IGHV3-64
- IGHV3-53
- IGHV4-39
- IGHV3-74
- IGHV3-15
- IGHV3-11
- IHGV3-7
- IGHV2-5
- IGHV1-46
- IGHV1-18
- IGHV1-8
- IGHV1-2

Mutated MCL

4-34 24%
3-15 12%
3-74 12%

Figure 4.1: *IGHV* usage and mutational status in MCL peripheral blood cohort

The immunoglobulin heavy chain variable region (*IGHV*) gene sequence was assessed from complementary (c) DNA using Sanger sequencing. (A) Pie chart demonstrating the *IGHV* gene usage in the peripheral blood MCL cohort (n=34) (B) Bias of *IGHV* 3 and 4 gene usage by the MCL cohort. (C & D) *IGHV* gene usage for the MCL cases when divided by mutational status.

4.3.4 Surface IgM expression and association with *IGHV* status in peripheral blood

Surface IgM/D expression was assessed in thirty-six patients with confirmed MCL using peripheral blood samples (**Table 4.2**). MCL populations were identified by forward and side scatter characteristics followed by gating on CD5/19 positive cells revealing heterogeneous expression of sIgM (**Figure 4.2 A and B**).
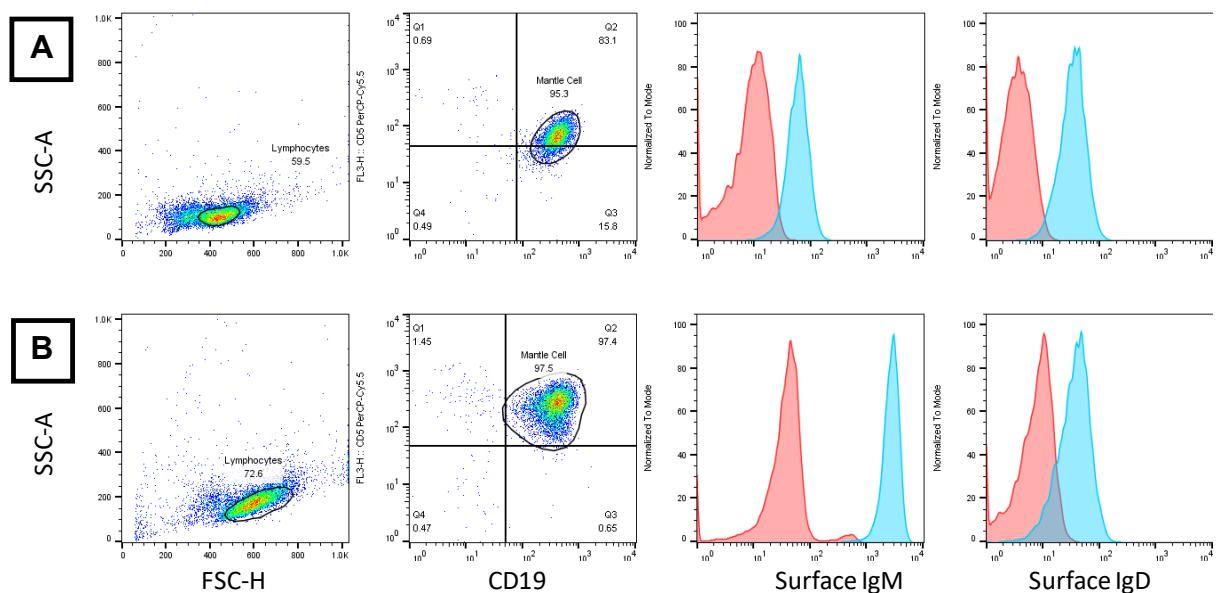
Figure 4.2: Immunophenotype of two samples demonstrating variable sIgM expression

Surface Ig M and D expression were assessed using a FACS Calibur flow cytometer and analysed by FlowJo software. Lymphocytes were identified through forward scatter (FSC-H) and side scatter (SSC-A) characteristics and the MCL cells were subsequently gated on CD5 positive and CD19 positive cells. Two representative samples are displayed (MCL 27 (A) and MCL 14 (B)). MFI was calculated as the difference between 'test antibody' (blue histogram) and isotype control (red). MCL 27 (A) has low sIgM (MFI 47) and sIgD (MFI 29) expression whilst MCL 14 (B) has high sIgM (MFI 2644) and low sIgD (MFI 28).

In the MCL cohort, sIgM expression was variable (MFI Range 47-2808, Median 942, coefficient of variation (CV) 74%). sIgD expression was low but showed more variable expression (MFI Range 1-253, Median 29, CV 106%). When investigating all U-MCL and M-MCL separately, sIgM levels were higher in U-MCL (Range 47-2808, median 1095) than M-MCL, although this was not significant, likely due to the variability amongst individual

patients and the low number of patients (Range 55-2644, median 573; p=0.16; **Figure 4.3 A**). There was no significant difference of sIgD levels in U-MCL compared to M-MCL (U-MCL, Range 1-136, Median 29 vs M-MCL, Range 3-253, Median 36, p=0.56).

Table 4.2: Phenotypic and functional phenotype of peripheral blood MCL samples.

PB MCL cohort (n=36)	PB MCL (n=36)		U-MCL (n=17)		M-MCL (n=17)		P
	Frequency (%)	Median (Range)	Frequency	Range (median)	Frequency	Range (median)	(U vs M)
Phenotypic Characteristics							
CD38 %	32 (86)	**98** (0-100)	15	**100** (30-100)	16	**87** (0-100)	0.02*
CD49D %	32 (86)	**39** (0-98)	15	**25** (4-92)	15	**55** (12-98)	<0.01*
CD23 %	30 (83)	**6** (0 – 84)	13	**2** (0-9)	15	**12** (0-84)	<0.01*
sIgM and sIgD expression							
sIgM MFI	36 (100)	**942** (47-2808)	17	**1096** (47-2808)	17	**573** (55-2644)	0.15
sIgD MFI	36 (100)	**36** (1-252)	17	**29** (1-136)	17	**36** (3-253)	0.55
Calcium mobilisation & Signalling capacity							
Peak sIgM (%)	23 (67)	**88** (20-100)	9	**80** (50-100)	14	**88** (20-100)	0.91
Mean sIgM (%)	23 (67)	**70** (5-100)	9	**68** (32-100)	14	**73** (5-100)	0.76
Peak sIgD (%)	23 (73)	**77** (16-100)	9	**77** (16-100)	14	**82** (24-96)	0.72
Mean sIgD (%)	21 (58)	46 (0-86)	7	55 (0-81)	11	45 (4-86)	0.55
Peak pSYK MFI	13	**15828** (7995-24750)	5	**21197** (7995-24750)	8	**10214** (1596-19481)	0.12
Peak pBTK MFI	10	**6835** (1517-19741)	4	**9803** (2382-19741)	6	**4919** (1517-6550)	0.11
Peak pERK MFI	10	**385** (65-2847)	5	**900** (177-2847)	5	**165** (65-463)	0.03*

Data compiled up to 18th November 2019. Clinical data extracted from electronic records from University Hospitals Southampton 2018 to November 2019. Statistics generated using GraphPad software. * denotes significance <0.05. Calculated if sufficient number. (PB, peripheral blood. U-MCL, unmutated mantle cell lymphoma. M-MCL, mutated MCL. LDH, lactate dehydrogenase. MIPI, Mantle cell International Prognostic Index. *IGHV*, immunoglobulin heavy chain variable. CD, cluster of differentiation. sIg, surface immunoglobulin. MFI, mean fluorescence intensity, n.s. Not significant)

However, when "aggressive" MCL were excluded (all cases excluding confirmed blastoid or pleomorphic MCL by histology, n=4) sIgM expression was significantly higher in U-MCL (U-MCL n=16, Median 1219, M-MCL n=14, Median 542, p=0.03). When assessing sIgM expression across MCL subtypes, conventional nodal MCL (n=19, Range 119-2808, Median 1200) had significantly higher expression than LNN MCL (n=13, Range 180-1571, Median 372, p=0.01). There was no statistically significant difference between sIgM expression in aggressive blastoid or pleomorphic cases (n=4, Range 47-2644, Median 795) with either nodal MCL (p=0.55) or LNN MCL (p=0.95), (**Figure 4.3 C**). In contrast to sIgM expression, sIgD levels were not different even when each MCL variant type was analysed separately (**Figure 4.3 D-F**).

These results point to differential levels of sIgM, but not of sIgD, between U-MCL and M-MCL in a fashion not dissimilar from those of CLL. In CLL, the low sIgM levels are suggested to be a reflection of engagement of sIgM/D by putative environmental antigen at lymph node sites,[340][351] which leads to sIgM, but not sIgD down-modulation. The low levels were more evident in the LNN M-CLL, raising the question if the behaviour of this subset was affected by antigenic-drive. To address this question Ig expression was then compared with CLL and in vitro properties and clinical behaviour explored to assess for associations with sIgM.
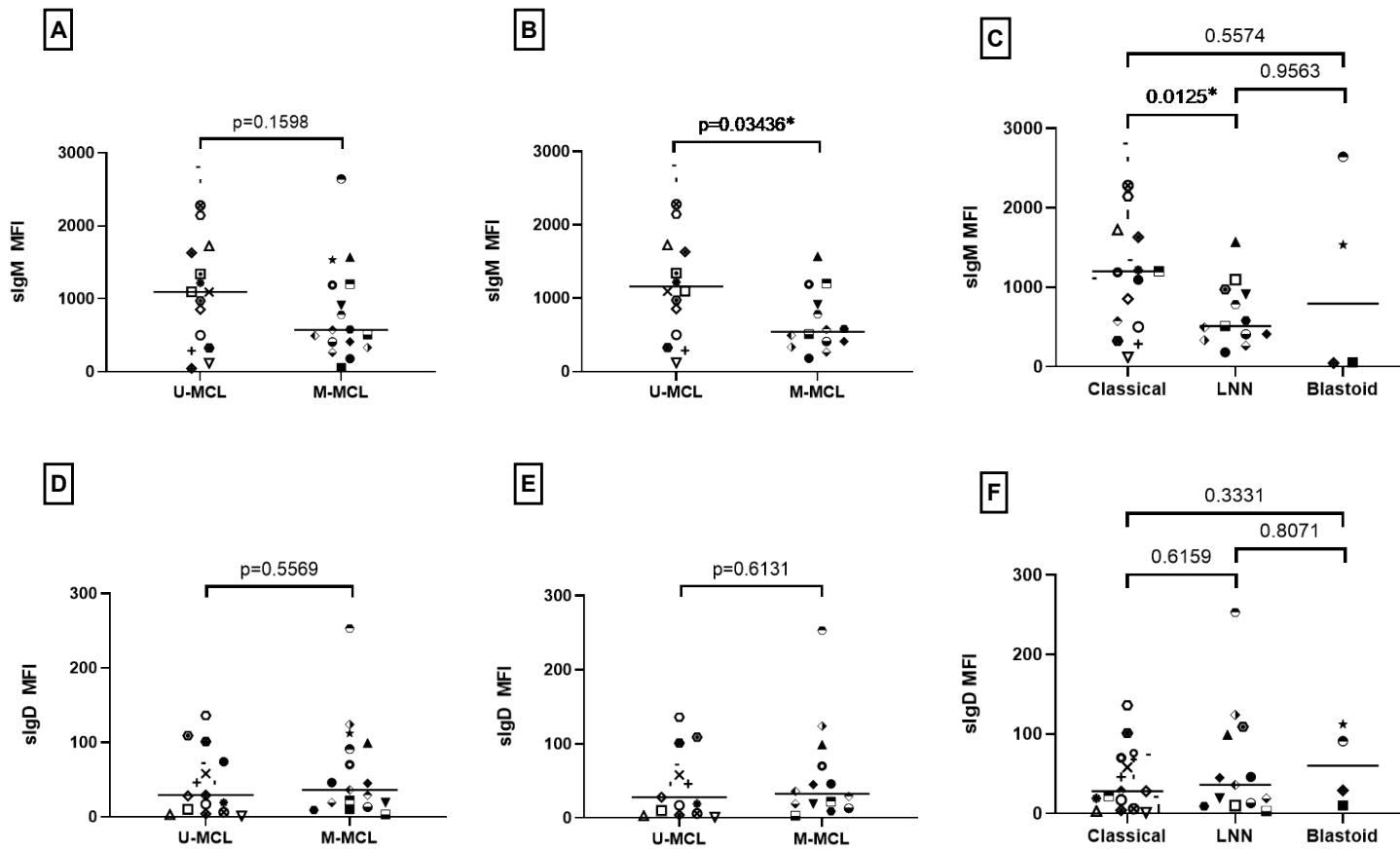
Figure 4.3: Surface Immunophenotype of MCL peripheral blood samples

Peripheral blood samples from MCL patients (n=36) were assessed for surface IgM and sIgD expression using a FACS Calibur flow cytometer and analysed using FlowJo software Surface Ig MFI was calculated following subtraction of isotype control MFI. MFIs for sIgM (A-C) and sIgD (D-F) were plotted against *IGHV* status of all samples (n=36, A and D), cases excluding blastoid histology (n=29, B and E) and by MCL sub diagnostic groups (n=36, C and F). Straight lines represent median value. The statistical significance of difference was calculated using the Mann-Whitney statistical test. ***** denotes significance p<0.05.

4.3.5 Comparison of sIgM levels in MCL vs CLL

To compare sIg expression in MCL against CLL, MCL data were analysed against a CLL cohort investigated under the same experimental methods (**Phenotypic analysis of MCL cells using flow cytometry**). SIgM levels were higher in U-MCL (n=17, Range 47-2808, Median 1096) than in U-CLL (n=43, Range 40-3931, Median 85; p<0.01; **Figure 4.4 A**). SIgM expression was significantly higher in M-MCL (n=17, Range 55-2644, Median 573) than in M-CLL (n=80, Range 9-3601, Median 54; p<0.01; **Figure 4.4 B**). Four cases (two U-MCL and two M-MCL (MCL 9 (▽), 27 (◇), MCL 3 (■) and 1 (●)) had low sIgM MFI (119, 47, 55 and 155) comparable to the main body of CLL sIgM MFI (**Figure 4.4 A**). There was no difference in median sIgD expression across U-(MCL n=16, Range 1-136, Median 29 vs U-CLL n=44, Range 3-308, Median 48, p=0.14) and M-(MCL n=17, Range 3-253, Median 36 vs U-CLL n=80, Range 3-488, Median 33, p=0.60) subgroups of MCL and CLL (**Figure 4.4 B**).
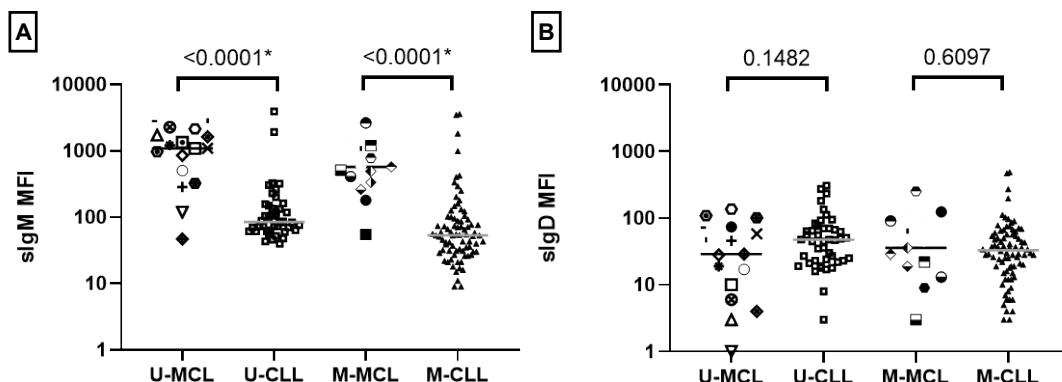
Figure 4.4: Surface IgM/D expression comparison in MCL and CLL

Peripheral blood samples from MCL patients (n=34) were assessed for surface IgM and sIgD expression using a FACS Calibur flow cytometer and analysed using FlowJo software. Comparison of sIgM expression (A) and sIgD expression (B) in unmutated (U) (MCL, n=17 CLL n=44) and a mutated (M) cohort (MCL, n=17 CLL n=80) - each symbol represents an individual patient sample. Horizontal lines represent median values. The statistical significance of difference was analysed using the Mann-Whitney statistical test. ***** denotes significance p<0.05.

These results indicate that sIgM levels tend to be higher in MCL than in CLL. However, like in CLL, sIgM levels are lower in M-MCL than U-MCL. Normal memory (IGHV mutated) B-

cells have instead higher levels than naïve (IGHV unmutated) B-cells. This suggests ongoing endocytosis of sIgM consequent to antigen engagement, prompting further investigation into the anti-IgM on the 2 U-MCL and M-MCL subsets.

4.3.6 Signalling capacity in MCL

Signalling capacity induced by BCR stimulation in the peripheral blood samples of MCL patients was measured by two methods: i) Assessment of intra-cellular calcium flux and ii) change in phosphorylated SYK and BTK in response to α-IgM. Quality of samples was checked by ionomycin-induced calcium release and samples were considered adequate for functional study when calcium release was more than 75% (**Figure 4.5 A and B**).

Anti-IgM induced calcium mobilisation was measured in twenty-three samples (**Table 4.2**). Median signalling capacity was high, although some samples revealed low levels (Range 11-100, Median 89%, CV 34%). Mean signalling capacity was lower than peak signalling capacity with more variability (Range 3-100, Median 84%, CV 57%).   Anti-IgD calcium signalling capacity was lower and less variable (Range 16-100, Median 74%, CV 39%) than sIgM induced signalling, consistent with the low sIgD levels. Mean IgD calcium signalling was lower than peak IgD (Range 0-86, Median 33%, CV 74%). Two representative examples are shown (**Figure 4.5**).
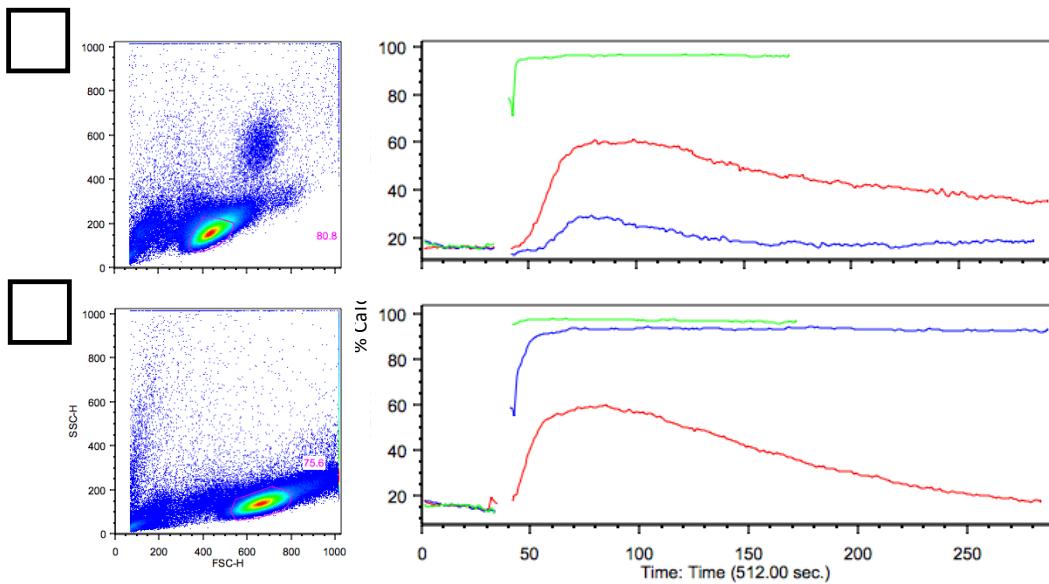
Figure 4.5: Intracellular calcium signalling of a 'low' signaller and 'high' signaller

MCL intracellular calcium release was assessed in response to α-IgM (blue), α-IgD (red) and ionomycin control (green) by flow cytometry gated upon the lymphocyte population and calculated as a proportion of CD5/19 positive lymphocytes. (A) MCL1 had low peak and median α-IgM signalling capacity (20% and 5% respectively). Response to α-IgD (red) is 69% (peak) and 45% (mean). Response to ionomycin represents a positive control (green line at 100%). (B) In comparison, MCL7 has high α-IgM signalling capacity (100% peak and mean), positive control (100%, green) and similar α-IgD (red) signalling 59% (peak) and 29% (mean).

In this MCL cohort, all cases signalled above 10% and sIgM positively correlated with peak and mean calcium signalling (**Figure 4.6 A and C**). There was a direct correlation between sIgM expression and signalling capacity (Spearman rank coefficient r=0.77, p<0.01).

The relationship between sIgM levels and calcium signalling between 0 and 1000 (MFI) was assessed separately as most cases signalled highly (80-100%) above this MFI and more variability was noted below 1000 (MFI). Surface IgM more strongly positively correlated with peak α-IgM induced signalling capacity in this subgroup (r=0.8449) than in the whole group (r=0.7681) (**Figure 4.6 A and B**). Mean α-IgM induced signalling capacity was similar (r=0.8322 and r=0.8615 respectively) in both limited (MFI<1000) and whole groups (**Figure 4.6 C and D**).
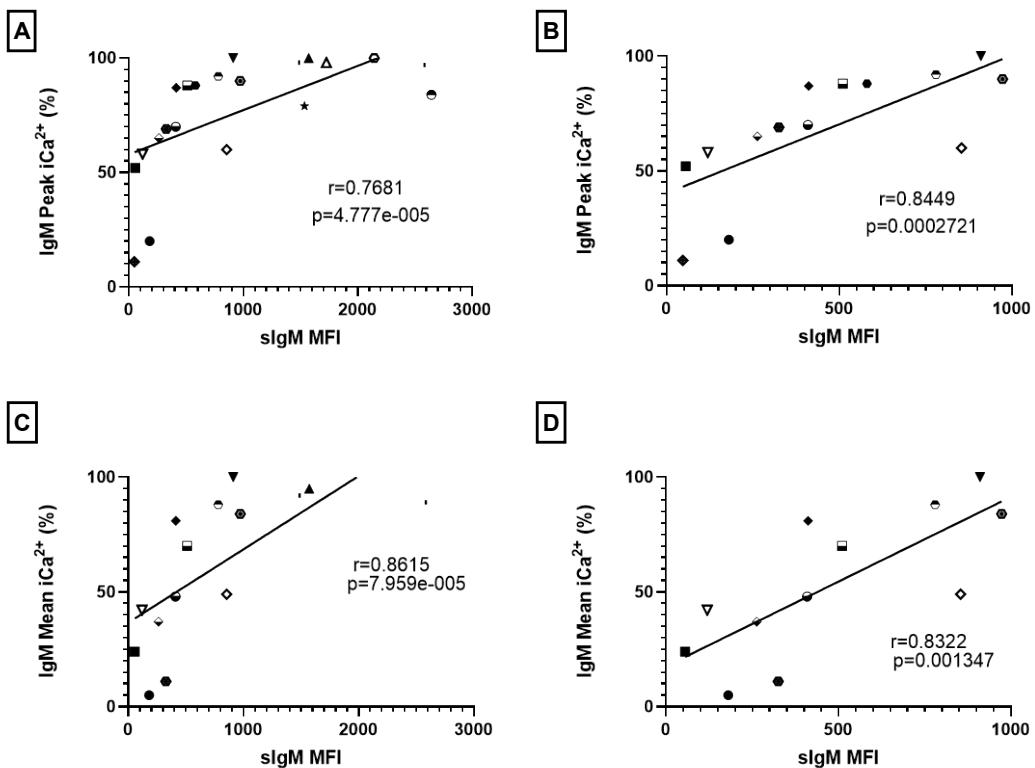
Figure 4.6: α-IgM induced calcium signalling capacity correlation with sIgM expression

Surface IgM expression (MFI) and sIgM signalling capacity were analysed by flow cytometry in 23 patient samples. Surface IgM positively correlates with peak and mean calcium signalling when assessing the whole available cohort (A and C) or a reduced MFI range (B and D).

Peak and mean α-IgM induced signalling capacity by intracellular calcium flux was not associated with *IGHV* mutation status (Peak U-MCL vs M-MCL; Median 54% vs 66%, p=0.74 and Mean U-MCL vs M-MCL; Median 37% vs 70%, p=0.18). There was no association of calcium signalling by WHO clinical subgroup (Median Peak signalling Nodal 80% vs LNN 88%, p0.64; Median mean signalling Nodal 68% vs LNN 76%, p=0.78).

4.3.7 Signalling capacity: Phosflow Validation

To further assess proximal BCR signalling a novel immunophenotyping assay was developed (**Phosflow phenotypic analysis**). Phosflow was initially performed on the Jeko MCL line concomitantly to Western blot (**Figure 4.7**). Four experimental conditions were used: i) Unstimulated, ii) unstimulated in the presence of a SYK inhibitor [Entospletinib 10μM], iii)

α-IgM stimulated and iv) α-IgM stimulated in the presence of SYK inhibitor [Entospletinib 10µM]). Phsophoprotein targets were pSYK, pBTK, pAKT and pERK.

The Phosflow assay demonstrated sensitivity to α-IgM stimulation (single curve shift to the right, blue to red) in pSYK, pBTK. pAKT and pERK. Unstimulated basal pSYK, pAKT and pERK were not visualised by Western blot but were apparent in Phosflow whilst pBTK was apparent in both. Subtle reductions in phosphorylated SYK and BTK were seen in both assays with Entospletinib incubation (dashed blue and green lines). SYK inhibition in stimulated cells compared well between Phosflow and Western blot in pAKT and pERK. Phosflow qualitatively appeared to reflect induced and inhibited signalling changes also seen by Western blot (**Figure 4.7**).
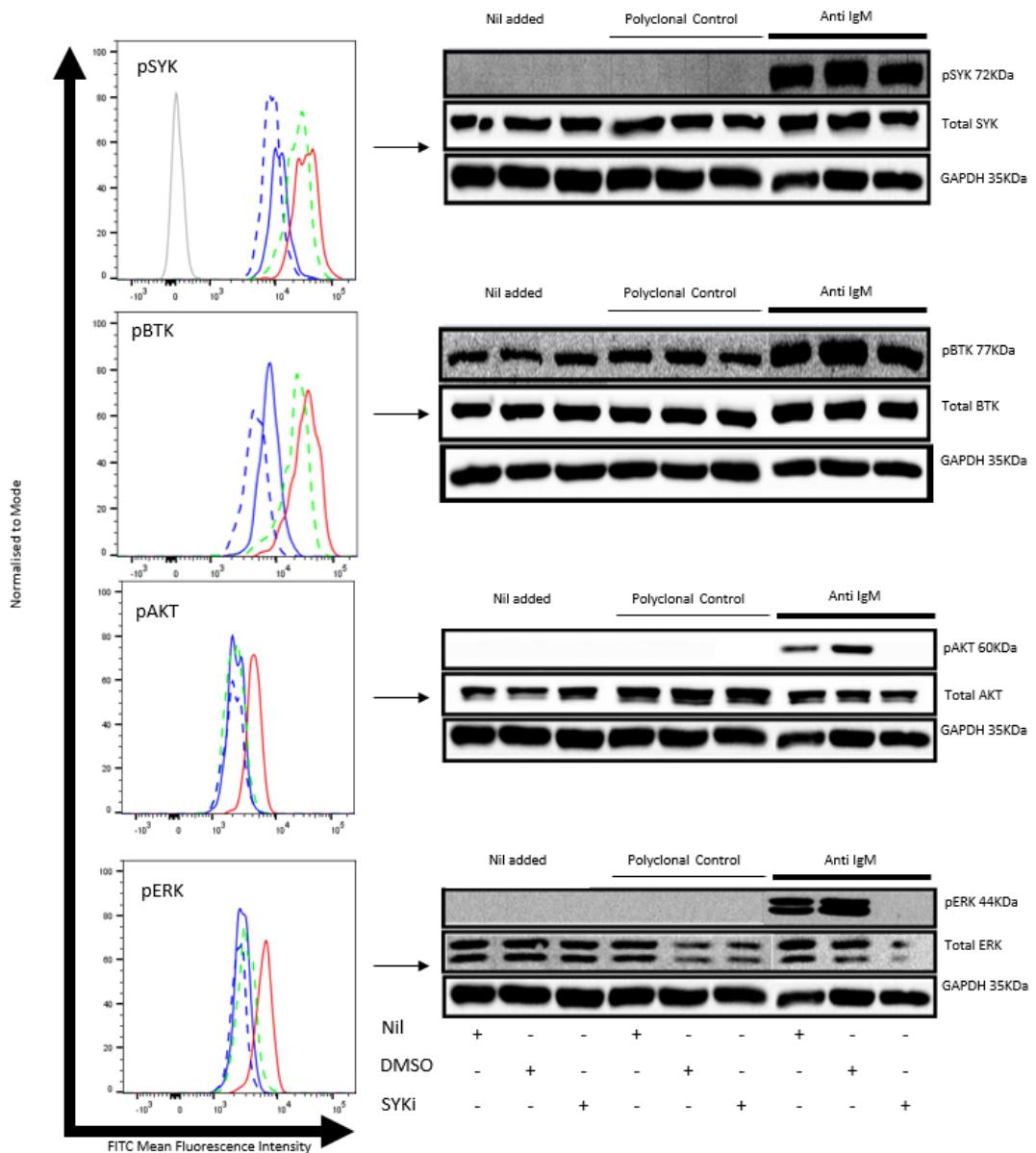
Figure 4.7: Phosflow validation by Western blot comparison in Jeko cell line

The cell line Jeko was assessed by Phosflow (Conditions were: 1 hour in complete RPMI, DMSO or Entospletinib 10μM untreated, plus 4 minutes 20μg/ml of α-IgM and 20μg/ml of Fab₂ polyclonal control for Western blot only. pSYK, pBTK, pERK and pAKT were assessed by Phosflow and Western blot: unstimulated in DMSO (blue line), unstimulated in Entospletinib 10μM (dashed blue line), stimulated with α-IgM for 4 minutes in DMSO (red line) and stimulated with α-IgM for 4 minutes in Entospletinib 10μM (dashed green line).

Signalling capacity in primary cells was then assessed by Phosflow. Cells were identified through 5 steps (**Figure 4.8 A**): (i) exclusion of doublets, (ii) gating on the lymphocyte population by SSC-A and FCH-H, (iii) gating of CD5/19 positive (iv) gating on CD3 negative cells and (v) identification of barcoded populations pertaining to the experimental condition. sIgM expression strongly positively correlated with α-IgM induced pSYK and pBTK (**Figure 4.8 B-C & E-F**). T-cells were used as a negative control; as expected, there was an absence of response to α-IgM stimulation in CD3/5 positive cells (**Figure 4.8 D**). Peak Phosflow signalling capacity was deemed to be of adequate quality if two conditions were met: the presence of single, clear peaks and, if sufficient T-cells were present, no response to α-IgM stimulation in the T-cell subset (**Figure 4.8 B, C and D**).

Phosflow analysis of anti-IgM stimulated MCL primary cells revealed that IgM levels positively correlated with α-IgM induced pSYK (r=0.8000, p=0.01), pBTK (r=0.8333 p<0.1), pAKT (r=0.5769 p=0.04), pS6 (r=0.9522 p<0.01) and pERK (r=0.8303 p<0.01) (**Figure 4.6 C and D**).
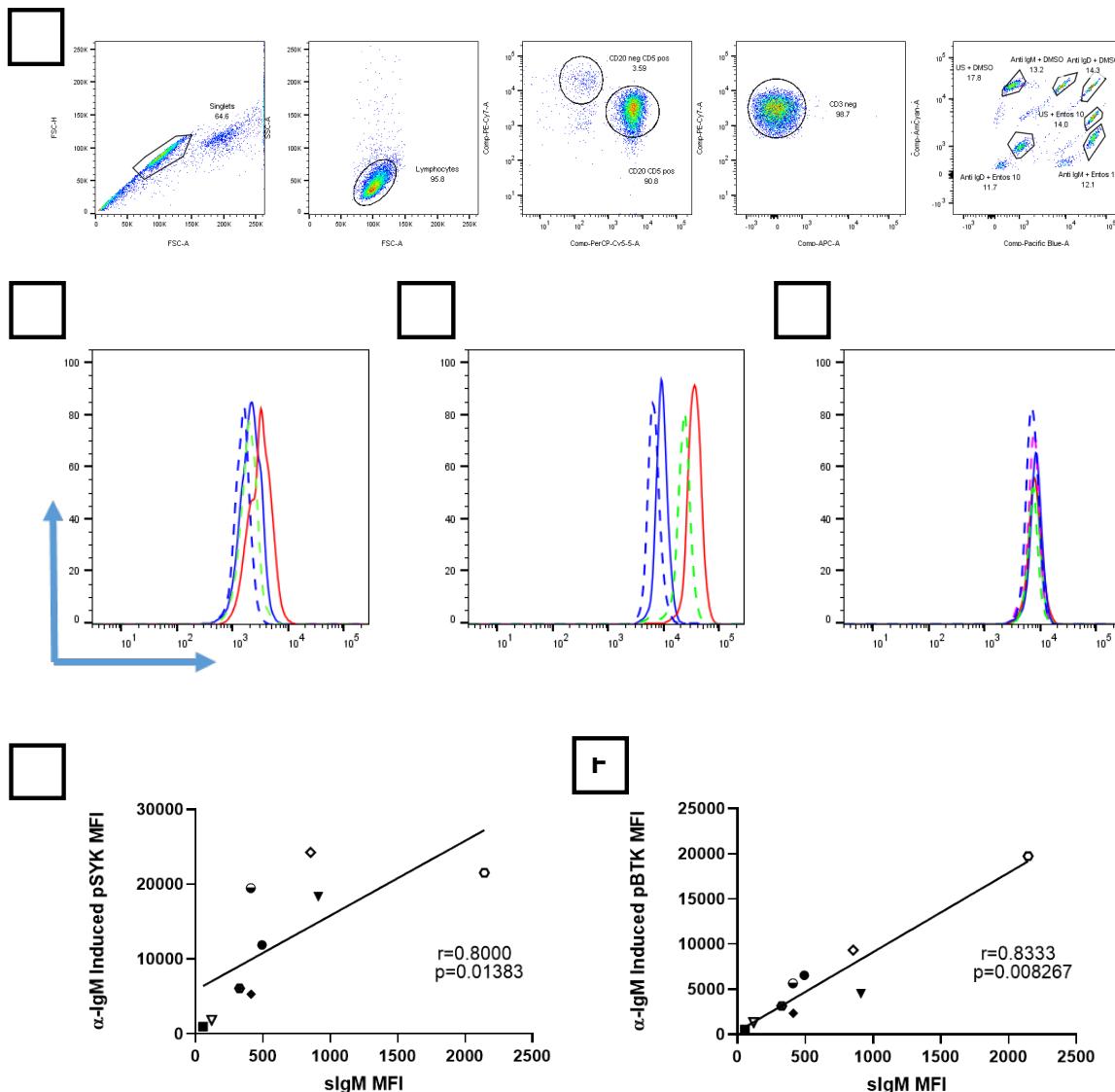
Figure 4.8: Phosflow α-IgM induced signalling and correlation with sIgM

Following stimulation, fixation, permeabilisation and incubation by appropriate antibodies, MCL cells were acquired on the CANTOII flow cytometer (Methods: Phosflow phenotypic analysis). Following doublet exclusion (A - Left through to right), gating Is demonstrated on the lymphocyte population of 5/19 positive, CD3 negative cells, identifying 6 conditions via barcoding (A: DMSO + Unstimulated, DMSO + α-IgM, DMSO + α-IgD, Entospletinib 10μM + Unstimulated, Entospletinib 10μM + α-IgM, Entospletinib 10μM + α-IgD). FlowJo software was used to analyse and compare conditions. Phospho SYK (pSYK) analysis is demonstrated in (B) MCL 3 (sIgM 55) and (C) MCL16 (sIgM 854) to show variation in signalling capacity. Unstimulated pSYK MFI (blue line), α-IgM stimulated pSYK MFI (red line), unstimulated pSYK MFI + Entospletinib 10μM (dashed blue line), α-IgM stimulated pSYK MFI + Entospletinib 10μM (green dashed line. T-cell control (D) gated upon CD19 negative, CD3/5 positive cells demonstrates no response to α-IgM. Surface IgM positively correlates with pSYK (E) and pBTK (F).

However, α-IgM induced signalling capacity was higher in U-MCL and in M-CLL [pSYK (n=13, p=0.13) or pBTK (n=11, p=0.11)] although numbers of samples were not sufficient to reach statistical significance. pERK levels were significantly higher in U-MCL than in M-CLL following stimulation (n=10, p=0.03) (**Figure 4.9 A-C**).
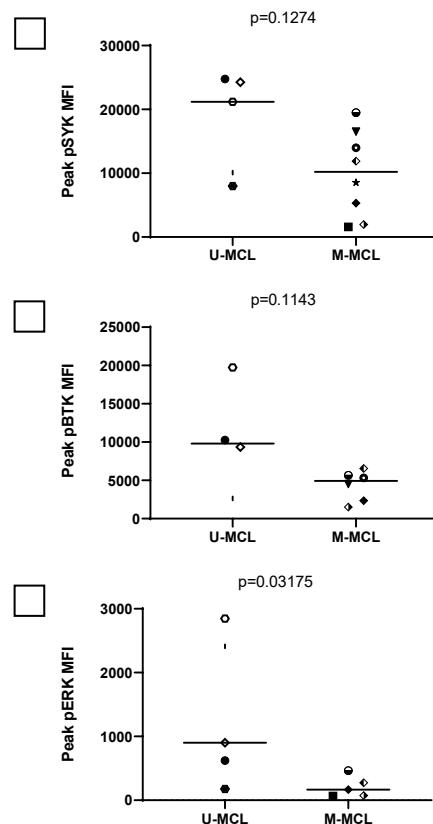
Figure 4.9: α-IgM induced peak PhosFlow signalling by *IGHV* status

Peak α-IgM induced PhosFlow signalling (Peak-basal MFI) was assessed in primary MCL samples and compared by *IGHV* subgroup. Peak pSYK (A), pBTK (B) and pERK (C) were analysed in this study.

Signalling capacity (Peak α-IgM induced Calcium flux) in the MCL cohort was compared with available CLL cohort calcium signalling data where sIgM and signalling capacity had been measured under identical procedures.   MCL signalling capacity (n=22, Range 11-100, median 86%) was significantly greater (p<0.01) than CLL (n=165, Range 1-100, Median 50%; **Figure 4.10 A-B**). Comparing the MCL cohort with CLL cases for sIgM expression and signalling capacity, a 'CLL-like' group of MCL was identified that fell within the 75th percentile (<73.5%) that warrant further investigation (Blue dashed circles; **Figure 4.10 A-B**).
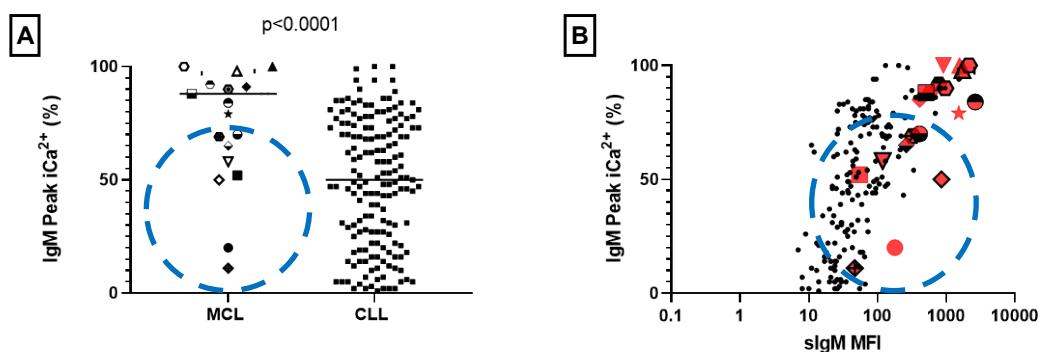
Figure 4.10: Comparison of MCL to CLL intracellular peak calcium signalling capacity

Anti-IgM signalling capacity was assessed in MCL and CLL cohorts by flow cytometry.   (A) Median peak signalling capacity between the two groups was compared by non-parametric statistics (Mann Whitney) and by (B) comparison of sIgM/Signalling capacity correlation. MCL samples are highlighted red. A low signalling capacity group in the MCL cohort (dashed blue circle) was identified with similar signalling capacity to CLL cells.

The group of low anti-IgM signalling MCL samples were comprised of four conventional (nodal) U-MCL and four LNN M-MCL (one of which was blastoid) (**Table 4.3**). The U-MCL low signallers group was overrepresented (50%) by *IGHV*3-21, characterised by paired λ V3-19 pairing and a tendency to improved prognosis. There was no use of *IGHV*4-34 in this subgroup.

Table 4.3: Characteristics of CLL-like signallers

MCL Sample	MCL Sub Type	IGHV	Homology (%)	sIgM (MFI)	iCa^{2+} α-IgM (%)	sIgD (MFI)	CD23 (MFI)	OS (Months)
1	LNN	3-15	95.23	180	20	46	53	180
3	LNN/Blastoid	4-39	97.58	55	52	10	17	157*
9	Classical	4-59	99.30	370	54	10	0	5*
16	Classical	3-21	99.25	854	60	28	3	18
27	Classical	3-21	100	47	11	29	0	39*
28	Classical	4-39	99.0	325	69	101	7	72*
53	LNN	3-64	95.8	409	70	13	0	41*
64	LNN	1-18	89.24	263	65	19	1	1

* Deceased, Mean Fluorescence intensity (MFI), Overall survival (OS)

4.3.8 CD23, CD38 and 49D expression in MCL

Expression of the surface markers CD23, CD38 and CD49d were assessed as part of the MCL phenotype. CD49d expression significantly associated with M-MCL (n=30, U-MCL vs M-MCL median %, 25 vs 55, p<0.01) (**Figure 4.11 G-I**). Conversely, CD38 (n=31, MFI Range 0-407, Median 163) was significantly lower in M-MCL than U-MCL (n=31 U vs M-MCL Median % 100 vs 87, p=0.02; **Figure 4.11 D-F**).
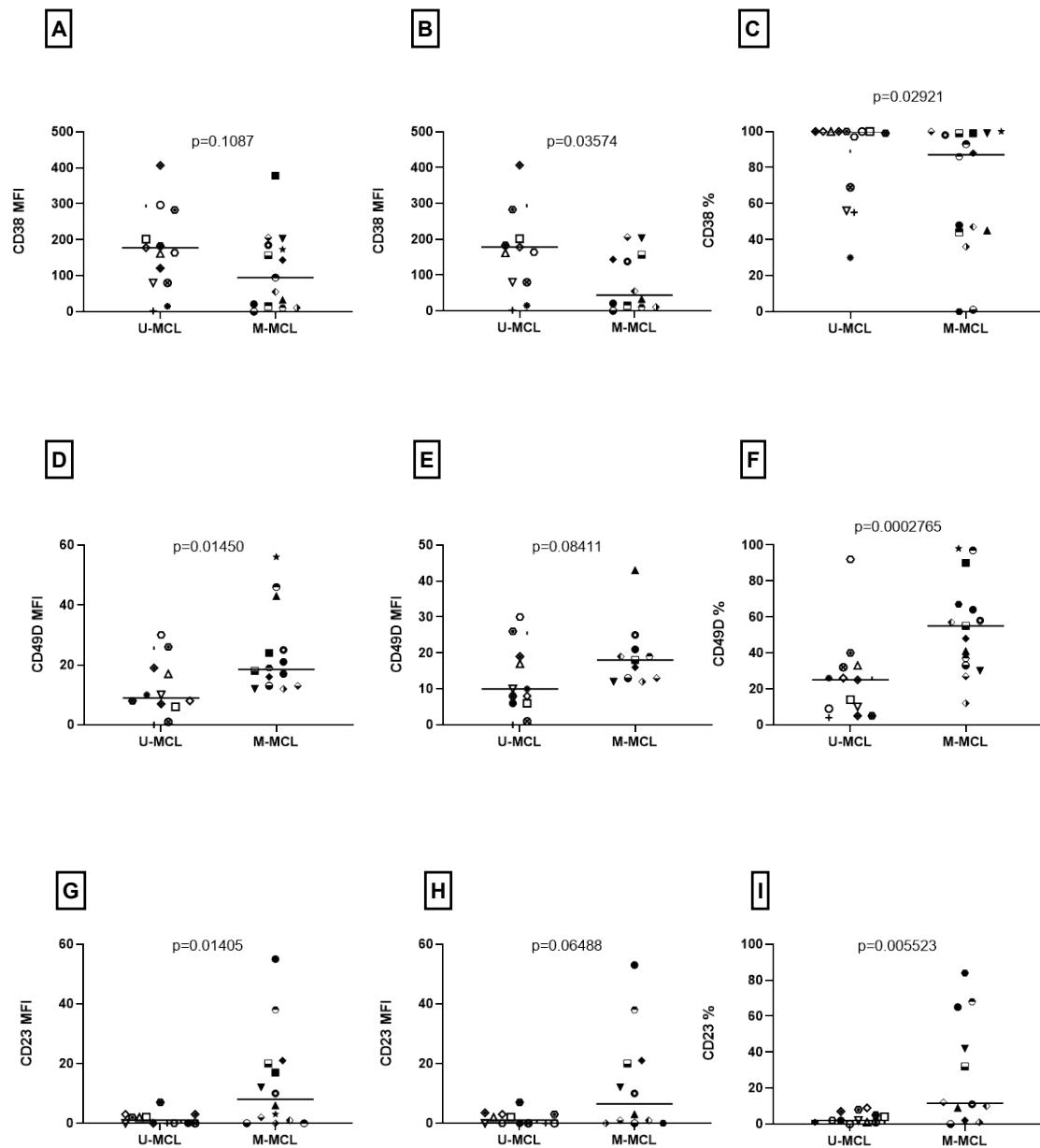
Figure 4.11: Surface CD38, CD49D, CD23 expression in MCL.

The expression of CD38, CD49d and CD23 expression was assessed in the MCL cohort (n=31) and divided by *IGHV* status (A, D, G), *IGHV* subgroup minus blastoid cases (B, E H) and expressed as a percentage of positive cells (C, F, I). Each symbol identifies an individual patient, horizontal lines indicate median values. The statistical significance of difference was calculated using the Mann Whitney test.

CD23 was generally low or absent (n=30, MFI range 0-53, Median 3). 10/27 cases had no expression of CD23 whilst a separate group of weak expressers were apparent in the M-MCL group (**Figure 4.11 G-I**). Percentage positivity of CD23 expression was significantly higher in M-MCL (n=25, U vs M-MCL blastoid excluded, Median % 2 vs 12, p=0.01).

A well-demarcated group of six cases expressed CD23 above all U-MCL cases and above the median in M-MCL. These cases were all LNN MCL (**Table 4.4**). This group was non-significantly associated with low sIgM expression (Median sIgM 1096 vs 148, p= 0.07).

Table 4.4: Characteristics of CD23 expressing MCL

MCL Sample	MCL Sub Type	IGHV	Homology (%)	sIgM (MFI)	iCa^{2+} α-IgM (%)	sIgD (MFI)	CD23 (MFI)	OS (Months)
1	LNN	3-15	95.23	180	20	46	53	180
3	LNN/Blastoid	4-39	97.58	55	52	10	17	157*
7	LNN	1-46	93.4	910	100	19	18	28*
11	LNN	4-34	97.8	412	87	45	21	286
52	LNN	3-7	97.6	780	92	253	38	73*
63	LNN	3-23	97.7	510	87	3	20	188

* Deceased, Mean Fluorescence intensity (MFI), Overall survival (OS)

4.3.9 Summary of main findings

To address the role of BCR structure, expression and function in MCL it was hypothesised that, like CLL, MCL surface immunophenotype and signalling capacity associates with *IGHV* mutation status and clinical subgroup. The main aims of the experiments were therefore to explore BCR *IGHV* structure and mutational status, describe MCL surface expression and assess for associations by *IGHV* mutation status and clinical subgroup. It also looked to explore signalling capacity in response to α-IgM and assess for associations by *IGHV* mutation status, clinical subgroup and to compare with CLL.

The data, in this small cohort, confirmed some previously known findings. The immunogenetic repertoire was restricted, with preferential use of *IGHV*4-34 and 3-21. Surface IgM expression was variable but generally high whilst sIgD was generally low. Signalling capacity correlated with sIgM expression. sIgM expression and signalling capacity was significantly higher in MCL than CLL. Surface CD38 expression was significantly higher in U-MCL whilst CD23 was higher in LNN MCL.

The new key findings were:

1. In leukaemic non-blastoid MCL, sIgM was more highly expressed in U-MCL than in M-MCL.
2. Its clinical counterpart, conventional, nodal MCL, expressed statistically significant higher sIgM.
3. sIgD expression did not vary by IGHV status or clinical subgroup.
4. A subpopulation of MCL had CLL-like sIgM expression and signalling capacity.
5. Analysis of cell surface adhesion molecules revealed a statistically significant association between M-MCL and CD23 and CD49D expression.

In relation to the chapter hypotheses, these results demonstrate a statistically significant association between sIgM expression and *IGHV* mutation status in leukaemic non-blastoid MCL supporting the first hypothesis. However, signalling data was less clear as calcium signalling was variable and did not associate with *IGHV* status or clinical subgroup. Maximal proximal kinase phosphorylation revealed a weak association between *IGHV* mutational status but was not sufficient to accept the signalling hypothesis.

4.4 Discussion

Phenotypic investigation of MCL sIg has revealed interesting variation in BCR structure and expression. Before discussing these features, it is important to state some limitations and weakness that are present in this study. First, the sample size was small. Attempts to maximally increase the sample size were made and all samples were analysed in the

available period. However, due to time and the scarcity of good quality material the sample size remained small. This means that, in particular, subgroup analysis was limited and statistical interpretation could be prone to mis-interpretation. Secondly, the cohort may have been affected by selection bias. This may have been towards the leukaemic form of MCL which preferentially has a M-*IGHV*. Thirdly, a significant majority of samples were acquired at relapse and/or after treatment, mainly immuno-chemotherapy. Multiple lines of prior treatment will have applied a selection pressure and expansion of genetic changes possibly affecting tumour behaviour; the relationship between genetic damage BCR expression and function was not assessed. Fourthly, some of the samples were from external centres where material was collected for genetic studies, not phenotypic or functional studies. In contrast to the local samples, those samples may have been less carefully controlled for cryopreservation procedures and hence for functional studies. In those samples, it was not known how cryopreservation and sample thawing would have affected MCL BCR characteristics. Indeed, several samples had such poor quality that no, or limited, characteristics were acquired. However, from studies in CLL from the local Southampton laboratory, the freezing/thawing procedures seemed not to affect sIgM/D levels or signalling when phenotypic and Ca release assays were used.[340]

Despite these limitations, this study does give interesting insight into MCL BCR immunogenetic structure. In this cohort, *IGHV*4-34 (21%), *IGHV*3-23 (9%) and *IGHV*3-21 (9%) were most common showing preferential use of *IGHV*3-21 and *IGHV*4-34 in U-MCL and *IGHV*4-34 in M-MCL respectively. This *IGHV* usage broadly reflected the MCL literature. *IGHV*4-34, 3-21 and 3-23 genes were identified in 46% of cases in one large study (n=807) and 39% in this study.[203 250 330 388] In comparison, datasets of normal healthy B-cells show *IGHV*4-34 gene frequency of less than 5%, *IGHV*3-21 in less than 6% and *IGHV*3-23 in up to 20%.[428-430] Biased usage of these *IGHV* genes imply a selective pressure among their clonogenic progenitors.

A biased *IGHV* gene repertoire is also seen in CLL employing *IGHV* genes 1-69 (12.7%), 3-23 (12.7%) and 4-34 (10.9%). [431] The enrichment for *IGHV*1-69 is not paralleled in this or other MCL repertoires perhaps suggesting a different selective pressure in MCL.[328] *IGHV*3-21 is enriched in CLL cases with an intermediate epigenetic signature and is generally regarded to have a poor prognosis.[152] Just under half of CLL cases with *IGHV*3-21 are unmutated and

the SMH is lower in this group than for CLL as a whole.[432][433] By contrast, in MCL there is some evidence for relatively improved outlook and, like in this study, MCL cases with *IGHV*3-21 were found only in the U-MCL sub-group.[392] *IGHV*3-21 MCL with paired λ light chain, has been used to argue for the presence of immunogenetic drive in early MCL ontogeny, as likely occurs in CLL.[434]   In this cohort, the *IGHV*3-21 cases paired with lambda light chains had variably low sIgM (MFI 47 and 854) whilst the one case paired with kappa light chains had high expression (MFI 2282) suggesting more BCR engagement and down-modulation in the lambda paired cases.

Further evidence for selective BCR driven ontogeny in CLL is BCR stereotypy. It is seen in approximately 30% of CLL cases, the majority of which are U-CLL.[434] This differs to MCL, where stereotyped sequence are seen in only 10.4% of MCL cases but predominantly in the 3-21 and 4-34 subtypes which are overrepresented in this cohort and others.[388] Sequence data was not assessed for stereotypy but the data confirmed only modest somatic hypermutation (SHM) with the majority of cases demonstrating an *IGHV* mutation level of >95%. Pronounced SHM was seen in only 6 cases reflecting the literature where M-MCL cells generally show less SHM than M-CLL.[152]

In addition to the analysis of *IGHV* use and structure, the measurement of Ig expression has contributed strongly to the understanding of CLL biology and natural history. In CLL, sIgM expression is variably low due to chronic (super) antigen induced down modulation of sIgM, but not D, and dependent upon cell of origin.[340] In contrast, this cohort has characterised generally high sIgM expression in MCL, although with significant variability. This implies that the MCL BCR is not undergoing strong down-modulation as seen in CLL that results in anergy. Indeed, high sIgM expression more closely resembles high-grade mature B-cell malignancies, such as diffuse large B-cell lymphomas and follicular lymphoma.[275] These are malignancies where the BCR is driven by a combination of extrinsic, constitutive and chronic activated B cell signalling.[114][377][435-437]

Despite significantly higher sIgM expression, this dataset has revealed Ig characteristics that are seen in CLL. First, sIgM expression was significantly higher U-*IGHV* MCL when aggressive blastoid and pleomorphic variants were excluded from the analysis. This small

biologically separate group has a more aggressive clinical path, having acquired more genetic damage, and can reasonably be analysed separately.[438] Higher sIgM expression was also significantly higher in its clinical correlate, conventional nodal MCL. In CLL, higher sIgM expression is associated with increased signalling capacity and shorter progression free survival due to a more proliferative phenotype.[340] In addition, surface IgD was expressed weakly or not at all and did not vary by *IGHV* mutation status or clinical subgroup. Moreover, sIgD expression was equal to that seen in the CLL cohort suggesting that external influence occurs through the D isotype. This Ig profile duplicates the pattern, though not the extent, found in CLL.[340] Intriguingly, this suggests that the MCL BCR could be under the same type of influence as seen in CLL. Despite this qualitative similarity, this quantitative effect on the IgM is weaker. This may be due to the nature of the putative driver or it may be negatively affected by other disease specific factors such as overexpression of cyclins or the tumour microenvironment.

The expression of surface molecules revealed associations between CD38 with U-MCL and CD23 and 49D with M-MCL. In one series, CD38 expression predicts a shorter survival in MCL and like CLL is thought to represent a micro-environmental proliferation marker.[203] CD38 expression falls following treatment with ibrutinib in MCL.[241] CD23 is known to be associated with LNN-MCL and can negatively affect BCR signalling.[130][199] It is not unsurprising, therefore, to find it associates with M-MCL and a potentially more indolent clinical picture.[173][439] The final adhesion molecule assessed was CD49D. This molecule permits trans-endothelial migration and is found in more aggressive CLL with nodal development.[359][360][440][441] A single study reports high CD49D in MCL but this is principally in cell lines.[192] Another demonstrates CD49D is lower in CLL than In MCL. [193] This data adds further nuance to show that CD49D is principally found in M-MCL. This is unexpected as the presence of CD49D promotes progression of lymphadenopathy in CLL. M-*IGHV*, usually LNN MCL, by definition does not possess, or have very little, lymphadenopathy. In the absence of proliferation centres, it is possible that this receptor facilitates lymphocyte migration in M-MCL leading to its clinical phenotype of LNN-MCL.

Interpretation of blastoid BCR characteristics was difficult due to the very small number of cases. Blastoid MCL sIgM expression was not statistically higher than conventional nodal or LNN MCL but had a very wide range of expression. This was surprising as it had been

hypothesised that sIgM down-modulation would be lost with increasing genomic complexity reflected by their blastoid morphology. In CLL, sIgM is higher in cases with increased genomic complexity.[340] Two cases, conventional U-MCL 27 and LNN case MCL 3 with very low sIgM expression (MFI 47 and 55) had pleomorphic and blastoid histology. Although no pre transformation levels were available to compare, it is clear that there was no significant rise in sIgM. These cases suggest that proximal BCR behaviour, low sIgM secondary to down-modulation, may be independent of distal kinome alterations (e.g. AKT or NF-κ B activation) that have been shown to be present in some blastoid cases.

As is already known, sIgM expression closely correlated with signalling capacity in MCL.[275] Given generally high sIgM expression, signalling capacity is therefore generally high in MCL. CLL sIgM also positively correlates with signalling capacity but, unlike MCL, signalling is much lower.[340] The majority of MCL cases retained strong signalling capacity. Despite a significant association between sIgM expression and IGHV status in non-blastoid cases and a positive correlation between sIgM and signalling, intra cellular calcium signalling itself did not associate with *IGHV* status. This may reflect insufficient sample size, functional assay bias (this functional assay was dependent upon live cells and may bias towards cells with higher signalling capacity) and limits of the assay (signalling capacity 'stops' at 100%). BCR phosphorylation nodes (pSYK, pBTK, pAKT, pS6 and pERK) positively correlated with sIgM at all MFIs, demonstrating that biochemical signalling continues beyond peak (100%) calcium flux. Phosflow assessment supported higher signalling capacity in U-MCL (statistically significant with pERK) but was again limited by small sample size and was insufficient for firm conclusions.

In CLL, high surface IgM and high signalling capacity associates with worse survival, likely due to increased proliferation at tissue sites.[340] It is tempting to speculate that MCL's more aggressive nature, in comparison with CLL, could, at least in part, be due to its increased signalling capacity and proliferative potential due to higher sIgM. However, the clear distinguishing feature between these two malignancies is cyclin dependency. It may be that this factor, combined with genomic instability, is a more important determining feature. Clinical correlates will be looked at in later chapters.

In contrast to the overall trend for high signalling, a small subgroup of MCL with low signalling capacity, akin to CLL, was identified. These cases were characterised by low sIgM and were generally mutated or unmutated employing *IGHVs* 3-21, 4-39 or 4-59. *IGHV*3-21 with paired λ light chain and BCR stereotypy, notable for poor prognosis in CLL but favourable outlook in MCL, is characterised by low sIgM in the U-MCL subgroup comparable to CLL expression.[426][433] Low sIgM in these subsets of MCL could, therefore, imply ongoing receptor down-modulation akin to CLL pathobiology. The next chapter explores the functional characteristics of these cases to determine experimental evidence for chronic BCR engagement.

Chapter 5 Functional characterisation of the MCL BCR

5.1 Introduction

Mantle cell lymphoma (MCL) is a clinically heterogeneous mature B-cell tumour characterised by cyclin overexpression, typically cyclin D1,[221] and high variable surface immunoglobulin (sIg) M expression.[158] MCL is clinically subdivided by presentation: conventional, nodal MCL with unmutated (U-) immunoglobulin (Ig) gene heavy chain variable region (*IGHV*) and generally aggressive natural history and a leukaemic non-nodal (LNN) form with mutated (M-) *IGHV* and more indolent disease.[158 250] Some cases can be clinically observed before treatment but most require early intervention.[281 282 284]

In chapter 4, a subgroup of MCL was identified, principally LNN MCL, with low sIgM and signalling capacity in a fashion resembling CLL, where sIgM levels and signalling are reduced *in vivo*, likely as a result of chronic antigen engagement at tissue sites. Unlike MCL, the CLL BCR Ig has been extensively investigated; studies from Southampton provide strong experimental support for ongoing engagement of the BCR by (super) antigen. This results in an anergic state defined by low sIgM, but not sIgD, and low signalling.[85 145 340 351 355]

Direct evidence for ongoing BCR engagement in CLL is principally inferred from reversible sIgM, but not sIgD, down-modulation *in vitro*. [145 351] Following *in vitro* culture, the CLL B-cells re-express sIgM whilst sIgD expression remains constant. This suggests release from exposure to antigen at tissue sites. In addition, *in vivo* recently egressed CLL cells with a CD5^{bright} CXCR4^{dim} immunophenotype are associated with an activated gene expression profile.[242 350] A similar phenomenon is identifiable in the lymphocytosis following ibrutinib therapy where the neoplastic B-cells lose a chronic antigen stimulation signature. This signature is characterised by high basal levels of phosphorylated Spleen tyrosine Kinase (pSYK) and an immature glycan pattern on the μ constant region identified through immunoblotting.[355] Normal B-cells bear a mature complex of glycans on the constant chain of their Ig protein but an immature, mannosylated form can also be expressed following persistent BCR engagement.[115 355]

When assessing for variation of BCR expression across compartments, CLL is a less useful guide. There is compartmental difference in CLL where lymph node CLL cells have a proliferative, CXCR4^{dim}CD5^{bright} phenotype similar to the recently egressed cells. These cells are highly positive for CD49d which permits trans-endothelial migration to the proliferation centres.[359] However, there is little direct data of sIgM or sIgD variation across lymph nodes and peripheral blood samples. In MCL studies on paired lymph node and peripheral blood samples reveal evidence of ongoing, active BCR signalling in the lymph node by gene expression profiling, BCR kinase activity e.g. pSYK and pERK, and by Ki67.[240] Variation in Ig expression across compartments is unknown.

It was therefore, hypothesised that the MCL BCR, in this low signalling group, would have similar functional characteristics to the CLL BCR. If demonstrated this would support tissue based chronic BCR engagement as a mechanism of BCR activation and ontogeny in at least a subgroup of MCL.

5.2 Overarching Hypothesis:

MCL with low 'CLL-like' signalling capacity possess features indicative of chronic BCR engagement

5.2.1 Sub-Hypotheses

1. 'CLL-like' MCL cells with down modulated sIgM, but not sIgD, will recover *in vitro* away from putative antigen
2. Chronically engaged 'CLL-like' MCL cells with low, down modulated, sIgM contain higher basal activation status of proximal kinases than unengaged sIgM.
3. 'CLL-like' MCL cells with BCR engaged sIgM possess both mature and immature constant chain surface glycans.
4. Surface IgM but not D will be higher in the peripheral blood compartment than the tissue compartment.

5.2.2 Aims:

1. To phenotype BCR Ig expression *in vitro* over a 72 hour period.
2. To explore BCR receptor modulation and basal signalling capacity.
3. To explore BCR receptor µ chain N-glycosylation patterns.
4. To explore sIg variation across tissue compartments.

5.3 Spontaneous *in vitro* recovery of sIgM, but not sIgD, occurred in MCL with low 'CLL-like' signalling.

Recovery experiments (**Phenotypic analysis of MCL cells using flow cytometry**) were performed on 24 primary MCL samples including all 8 'CLL-like' low signallers (**Table 5.1**). CD5+ CD19+ populations were identified and assessed for sIgM, sIgD and CXCR4 expression at 0, 24, 48 and 72 hours (**Fi**gure 5.1 **A**). CXCR4 is a chemokine receptor involved in B-cell trafficking known to be present on MCL cells with reduced expression in tissue compartments.[192 241 442] In CLL it is rapidly endocytosed in tissue and recycled to the cell surface acting as a useful control in recovery experiments. Cell viability, assessed by gating on live cells (forward/side scatter), was used to check quality of MCL population at each time point. Phenotypic results were excluded if the 'live' population was indiscrete from 'dead' cells or fewer than 10% (**Fi**gure 5.1 **B**). Five cases (2 Conventional, 2 LNN and 1 blastoid, previously LNN) were excluded due to cell death.
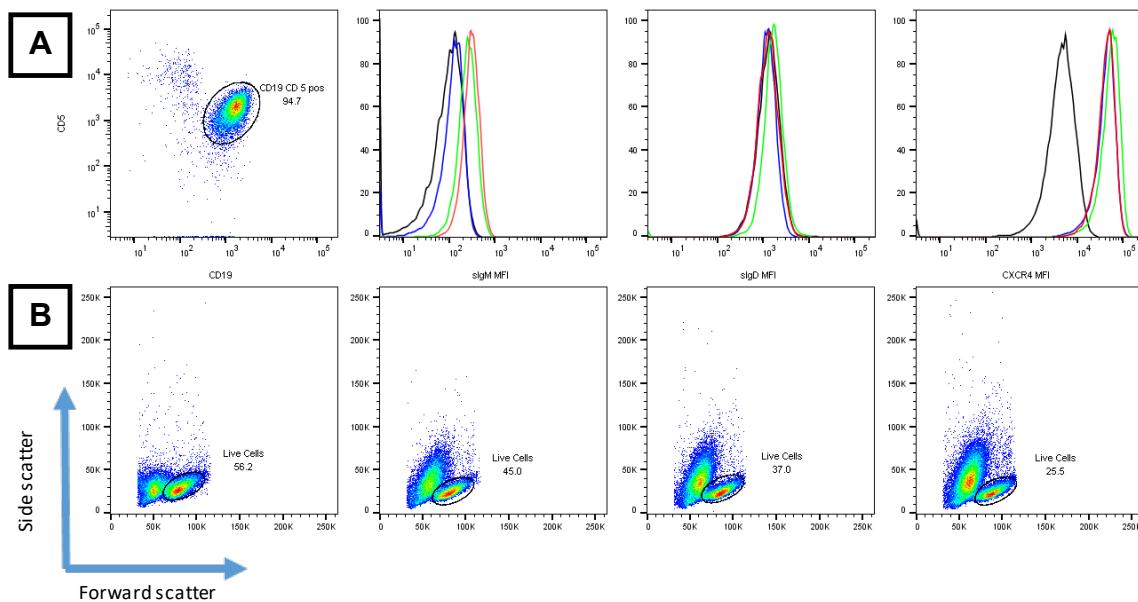
Figure 5.1: Recovery of sIgM, CXCR5 but not sIgD in CLL-like signaller MCL27

Primary MCL cells were incubated at 37°C/5% CO_2 in complete RPMI and immunophenotyped at 3, 24, 48 and 72 hours assessing IgM (PE), IgD (FITC) and CXCR4 (APC) MFI and isotype controls (not shown) (Panel A). Live (by forward and side scatter [Panel B]) CD5/19 positive malignant cells (A) were gated upon and IgM/D and CXCR4 plotted at 3 hours (black line), 24 hours (blue), 48 hours (green) and 72 hours (red) in graphs left to right. In this example, sIgM increases at each time point. Surface IgD, in contrast, remains constant whilst CXCR4 rises rapidly at 24 hours as proof of recovery competence (Panel A). Cell death was assessed by forward/side scatter at each time point to ensure viability of acquired cell population (panel B).

Surface IgM expression recovered to at least 130% of baseline (3 hours) in 7/8 (88%) of 'CLL-like' low signalling samples. MCL3, 16, 27, 28 and 63 increased sIgM expression at each time point (up to a maximum of 160%, 75%, 196%, 149% and 85% respectively) (**Figure 5.2 A and D**). Maximum sIgM recovery occurred at 72 hours in all samples. In three cases (38%), sIgM expression fell at 24 hours before increasing again. One sample did not recover above baseline at any time point (MCL 9, ($\bigtriangledown$) Conventional U-MCL, sIgM 119, Peak iCa flux 58%).

Surface IgD expression stayed at, or below, baseline in 6/8 (75%) of CLL-like signalling samples (**Figure 5.2 B and E**). Two samples, (MCL 3 ($\blacksquare$), Blastoid M-MCL, sIgM 55, Peak iCa flux 52%, and MCL 9, ($\bigtriangledown$) Conventional U-MCL, sIgM 119, Peak iCa flux 58%) exceeded baseline expression at 72 hours by 27% and 40% respectively.

Baseline CXCR4 expression was variable (MFI Range 3879-17309, Median 9855, CV 42.6%) (**Figure 5.2 C**). CXCR4 expression exceeded baseline at 24 hours in 7/8 (88%) samples (**Figure 5.2 F**) demonstrating recovery competence in this group.   CXCR4 recovered to peak MFI at 24 hours in 5/8 cases and in 2/8 at 48 hours but did not recover at all in 1 case (MCL 3). There was weak CXCR4 recovery (<100% at peak recovery) in 3 samples (MCL 28 ($\bullet$) Conventional U-MCL, sIgM 325, Peak iCa flux 52%; MCL 9, ($\bigtriangledown$) Conventional U-MCL, sIgM 119, Peak iCa flux 58% and MCL 53 ($\ominus$) LNN M-MCL, sIgM 409, Peak iCa flux 70%). Baseline CXCR4 (MFI) in MCL 3, 9, 28 and 53 was 14435, 17309, 8994 and 10173 respectively. Median CXCR4 peak increase from baseline at 24 hours was 150% (Range 0% - 860%, CV 85.4%) (**Figure 5.2 C and F**).
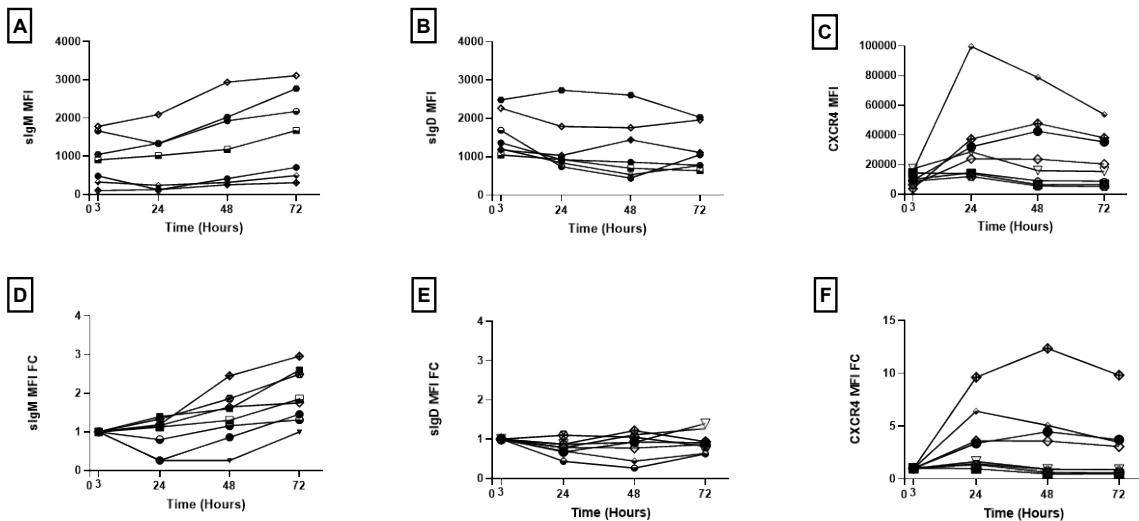
Figure 5.2: sIgM and sIgD recovery in low 'CLL-like' MCL

Surface IgM/D and CXCR4 immunophenotypic analysis of 8 'CLL-like' signallers was assessed after 3, 24, 48 and 72 hours *in vitro* incubation. MFI (Test-Isotype control) was plotted against Time (Hours) for sIgM (A), sIgD (B) and CXCR4 (C). Fold change (FC) MFI difference to baseline (MFI at 3 hours set to 1) for sIgM (D), sIgD (E) and CXCR4 (F) is displayed to demonstrate change in expression over time.

In vitro sIg recovery was assessed in 11 'high signalling' MCL cases (**Table 5.1**). Surface IgM expression recovered above baseline in 3 cases: 18% above baseline in 1 case (MCL 7 (▼), LNN M-MCL, sIgM 910, Peak iCa flux 100%), above 50% (MCL 13, (★) Blastoid, sIgM 1534, Peak iCa flux 79%) and at each time point to a maximum of 85% in one further case (MCL 63, (◼) LNN-MCL, sIgM 510, Peak iCa flux 88%) (**Figure 5.3 A and D**). Surface IgM expression did not recover, staying at (to a maximum of 8% above baseline at any time point) or below baseline, throughout the 72 hour experiment in the other 9 samples.

Surface IgD expression did not exceed baseline expression in 9/11 (82%) of samples. Surface IgD expression recovered above baseline in 2 samples: by 28% at 72 hours (MCL 5 (△), Conventional U-MCL, sIgM 1726, Peak iCa flux 98%) and by 146% (MCL 13, (★) Blastoid, sIgM 1534, Peak iCa flux 79%) (**Figure 5.3 B and D**).

CXCR4 expression exceeded baseline at 24 hours in 10/11 (91%) samples (**Figure 5.3 C and F**) demonstrating themselves to be recovery competent. CXCR4 reached peak MFI in 4/11

at 24 hours, 4/11 at 48 hours and 2/11 at 72 hours but did not exceed baseline in 1 (MCL 7). Median CXCR4 peak increase from baseline at 24 hours was 250% (Range 50% - 1223%, CV 78.1%). Baseline CXCR4 expression in the high signalling group was lower (Not significant, p=0.3950) and more variable than the 'CLL-like' group (Range 1136-20409, Median 6764, CV 68.4%).
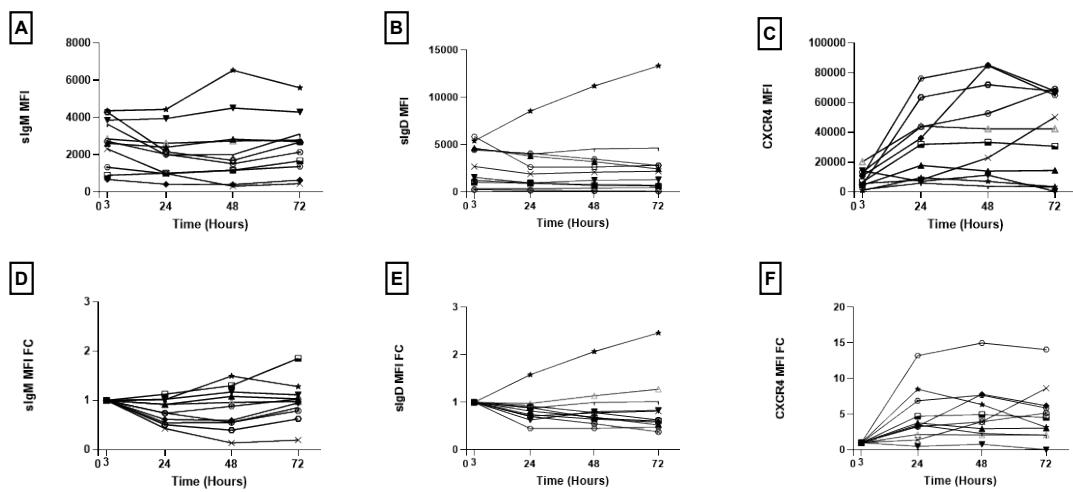
Figure 5.3: sIgM and sIgD recovery in non-'CLL-like' MCL

Surface IgM/D and CXCR4 immunophenotypic analysis of eleven 'high' signallers was assessed following 3, 24, 48 and 72 hours *in vitro* incubation. MFI (Test-Isotype control) was plotted against Time (Hours) for sIgM (A), sIgD (B) and CXCR4 (C). The Fold change (FC) MFI difference to baseline (MFI at 3 hours set to 1) for sIgM (D), sIgD (E) and CXCR4 (F) is displayed to demonstrate change in surface expression over time.

Table 5.1: Characteristics of peripheral blood samples undergoing *in vitro* recovery

MCL Sample 'CLL-like' in <u>bold</u>	MCL Sub Type	*IGHV*	Homology (%)	sIgM	Peak iCa^{2+}	CXCR4 (MFI)	'Recovery competent'
Isolated sIgM Recovery n=7 sIgM (Range 47-854) median 325 (Vs no Ig recovery p<0.01), Ca flux (%) (Range 11-88) Median 69 (Vs no Ig recovery p=0.03)							
<u>**1**</u>	LNN	3-15	95.23	180	20	9537	✓
<u>**16**</u>	Conventional	3-21	99.25	854	50	6646	✓
<u>**27**</u>	Conventional	3-21	100	47	11	3879	✓
<u>**28**</u>	Conventional	4-39	99.0	325	69	8994	×/✓
<u>**53**</u>	LNN	3-64	95.86	409	70	10173	×/✓
<u>**63**</u>	LNN	3-23	97.74	510	88	6764	✓
<u>**64**</u>	LNN	1-18	89.25	263	65	15565	✓
No surface immunoglobulin recovery n=8 sIgM (Range 412-2584) median 1571, Ca flux (%) (Range 76-100) Median 98							
4	LNN	4-34	97.19	1571	100	4748	✓
7	LNN	1-46	93.4	589	100	14375	×
11	Conventional	4-34	97.88	412	87	10927	✓
17	Conventional	4-59	99.65	2144	100	6864	✓
18	Conventional	3-11	100	2311^	76	4811	✓
60	LNN	4-34	99.65	973	90	4132	✓
61	Conventional	3-31	100	2282	-	11067	✓
65	Conventional	-	-	1487	99	13380	✓
Dual sIgM and sIgD Recovery							
<u>**3**</u>	Blastoid	4-39	97.58	55	52	14435	×
13	Blastoid	3-74	97.57	3857	79	1136	✓
Isolated D recovery							
5	Conventional	3-23	100	1726	98	20409	✓
<u>**9**</u>	Conventional	4-59	99.30	370	54	17309	×/✓

LNN, Leukaemic Non-nodal, ^ analysed by CANTOII flow cytometer, Rx, Treatment). (×) no CXCR4 recovery above baseline, ×/✓ weak CXCR4 recovery above baseline, ✓ Strong CXCR4 recovery above baseline. sIgM (MFI), Peak iCa^{2+}(%). Statistics calculated on GraphPad Prism software using non-parametric analysis.

5.4 MCL 'CLL-like' low signallers associated with high basal pSYK

CD5/19 positive cell gating was used to identify MCL cells and were assessed by Phosflow for their expression of pSYK and pS6 (a downstream AKT target) in their resting, 'basal' state, in the presence of a highly selective SYK inhibitor, Entospletinib, and with or without α-IgM stimulation.

Phosphorylation of SYK at codon Y525 was measured by Phosflow in thirteen samples to determine basal kinase activity status. Median pSYK level was 3318 (Range 1515-8774, CV 61.4%), whilst in the presence of Entospletinib (10μM) this reduced to 2258 (Range 919 - 6531, CV 71.5%). Median pS6 (Ser235/236) was 7179 (Range 3926 - 27713, CV 71.8%) falling to 5063 (Range 2946 - 21065, CV 65.5%) in the presence of Entospletinib (10μM). The difference between basal and inhibited phosphorylated kinase was calculated to measure background BCR engagement activity. Analysis was performed with and without blastoid cases as they are known to upregulate BCR signalling pathways having undergone kinome remodelling.[398]

Basal pSYK was significantly higher in the 'CLL-like' low signallers in both all (Median 7413 vs 2725, p=0.01) and non-blastoid cases (Median 6647 vs 2457, p=0.04) (**Figure 5.4 A and B**). Basal pSYK negatively correlated with sIgM expression (Not shown, r=-0.6088, p=0.01). Measurable SYK inhibition (the difference between basal pSYK and pSYK in the presence of an inhibitor) was significantly greater in the low signalling group in all (Median 2292 vs 827, p=0.01) and non-blastoid cases (Median 2367 vs 676, p=0.04) (**Figure 5.4 C and D**). Although basal pS6 was higher in the 'CLL-like' group in all samples (Median 15735 vs 6185, p=0.10) and when blastoid cases were excluded, this was not statistically significant (Median 16726 vs 6185, p=0.06) (**Figure 5.4 E and F**). Similarly, measurable pS6 inhibition was non-significantly greater in the 'CLL-like' low signalling group (Median 16309 vs 6185, p=0.10) and with the exclusion of blastoid cases (Median 4066 vs -104, p=0.11, not shown).
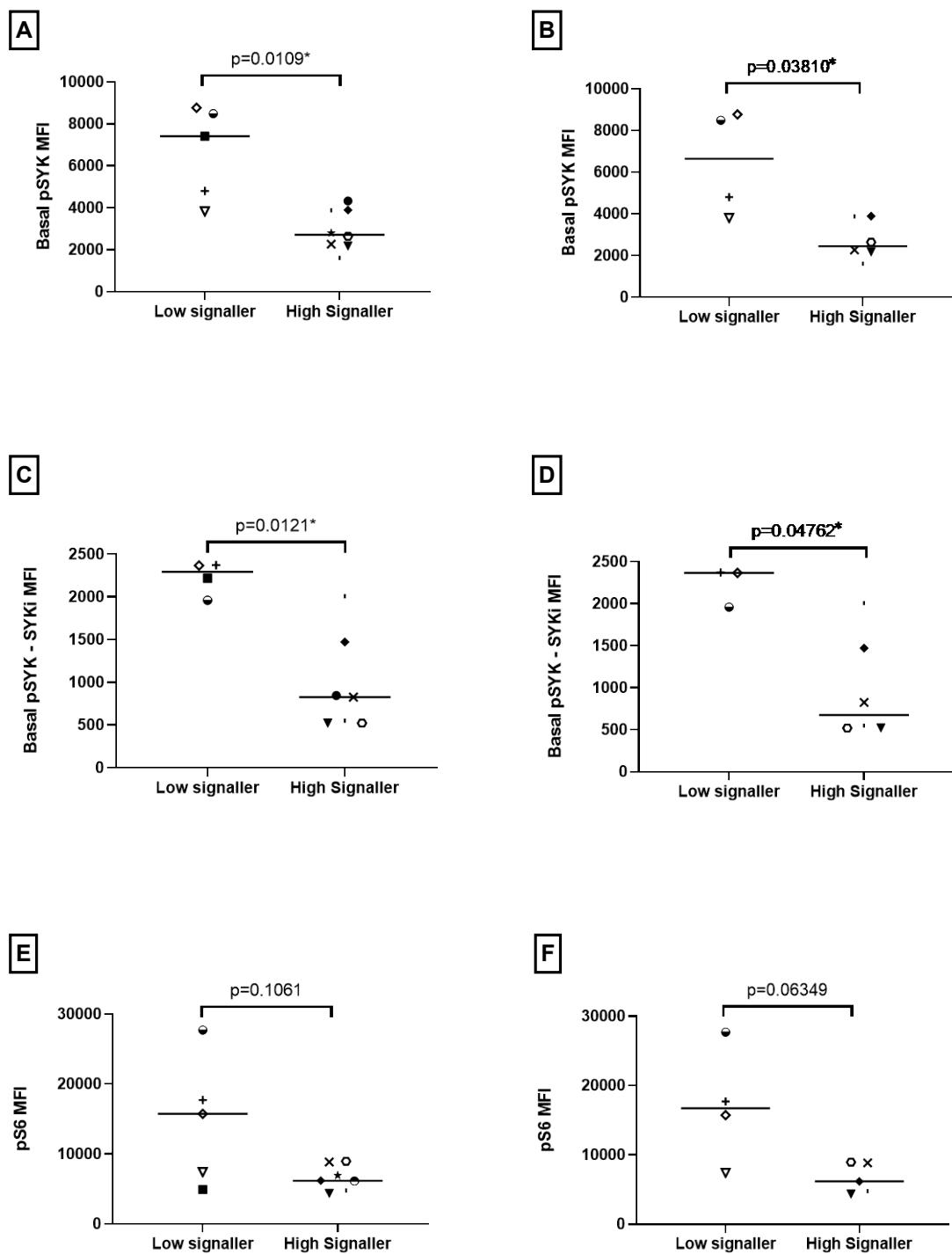
Figure 5.4: Basal pSYK, SYK inhibition and basal pS6 associate with low 'CLL-like' signallers

Tumour cells were isolated via CD5/19^{+}, CD3^{-} staining to exclude normal B and T-cells. MFI was calculated as basal pSYK/S6 (Y525/s235/236) less the unstained MFI. (A) Basal pSYK in all recorded samples and (B) basal pSYK excluding BL-MCL. (C) The difference between basal pSYK and basal SYK incubated with SYK inhibitor in 'CLL-like' low signallers compared with high signallers and (D) with BL-MCL excluded. (E) Basal pS6 expression in 'CLL-like' low signallers compared with high signallers and (F) with BL-MCL excluded. Straight lines represent median values. Statistics calculated using non-parametric Mann-Whitney statistical tests.

5.5 The glycosylation pattern of 'CLL-like' MCL signallers is suggestive of BCR engagement *in vivo*

Eleven peripheral blood MCL samples were available for analysis of the glycosylation pattern of the BCR μ chain (**Table 5.2**). The immunoblots of the 'CLL-like' signallers contained two bands of variable proportions in the absence of EndoH or PNGase digestive enzymes (**Figure 5.5 A - D**). The upper band (83KDa) corresponds to the mature forms whilst the lower band (78KDa) corresponds to the immature glycoforms.[351][355] The lower immature bands disappeared following Endo H digestion as it cleaves oligomannose rich glycans. EndoH is unable to digest highly processed complex oligosachharide from glycoproteins, leaving the upper (undigested) band visible. This confirmed the presence of the immature, mannosylated, glycoforms. PNGase enzymatic digestion cleaves all N-linked oligosaccharides; the upper mature band disappeared leaving a faster band in the presence of PNGase in all cases (**Figure 5.5 A - C**).

Table 5.2: Characteristics MCL samples undergoing μ chain glycosylation analysis

MCL Sample 'CLL-like'	MCL Sub Type	*IGHV*	Homology (%)	sIgM (MFI)	Ca²⁺ Flux M (%)	sIgM Recovery	M:I Ratio (0 Hours)
<u>3</u>	LNN/Blastoid	4-39	97.58	55	52	✓	3.4
7	LNN	1-46	93.4	910	100	×	20.26
<u>9</u>	Conventional	4-59	99.30	370	54	×	0.96
11	LNN#	4-34	97.88	412	91	×	8.15
12	LNN	3-74	93.75	580	88	NA	13.7
13	Blastoid	3-74	97.57	3857	79	✓	3.99
<u>16</u>	Conventional	3-21	99.25	854	60	✓	2.92
<u>27</u>	Conventional	3-21	100	47	11	✓	.28
45	Conventional	6-1	98.99	2584	96	NA	6.18
<u>53</u>	LNN	3-64	95.86	409	70	✓	4.14
61	Conventional	3-21(κ)	100	2282	-	×	9.46

M:I ratio = Mature:Immature ratio, # Splenectomised, - NA = Not available/Not performed
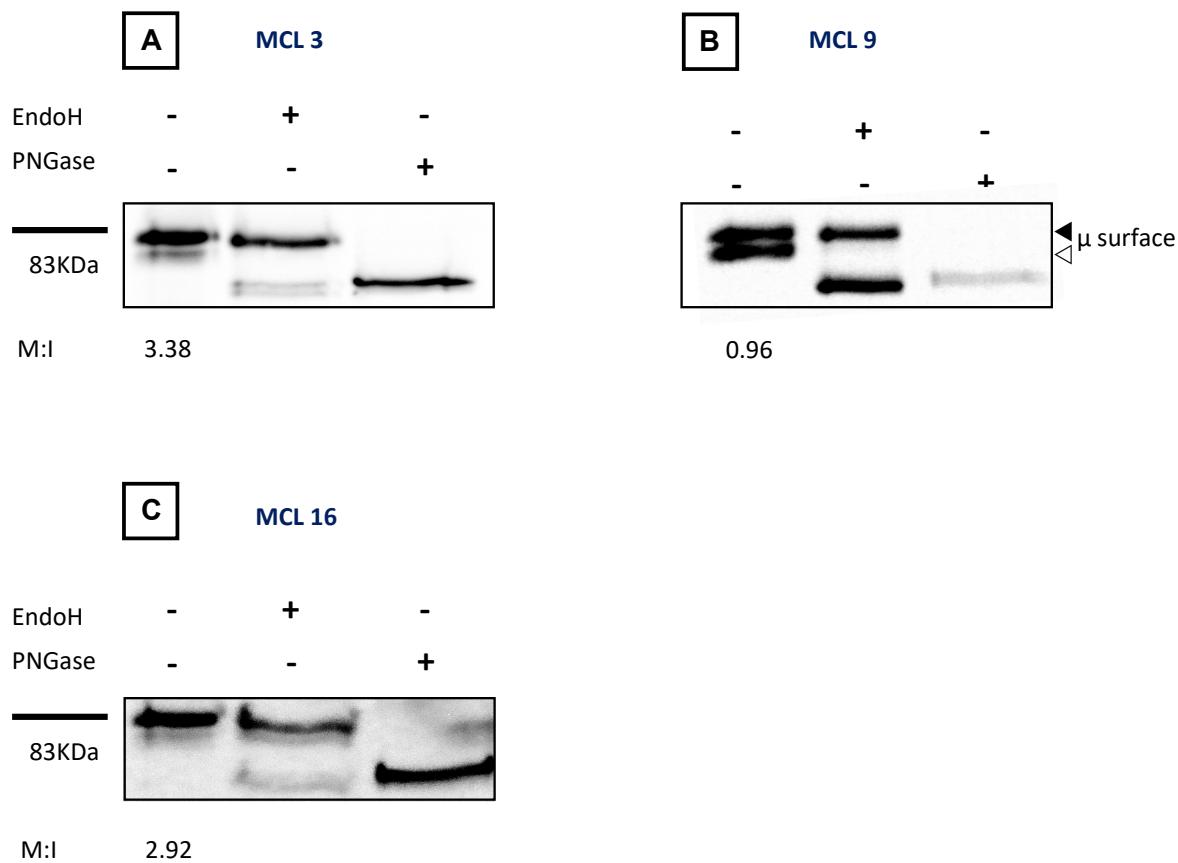
Figure 5.5: Analysis of μ chains expressed by surface IgM in 'CLL-like' signallers

Surface proteins were isolated by biotinylation from MCL samples with 'CLL-like' low signalling. μ chains were analysed by immunoblotting following no treatment or digestion with Endo H or PNGase. Endo H digests the immature mannosylated glycan structure and PNGase cleaves at the asparagine core digesting both mature (83KDa) and immature glycan band. Results are shown for three MCL samples MCL 3 (A), 9 (B) and 16 (C). The mature (◀) and immature (◁) μ chain glycoforms and their mature:immature (M:I) glycoforms ratio are shown for each sample.

In contrast, the immunoblots of high signallers consistently revealed a single band (83KDa) representing the mature glycoforms (**Figure 5.6 A - D**). Endo H digestion had no effect with persisting mature band and the absence of a faster band. PNGase digestion revealed a faster band confirming the presence of mature glycan forms.
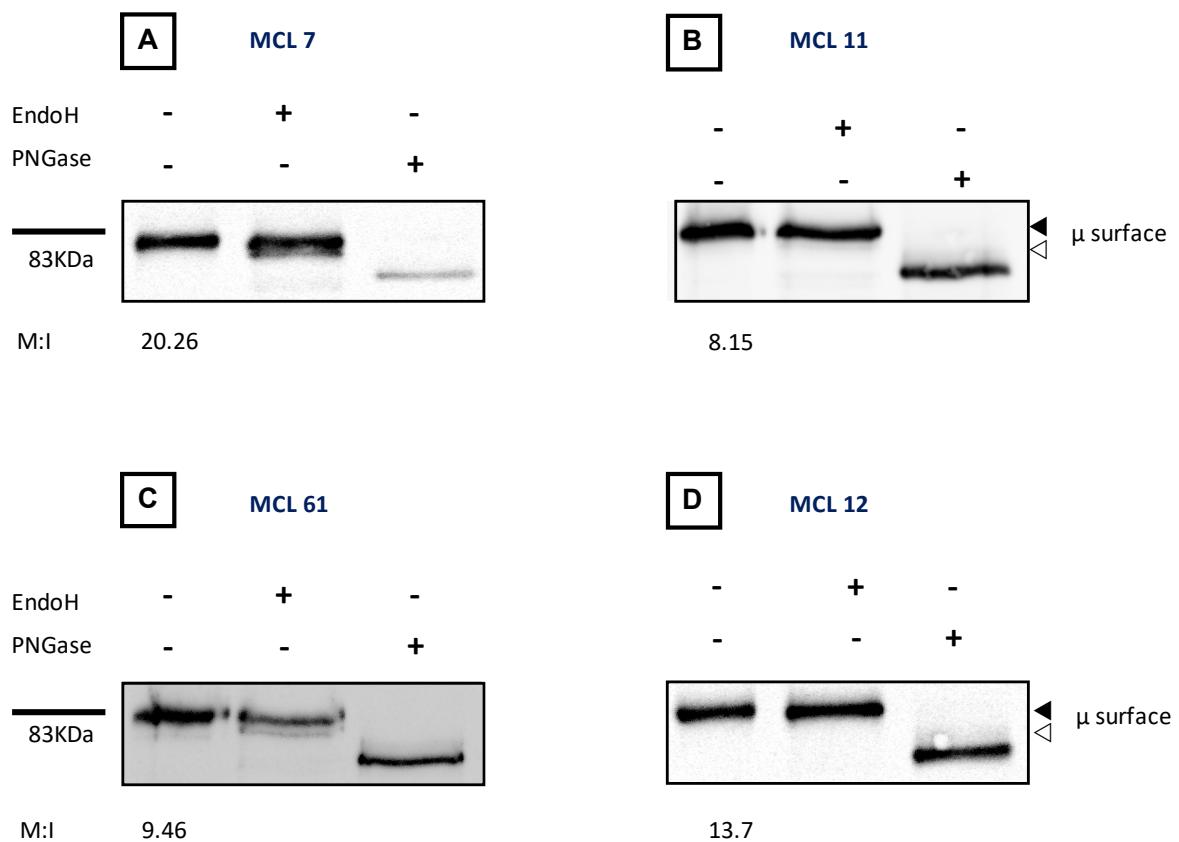
Figure 5.6: Analysis of μ chains expressed by surface IgM in non 'CLL-like' high signallers

Surface proteins were isolated from MCL samples by biotinylation of samples with 'high' signalling. μ chains were analysed by immunoblotting following no treatment or digestion with Endo H or PNGase. Endo H digests the immature mannosylated glycan structure and PNGase cleaves at the asparagine core digesting both mature (83KDa) and immature glycan band. Results are shown for four MCL samples MCL 7 (A), 11 (B), 61 (C) and 12 (D). The mature (◀) and immature (◁) glycoforms are shown for each sample and the ratio of mature:immature (M:I) glycoforms.

The 'CLL-like' signallers had significantly lower M:I ratio (n=5, Range 0.28 – 4.1, Median 2.9) than the higher signallers (n=6, Range 3.99 – 20.26, Median 8.8; p<0.01) including and excluding blastoid cases (p=0.02) (**Figure 5.7 A and B**).
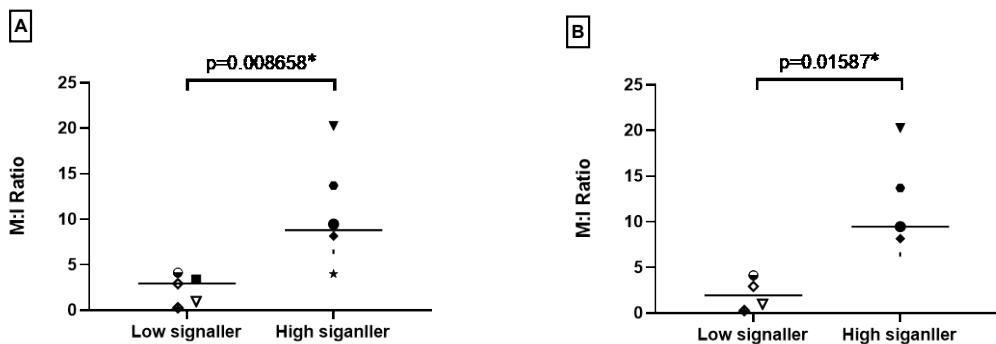
Figure 5.7: CLL like signallers associate with immature glycoforms

The mature: immature (M:I) ratio was calculated by densitometry analysis (ImageJ software) of immunoblot mature and immature bands. M:I ratios for all available samples (A) and excluding blastoid MCL (B) are displayed. Non-parametric statistics were performed using GraphPad PRISM software.

5.6 Surface IgM expression and signalling vary across anatomical compartment

Surface IgM/D expression was assessed in thirty-six patients with confirmed MCL using peripheral blood samples. The tumour Ig, sIgM and D levels and signalling capacity were determined in samples obtained from the lymph node (LN) (n=15) or spleen (n=6) of 21 patients (**Table 5.3**) with MCL using the same methods. When assessed across histology types, lymph node (LN) sIgM expression (MFI Range 88-1766, median 409) was non-significantly lower than peripheral blood (PB) sIgM (Range 47-2808, median 942, p=0.09) and splenic sIgM expression (Range 40-1796, Median 1036, p=0.6222). All six splenic samples were classified as conventional nodal MCL.   There was no statistically significant different sIgD expression across the three compartments (PB Range 1-253, Median 29; LN Range 1-125, Median 27; Spleen Range 15-136, Median 26; PB vs LN p=0.67; PB vs Spleen p=0.8421; LN vs Spleen p 0.89) (**Figure 5.8 A and C**).

Table 5.3: Immunogenetic and phenotypic characteristics of MCL tissue samples

MCL Sample	Tissue Type	*WHO Subtype*	*IGHV*	Aggressive histology	Ki67%	Homology (%)	sIgM (MFI)	Ca²⁺ Flux α-IgM (%)	sIgD (MFI)
19	Lymph Node	Nodal	4-39	×	40%	97.59	295	36	NA
21	Lymph Node	Nodal	NA	×	40%	NA	369	NA	30
22	Lymph Node	Nodal	3-7	×	NA	99.31	1715	26	125
23	Lymph Node	Nodal	1-2	×	NA	100.00	109	19	23
24	Lymph Node	Nodal	3-13	×	NA	99.30	687	NA	10
25	**Lymph Node**	Nodal	**3-23**	×	**35%**	**98.20**	**265**	**NA**	**49**
27	Lymph Node	Nodal	3-21	✓	53%	100.00	88	0	19
29	Lymph Node	Nodal	4-39	×	NA	99.66	251	NA	16
31	Lymph Node	Nodal	1-8	×	NA	98.26	1108	52	27
36	Lymph Node	Nodal	NA	✓	NA	NA	1766	8	24
39	Lymph Node	Nodal	3-7	✓	90%	100.00	967	NA	40
50	Lymph Node	Nodal	NA	×	Low	NA	409	NA	27
51	Lymph Node	Nodal	NA	×	30%	NA	323	NA	56
71	**Lymph Node**	Nodal	**NA**	**NA**	**NA**	**NA**	**1055**	**NA**	**1**
72	**Lymph Node**	Nodal	**4-34**	**NA**	**NA**	**NA**	**859**	**NA**	**65**
20	Spleen	Nodal	3-21	×	Low	98.87	40	88	16
28	**Spleen**	Nodal	**4-39**	×	**NA**	**99.00**	**172**	**35**	**41**
39	Spleen	Nodal	3-7	✓	90%	100.00	1036	56	35
48	Spleen	Nodal	4-59	✓	70%	98.48	1035	100	136
68	**Spleen**	Nodal	**5-51**	×	**38%**	**NA**	**1796**	**NA**	**15**
70	Spleen	Nodal	NA	×	Low	NA	1585	77	16
28	**Stomach**	Nodal	**4-39**	×	**Low**	**99.00**	**210**	**NA**	**39**

Aggressive variant – Blastoid or pleomorphic histology - × absent, ✓ present. Low Ki67 <30%. NA – not available. Paired samples in **bold**.

As in previous chapters, an analysis of cases excluding aggressive variants (Blastoid and pleomorphic but termed 'non-blastoid') was made. Non-blastoid MCL PB sIgM (n=32, Range 119-2808, median 942) was significantly higher than LN (n=12, Range 109-1715, median 389, p=0.03) but not spleen (n=4, Range 40=1796, Median 878, p=0.59) (**Figure 5.8 B and D**). LN and spleen sIgM was statistically similar (p>0.99). Mimicking the pattern documented in CLL and MCL by *IGHV* mutation status, sIgD expression remained low and statistically similar across the three compartments (PB n=32, Range 1-253, median 29 vs LN n=11, Range 1-125, median 30, p=0.95; PB vs Spleen n=4, Range 15-41, Median 16, p=0.41; LN vs Spleen p=0.34).
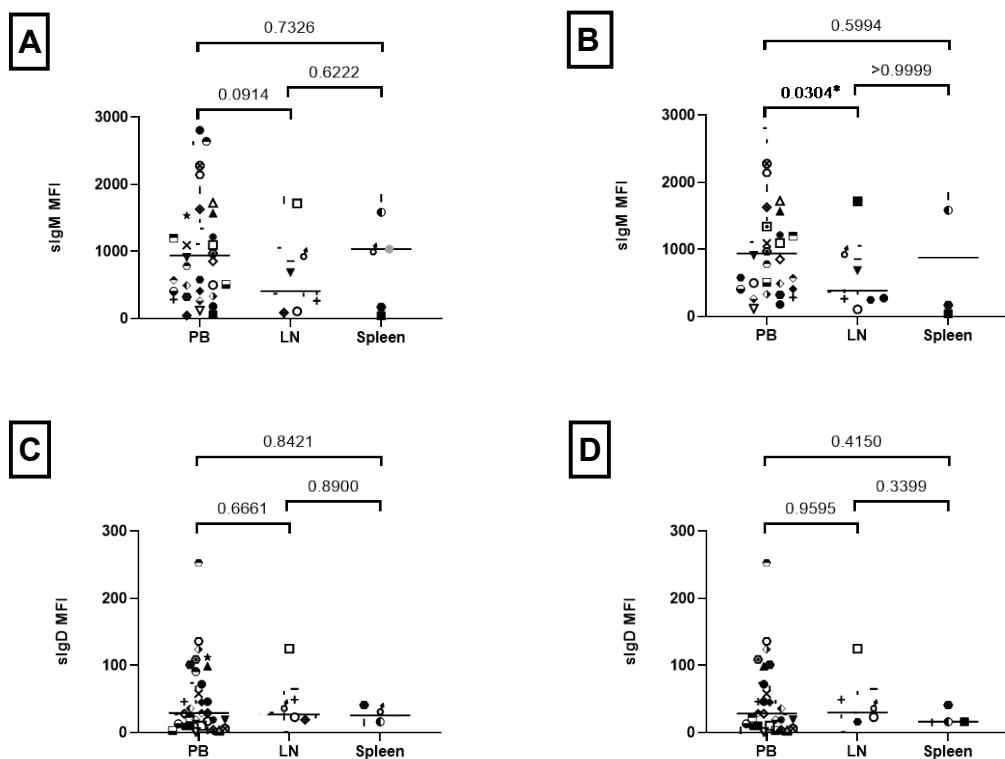
Figure 5.8: Surface IgM/D expression across anatomical compartments.

Peripheral blood (n=36), lymph node (n=15) and spleen (n-6) samples from MCL patients were assessed for surface IgM and sIgD expression using a FACS Calibur flow cytometer and analysed using FlowJo software. Surface Ig MFI was calculated following subtraction of isotype control MFI. MFIs for sIgM (A-B) and sIgD (C-D) were plotted by compartment including all samples (A&C) and cases excluding blastoid or pleomorphic histology (B and D). Straight lines represent median value. The statistical significance of difference was calculated using the Mann-Whitney statistical test. * denotes significance p<0.05.

Signalling capacity (Anti IgM Peak intracellular calcium %) was significantly higher in PB (n=27, Range 3-98, Median 80) than LN (n=6, Range 0-52, Median 23, p<0.01) but not spleen (n=5, Range 35-100, Median 71, p=0.85) when measured in all cases and when aggressive variants were removed. Splenic signalling capacity was significantly greater than LN (p=0.02) (**Figure 5.9 A – B**). Six paired samples from five patients (MCL 25 LN, MCL 28 Spleen and stomach, MCL 68 Spleen, MCL 71 LN and MCL 72 LN **Table 5.3**) demonstrated a statistically significantly lower sIgM expression in tissue compared to PB (**Figure 5.9 C –D**) (Median percentage reduction 23%, Range -5 to -47%, p=0.03).
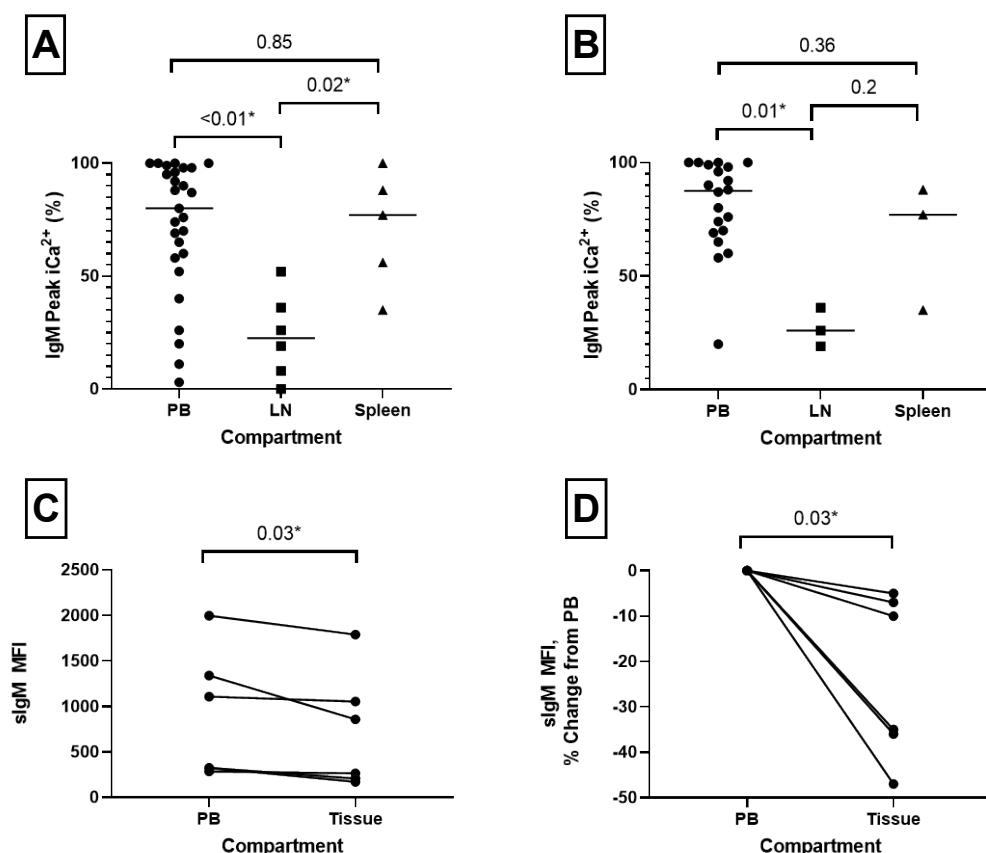
Figure 5.9: sIgM expression by tissue compartment and in paired samples.

Peripheral blood (PB, n=27), lymph node (n=6) and spleen (n=5) samples from MCL patients were assessed for anti-IgM signalling capacity and compared across tissue compartments (A). Analysis without aggressive blastoid variants (B). Five patients provided paired MCL PB and tissue (n=6; tissue [LN 4, Spleen 1, Stomach 1]). Surface IgM was compared using a FACS Calibur flow cytometer and analysed using FlowJo software. Surface Ig MFI was calculated following subtraction of isotype control MFI. Comparison of sIgM expression (C) and percentage change in sIgM expression using PB as 0 (D). Straight lines represent median value. The statistical significance of difference was calculated using the Mann-Whitney statistical test (A-B) and the paired Wilcoxon test (C-D). ***** denotes significance p<0.05.

5.7 Summary of main findings

To further investigate the role of the MCL BCR it was hypothesised that cases with low 'CLL like' signalling capacity would possess features indicative of chronic BCR engagement. The main aim was to gather experimental evidence of prior BCR engagement and compare samples with low and high signalling capacity. Samples were therefore investigated for the presence of a functional BCR activation signature based upon extensive data found in CLL. The main determinants of this signature are: recovery of sIgM expression *in vitro*, increased proximal basal signalling activity and an immature Ig constant chain carbohydrate glycosylation pattern. This would be supported by data comparing compartmental variation in Ig expression. It was hypothesised that surface IgM, but not D, would be higher in the peripheral blood compartment than the tissue compartment reflecting tissue based BCR engagement.

The experimental data found that:

1. MCL with low, 'CLL-like', signalling recovered sIgM, but not sIgD, *in vitro* over time.
2. MCL with high signalling capacity did not recover sIgM or sIgD expression *in vitro*.
3. Non-blastoid MCL with low 'CLL-like' signalling capacity significantly associated with high basal pSYK.
4. The immunoglobulin μ constant region of low 'CLL-like' MCL signallers possessed an immature glycosylation pattern.
5. The μ constant region of 'high' MCL signallers possessed a mature glycan profile.
6. PB blood sIgM expression was significantly higher than lymph node sIgM expression in non-blastoid MCL.
7. There is compartmental variation in calcium induced signalling capacity: PB and splenic MCL cells retain signalling capacity whilst LN MCL have lower signalling capacity

Overall, these findings suggest that a subset of MCL BCRs do possess features of prior, or ongoing, engagement akin to the findings in CLL supporting the overarching hypothesis. These findings support a role for putative tissue based antigen-BCR interactions reflecting the immunogenetic bias that is present in larger datasets.[168 250 388]

5.8 Discussion

The B-cell receptor (BCR) can define B-cell fate via antigen-induced and antigen-independent (constitutive) signals.[114] Constitutive signalling plays a crucial role in BCR-dependent maintenance of resting mature B-cells and some lymphomas. These typically express high surface IgM (sIgM) levels and can respond relatively well to PI3K/AKT pathway inhibtors.[443 34 444 445] Chronic antigen induced BCR signalling is key to survival and proliferation of chronic lymphocytic leukaemia (CLL) cells which are characterised by low sIgM and anergy.[351 446]

In CLL, a state of low signalling capacity is induced by chronically engaged down-modulated sIgM evidenced by reversibility of sIgM expression, immature µ chain glycosylation and high basal pSYK. In chapter 4, it was demonstrated that MCL maintained the association between *IGHV* mutations status and expression but expressed much higher levels of sIgM than CLL. Within the spectrum of sIgM expression, this cohort of MCL incorporated a sub group with CLL-like features including low signalling. This group was generally composed of LNN M-MCL and *IGHV* specific U-MCL cases. This chapter, focused on this small subset, has provided evidence that the sIgM of these MCL shares functional similarities with the CLL BCR.

This study is restricted by some experimental limitations. MCL is a relatively rare but heterogeneous disease and good quality pre-treatment samples are uncommon. MCL often presents to clinicians at a point that requires relatively urgent treatment precluding the long period of managed observation that allows for repeated sample collection. Therefore, sample numbers in these experiments were again low. Moreover, some samples had previously undergone treatment with systemic chemotherapy likely inducing some form of genetic damage. Unfortunately, well-characterised markers of genetic damage were not available for all samples. Cellular viability and sample bias will have affected which samples were available, meaning this cohort may not reflect the true spectrum of MCL.

In particular, the recovery experiment depended upon lymphocyte viability as they were analysed *in vitro* at up to 72 hours. Following the freeze/thaw process, this was a metabolically 'demanding' process. It is possible that cellular death 'favoured' some samples and, therefore, selected those with certain 'survival' characteristics such as sIgM expression. However, conditions were the same for all samples and reproducible. Moreover, typical of CLL but not previously well documented in MCL, rapid increases in CXCR4 expression acted as a positive control reflecting expected behaviour allowing confidence in cells to be classed as 'recovery competent'.[350] A variable that could not be controlled was length of time between blood sampling, processing and freezing. Some samples will have rested for longer periods 'away from tissue' and may have already 'recovered' some of their expression. It is possible, therefore, that some recovery is underestimated or cell death has occurred skewing results.

Finally, basal signalling is difficult to assess by flow cytometry. There are no accepted isotype controls. Although, unstained MFI was taken into account, it is possible that non-specific antibody binding interfered with interpretation exaggerating or masking true basal phosphorylation. However, the addition of a high concentration of signalling inhibitor to fully inhibit basal expression within samples (treated and not treated) aimed to account for any non-specific binding error.

In this functionally defined, low signalling MCL subset, it was shown that sIgM expression, but not sIgD, recovered *in* vitro. This infers ongoing *in vivo* down-modulation of the MCL sIgM. The dichotomy in MCL BCR isotype recovery mirrored the Ig phenomenon that occurs in HEL expressing transgenic mice and CLL.[85 134 138 145] MCL samples with high signalling capacity were unable to recover sIgM expression, supporting the absence of prior, or ongoing, sIgM down-modulation in this larger, generally unmutated, subset of conventional MCL. Both U- and M-MCL recovered sIgM expression, but with preference for the *IGHV*3-21 subset in U-MCL. This could explain some sIgM expression variability in MCL and why the distinction between U- and M-MCL sIgM levels, and perhaps clinical outcome, may be small.[250] Caution must be applied to these conclusions as the case numbers were small.

Further evidence to support ongoing BCR engagement arose from BCR signalling data. Significantly, higher basal pSYK was seen in the 'CLL-like' low signalling group in all cases and non-blastoid MCL. Blastoid MCL is a genetically complex disease, often characterised by upregulated BCR kinase activity, and was again separated in the analysis.[307 401 438] It is known that median basal pSYK is higher in CLL than in MCL, and higher in pre-ibrutinib treated CLL, as compared to post ibrutinib, cells.[275 351] The theoretical underlying mechanism is chronic, tissue based BCR engagement with ongoing SYK phosphorylation; as CLL sIgM is continuously down-modulated, the BCR is chronically activated increasing SYK phosphorylation levels. The addition of ibrutinib inhibits cellular adhesion molecules releasing CLL from tissue exposure, BCR engagement and hence activation, reducing SYK phosphorylation.[352-354] Mirroring this phenomenon, MCL 'low' signallers had higher phosphorylated SYK levels. Further support was added by determining basal pSYK levels through maximal SYK inhibition, using high concentrations of the highly selective SYK inhibitor entospletinib. Statistically significant reductions in pSYK in the 'low' signalling group suggested ongoing, prior SYK phosphorylation through engagement of the MCL BCR.

The identification of a, non-significant, difference in S6 phosphorylation between the two groups was intriguing. S6, a protein component of the 40S ribosomal unit, contributes to selective mRNA translation. Although the overall numbers of cases examined was low, it could infer different translation and proliferation profiles warranting further investigation.

Further supporting the BCR engaged model was the finding of characteristic changes in the glycosylation profile of the 'low' signalling group. The M:I ratio on the BCR μ constant region was significantly lower in MCL 'CLL-like' low signallers. Immature glycans are present in normal chronically stimulated healthy B-cells and in CLL, which is characterised by ongoing (super) antigen engagement.[355] This data revealed μ-chain glycan modification is also present in some MCL. This may be as a consequence of ongoing BCR engagement, highlighting the environmental influences acting across the MCL BCRs *in vivo*.

The comparison of sIgM expression between tissue compartments demonstrated a significantly lower sIgM expression in the lymph node compartment in non-aggressive MCL.

Splenic sIgM expression, however, was similar to peripheral blood (PB). Surface IgD expression was generally low and did not vary between compartments. Furthermore, paired PB and tissue samples demonstrated variable reduction in sIgM. Three samples from one patient (Conventional nodal U-MCL28, *IGHV*4-39) revealed variably lower sIgM expression across three compartments. Paired spleen (MFI 172), stomach (MFI 210) and PB (MFI 325) inferring tissue BCR engagement and down-modulation including the gut. Variation in sIgM expression reflected signalling capacity as lymph node MCL cells had low peak anti-IgM induced calcium signalling. This generally supports the hypothesis that BCR engagement occurs within the secondary lymphoid organs through the sIgM molecule preferentially in the tissue (lymph node) compartment.

However, there was no difference when comparing splenic MCL BCR expression, or signalling, with either peripheral blood or lymph node samples. The number of samples were particularly low given its rarity; all splenic samples were conventional nodal U-MCL. Moreover, splenic sIgM expression was highly variable with particularly low sIgM expression in samples with BCRs encoding U-IGHVs identified in the 'CLL-like' low signalling group (*IGHV*3-21 - sIgM 40; *IGHV*4-39 - sIgM172 & 210; *IGHV*4-59 - sIgM 1035). Although there is some experimental evidence that the spleen is a site for BCR engagement in experimental mice, this suggests that splenic BCR engagement is also dependent upon its immunogenetic profile.[248] The expression range of sIgM was similar across lymph node (88-1766) and spleen Range (40-1796) in this cohort. This may point to the spleen's equal importance as a site for BCR engagement and down modulation not picked up due to the small numbers in this study.

In CLL, chronic antigen engagement results in down-modulated sIgM with variable degrees of anergy. Subsequent sIgM and signalling capacity, strongly associated with *IGHV* mutation status, drives either a proliferative or a more indolent clinical phenotype.[85 115] This data has demonstrated that, at least in part, MCL BCRs are also under the influence of BCR engagement and that tissue based interactions play a significant role in MCL biology. In CLL, the BCR is clinically important helping prognosticate and guide treatment.[447 448] The next chapter will explore the association between MCL BCR phenotype and clinical significance.

Chapter 6 The clinical significance of the MCL BCR

6.1 Introduction

MCL is a clinically heterogeneous disease but with generally poor outcomes. Most patients require treatment though a small minority can be actively monitored. Indolent MCL is characterised by the leukaemic non-nodal (LNN) subtype, the absence of proliferative features (high tumour burden, raised lactate dehydrogenase (LDH), Ki67<30%), non-aggressive histology (non-blastoid, non-pleomorphic) and the absence of B symptoms. The MCL International Prognostic Index (MIPI) is capable of stratifying patients into immune-chemotherapy response groups using four independent prognostic factors: age, performance status, LDH, and leukocyte count.[257][258] This was refined further with Ki-67 expression as the MIPI-biological (MIPI-b) and MIPI-combined (MIPI-c) scores and validated in the European MCL Younger and Elderly trials.[179]

Although not employed routinely in clinical practice, the mutation status of the tumour *IGHV* in MCL has shown to have prognostic significance in some studies.[449] In one large study, using a 97% cut-off, those MCL with an identity to germline higher than 97% (U-MCL) had a worse prognosis than patients with lower identity (M-MCL), with a 5 year OS of 40% and 59%, respectively.[250] However, conflicting studies have been published where no relationship was identified; these used the more conventional 98% cut off and the studies were small and perhaps underpowered.[168][392][450-452] More recently, a BCR gene expression panel, including CD79B, SYK, BTK and AKT, was associated with shorter progression free (PF) and overall survival (OS).[276]

In CLL, clinical outcomes are strongly associated with BCR phenotype and the *IGHV* mutation status can aid clinical decision-making.[340][453] Early studies in CLL revealed striking variation in clinical outcome by mutations status; median OS for early stage (Binet A) U-CLL was 95 months compared to 293 months for M-CLL.[328] This key finding remains useful today and informs time to progression from diagnosis and duration of response to immune-chemotherapy informing treatment decisions.[340][454] Patients with M-CLL respond

extremely well to combination chemo-immunotherapy (5 year OS 86.3% Fludarabine, cyclophosphamide, rituximab) and BH3 mimetics in combination with anti-CD20 antibodies.[454 455] BCR-associated kinase inhibitors and BH3 mimetics are now being used increasingly in clinical trials for MCL with striking efficacy.[312 315 316 318] However, it is not yet known how *IGHV* status, or its associated phenotype, informs progression in MCL.

In chapter 4, it was demonstrated that non-blastoid MCL sIg expression was extremely variable and, unlike CLL, often high. Surface IgM expression mirrored the dichotomy seen in CLL with higher sIgM levels in U-MCL compared with M-MCL. This association was also present in the clinical subgroups where low sIgM associated with leukaemic non-nodal (LNN) MCL. Signalling capacity correlated strongly with sIgM expression. In chapter 5, supportive evidence of ongoing BCR engagement was documented principally, though not exclusively, in M-MCL with low sIgM.

Therefore, this chapter aims to assess MCL for clinical prognostic subgroups based upon *IGHV* status, WHO clinical phenotype with a focus on M-MCL with low sIgM expression. It will further assess for common clinical characteristics and contextualise treatment strategy within the limitations of a retrospective study with small numbers of samples.

6.2 Hypothesis

MCL BCR characteristics are clinically significant.

6.2.1 Aims:

1. To document BCR characteristics and clinical parameters of the MCL peripheral blood cohort, comparing treated and untreated groups.
2. To investigate the association between *IGHV* status and WHO clinical subgroups with parameters of disease activity.
3. To investigate the association between sIgM expression and signalling capacity with parameters of disease activity.

6.3 Clinical outcomes of peripheral blood Mantle Cell Lymphoma cohort

The cohort of peripheral blood MCL patients (**Table 6.1**) were taken from samples biopsied 2003 - 2019. At the end of investigation (January 2020), thirty-two of the thirty-six had initiated treatment and twenty patients were still alive. Of the patients who had started therapy, nineteen (59%) had received one line of therapy, six (19%) received two lines, six (19%) received three lines and one patient had received the maximum of six lines. The most frequent first line regime used was the anthracycline containing regime R-CHOP (n=7, 22%, see **Table 6.1** for acronym definition) followed by the NORDIC, cytarabine containing, regime with autologous stem cell transplant consolidation (n=6, 19%). Less frequently used regimes included BR (n=5, 16%), R-CVP (n=4, 13%), FC(R) (n=3, 9%), IR (n=2, 6%), R-BAC (n=1, 3%) and R-Chlor (n=1, 3%). Bruton's tyrosine kinase inhibitors were used in the second line for five cases (16%), principally ibrutinib. One patient received acalabrutinib; venetoclax was not used in any patient.

The median time to first treatment (TTFT) was 2 months, median event free survival (EFS - time from treatment to retreatment or death due to any cause) was 23 months and median overall survival (OS - time from diagnosis to death from any cause) was 73 months (**Figure 6.1 A-C**). Median follow up for this cohort was 39 months.
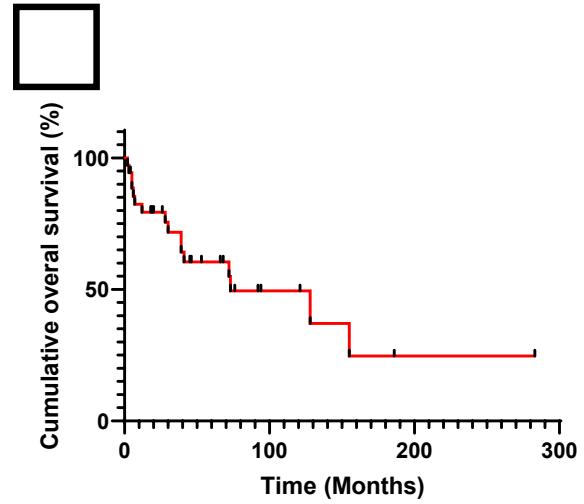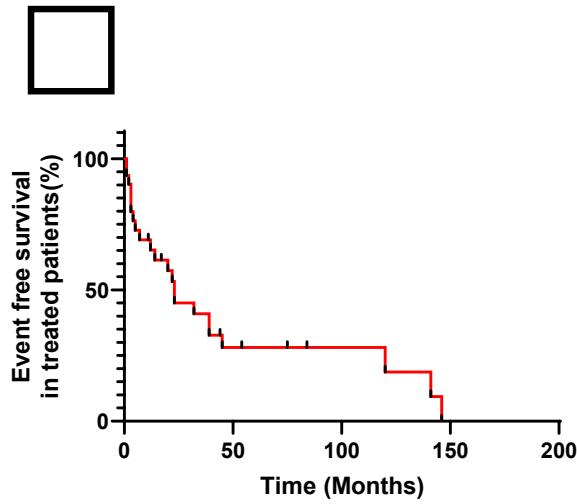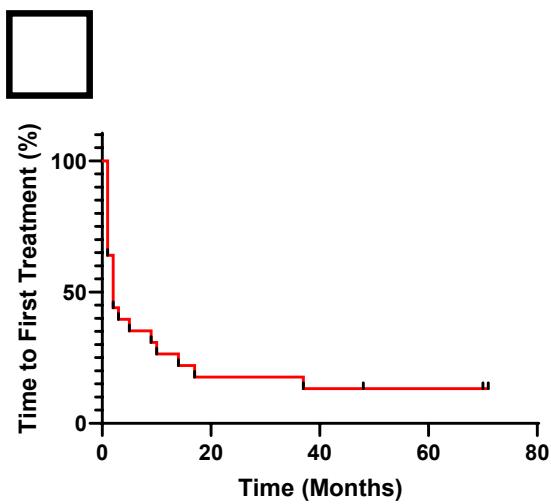
Figure 6.1: Time to first treatment, event free survival and overall survival of MCL cohort.

Patients were investigated for time from diagnosis to requirement of treatment for TTFT (n=32) (A), time from first treatment to first event (retreatment or death) (n=32) (B) and time from diagnosis to death (n=36) (C). 95% confidence interval of ratio of medians calculated with graph-pad software. Survival analysis was performed by Kaplan-Meier algorithm using Log-rank (Mantel-Cox) test.

Table 6.1: Clinical characteristics of MCL peripheral blood cohort

MCL No.	Biological Sex	Diagnostic Age (years)	Performance status (ECOG)	mIPI	WHO Sub Type	*IGHV*	Homology (%)	IGHV mutated	sIgM (MFI) Low in bold^	Peak iCa^{2+} (%)	Treatment	OS Months
3	Male	62	1	7.10	LNN/ Blastoid	4-39	97.59	M	**55**	52	CHOPx6, R-CVP, Bendamustine	155*
1	Male	72	0	6.70	LNN	3-15	95.24	M	**180**	20	R-CHOP + maintenance R	121
64	Male	88	1	7.00	LNN	1-18	89.24	M	**263**	65	**UNTREATED**	12
73	Male	62	1	7.50	LNN	4-34	92.28	M	**334**	74	NORDIC	20
53	Male	66	0	6.60	LNN	3-64	95.86	M	**409**	70	FC, R-Velcade	41*
11	Male	45	0	-	LNN	4-34	97.89	M	**412**	87	Splenectomy, Chlor/Flu/R-CHOP, Ibrutinib, R-Cyt + Auto	283
66	Male	76	0	8.00	LNN	3-7	93.60	M	**493**	100	BR, Ibrutinib	3*
63	Male	61	0	5.60	LNN	3-23	97.74	M	**510**	88	FCR, ACP-196, Anti CD32B	186
32	Male	79	1	7.10	Nodal	4-34	96.84	M	**573**	N/A	R-CVP	5*
12	Male	67	0		LNN	3-74	93.75	M	**580**	N/A	**UNTREATED**	71
52	Female	50	1	7.20	LNN	3-7	97.57	M	**780**	92	NORDIC, Ibrutinib, Allograft	73*
7	Female	85	2	9.70	LNN	1-46	93.40	M	**910**	100	R-CVP, BR	28*

65	Male	75	0	7.10	Nodal	3-48	95.63	M	1188	99	Ibrutinib-Rituximab	5
30	Male	63	1	7.50	Nodal	1-8	96.53	M	1200	N/A	RCHOP	7*
13	Male	75	1	-	Blastoid (LNN)	3-74	97.57	M	1534	95	R-CHOP, Ibrutinib, , Benda-Cytarabine	30*
4	Female	57	0	6.20	LNN	4-34	97.19	M	1571	100	**UNTREATED**	94
14	Male	82	2	9.30	Blastoid (LNN)	3-15	91.16	M	2644	98	BR + Maintenance (Main) R	19
27	Male	71	1	8.30	Pleomorphic (LNN)	3-21	100.00	U	**47**	11	R-BAC	39*
9	Male	81	1	7.60	Nodal	4-59	99.30	U	**119**	58	BR+ Ibrutinib	6*
25	Female	72	1	7.20	Nodal	3-23	98.20	U	**286**	NA	R-CHOP	2*
28	Male	68	1	6.80	Nodal	4-39	99.00	U	**325**	69	R-CVP	72*
2	Male	65	1	7.60	Nodal	1-8	98.61	U	**502**	NA	CHOP/R-Cytarabine Temsirolimus	12*
16	Male	60	0	6.30	Nodal	3-21	99.25	U	**854**	60	NORDIC + Main R	18
60	Male	46	0	6.10	LNN	4-34	99.65	U	973	90	NORDIC	4
18	Male	76	0	-	Nodal	3-11	100.00	U	1093	76	R-CHOP	39*
10A	Male	62	0	5.90	LNN	2-5	98.63	U	1096	NA	BR + main R	53
26	Female	91	1	7.50	Nodal	1-2	98.61	U	1219	NA	R-CVP	5*
72	Female	69	0		Nodal	4-34	99.30	U	1341	NA	NORDIC + main R	76
33	Male	75	0	6.70	Nodal	3-53	99.65	U	1630	NA	R-CHOP	92

5	Female	69	0	6.70	Nodal	3-23	100.00	U	1726	98	BR+- Ibrutinib	68
17	Female	83	0	-	Nodal	4-59	99.65	U	2144	100	**UNTREATED**	45
61	Male	87	0	-	Nodal	3-21	100.00	U	2282	NA	R-Chlor, Ibrutinib	26
45	Male	52	0	6.40	Nodal	6-1	98.99	U	2584	96	FC, R-CHOP, PACE-BO-R	128*
69	Male	70	2	7.70	Nodal	4-34	98.24	U	2808	80	Ibrutinib Rituximab	2
71	Female	50	0	NA	Nodal	NA	NA	-	1110	NA	NORDIC	94
68	Male	79	0	NA	Nodal	NA	NA	-	1916	NA	Splenectomy, BR+ Main R	66

Table categorised by *IGHV* mutational status and placed in order of increasing sIgM expression.

LNN, Leukaemic Non-nodal, CHOP (Cyclophosphamide, doxorubicin, vincristine, prednisolone), R (Rituximab), NORDIC (Alternating R-maxi CHOP/R-Cytarabine with autologous stem cell transplantation); FC (Fludarabine, Cyclophosphamide); Chlor (Chlorambucil); CVP (Cyclophosphamide, vincristine, prednisolone); BR (Bendamustine Rituximab); R-BAC (Rituximab-Bendamustine, Cytarabine); PACE-BO-R (Cisplatin, doxorubicin, cyclophosphamide, etoposide, bortezomib, rituximab). * Deceased. ^ Low sIgM defined as MFI <946 (**6.5**).

Of the four untreated patients three (MCL4, 12 and 64) had a M-*IGHV* and were the leukaemic non-nodal clinical subtype. No aggressive histology was present but one patient (MCL 12) was *TP53* disrupted. Surface IgM was variable (Median sIgM 1076 MFI; Range 263-2144; n=4) and equal to the treated group (Treated median sIgM 941, p=0.79). Surface IgD was also variable (Median sIgD 59 MFI; Range 9-136) and equal to the treated group (Treated median sIgD 29, p=0.59). Signalling capacity was high in the untreated group (available in n=3, median 100%, Range 65-100%) and not statistically different to the treated group (Median 86%, p=0.34).

Within the M-MCL cohort, untreated cases (n=3) had similar sIgM expression (Treated median sIgM 542 vs Untreated sIgM 580, p=0.86), similar sIgD expression (Treated median sIgD 41 MFI vs Untreated sIgD 19 MFI p=0.52) and anti-IgM intracellular signalling capacity (Treated median iCa 88% vs Untreated iCa 83% p=0.90) compared to patients receiving treatment. There was no statistical difference in the cell adhesion markers CD38 (Treated median 88% vs Untreated 45%, p=0.62), CD49D (Treated median 56% vs Untreated 41%, p=0.63) or CD23 (Treated median 13% vs Untreated 12%, p=0.63). There were insufficient untreated U-MCL cases (n=1) for statistical analysis.

Table 6.2: Clinical and functional characteristics of untreated MCL cases.

MCL Sample	Diagnostic Age	Sex	MCL Sub Type	LDH (Units/ Litre)	TP53 disruption	IGHV	Homology (%)	sIgM (MFI)	Ca²⁺ Flux M (%)	sIgM Recovery	CD23%	CD49D%	CD38%	Follow up (Months)
4	57	Female	LNN	367	-	4-34	97.19	**1571**	100	×	9	41	45	94
12	67	Male	LNN	249	Del17p	3-74	93.75	**580**	-	-	84	67	0	45
17	83	Female	Nodal	400	No	4-59	99.65	**2144**	100	×	2	92	97	71
64	88	Male	LNN	565	No	1-18	89.24	**263**	65	✓	12	27	100	12

LNN: Leukaemic non-nodal. LDH: Lactate dehydrogenase. IGHV: Immunoglobulin V gene heave chain. – Not known

6.4 Mutational status, signalling capacity , WHO subgroup and clinical outcome

Within the peripheral blood cohort, *IGHV* mutations status was available in thirty four of thirty six cases. Using the log-rank (Mantel-Cox) test *IGHV* mutational status (Cut off 98%) was not associated with TTFT (Median (U) two months vs (M) two months, p=0.31), EFS (Median (U) twenty-three months vs (M) twenty months, p=0.87) or OS (Median (U) seventy-two months vs (M) seventy-three months, p=0.53). At 3 year 35% of M-MCL had not been treated whilst 7% of U-MCL were still untreated **(Figure 6.2 A-C)**.

WHO clinical subgroup was available in all thirty-six cases. Using the log-rank (Mantel-Cox) test clinical phenotype was not associated with TTFT (Median (LNN) one and a half months vs (Nodal) one months, p=0.82). There was a non-significant trend to improved outcomes in LNN MCL for EFS (Median forty-five months vs (Nodal) twenty-two months, p=0.14) and OS (Median one hundred and fifty five months vs (Nodal) seventy-two months, p=0.16) **(Figure 6.3 A-C)**.
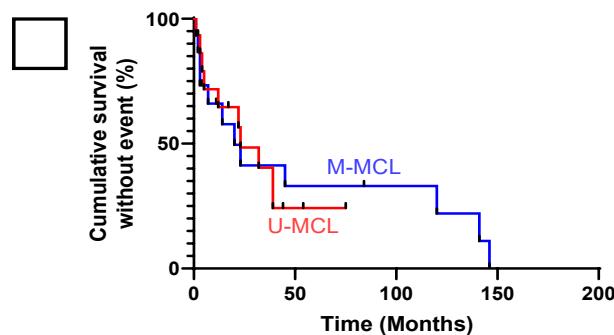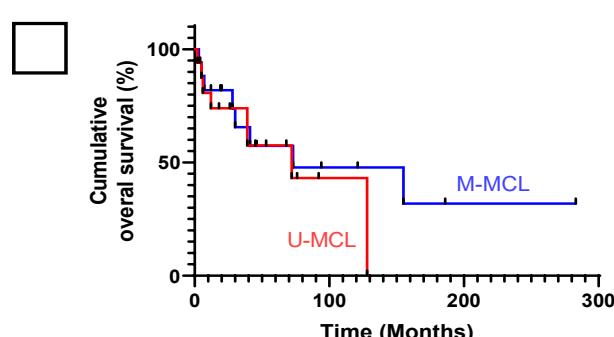
Figure 6.2: The clinical significance of *IGHV* mutation status in the MCL cohort.

Patients were investigated TTFT (A), EFS (B) and OS (C). 95% confidence interval of ratio of medians calculated with graph-pad software. Survival analysis was performed by Kaplan-Meier algorithm using Log-rank (Mantel-Cox) test.
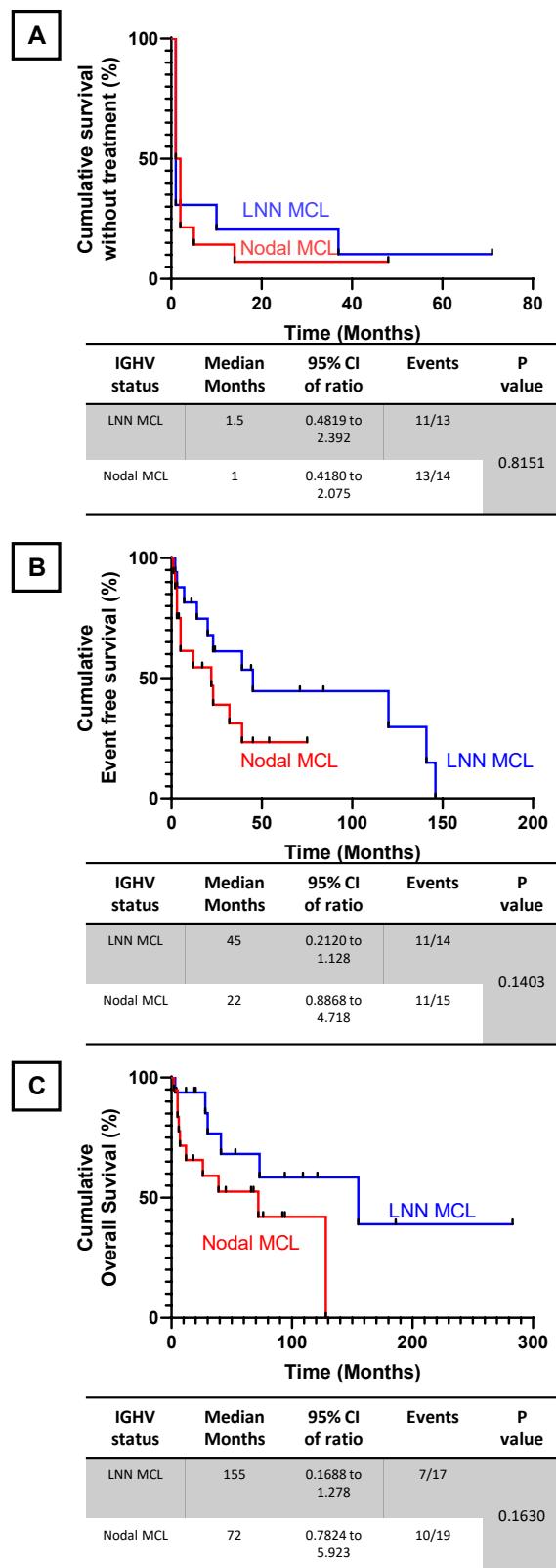
Figure 6.3: The clinical significance of WHO subgroup in the peripheral blood MCL cohort.

Patients were investigated for TTFT (A), EFS (B) and OS (C). 95% confidence interval of ratio of medians calculated with graph-pad software. Survival analysis was performed by Kaplan-Meier algorithm using Log-rank (Mantel-Cox) test.

6.5 M-MCL with low sIgM expression associates with improved clinical outcome

As the entire MCL cohort was phenotypically characterised for sIgM expression, but not signalling capacity, a surface IgM expression cut off was determined to reflect low signalling capacity. Using the CLL cohort data it was demonstrated that 95% of all CLL cases have peak α-IgM calcium signalling capacity at or below 94% (**Figure 6.4A**). This signalling capacity equates to a sIgM MFI cut-off of 946 (**Figure 6.4B**).
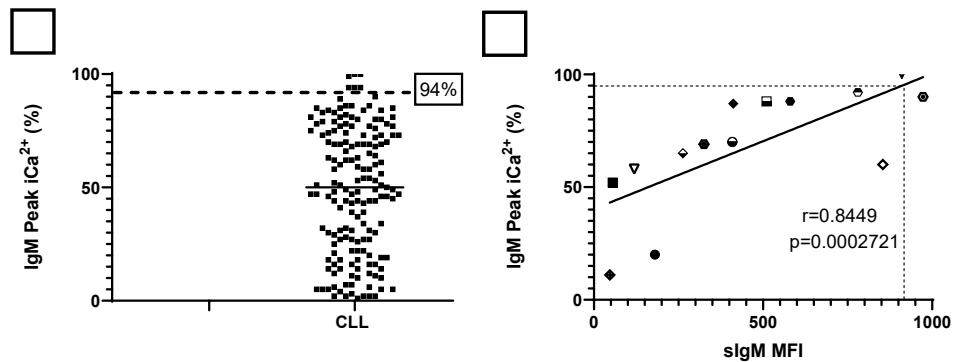
Figure 6.4: Calculation of sIgM expression cut off.

'CLL like' sIgM expression cut off was calculated from the CLL cohort data (n=165, median peak iCa 50%) with value 94%. This cut off was used to calculate the upper limit of 'CLL-like' signalling encompassing the majority (95%) of CLL cases. The surface IgM cut off was calculated from the equation of linear regression (Y = 0.05367*X + 43.20) resulting in a sIgM MFI of 946.

In patients requiring treatment, there was no difference in EFS between high and low sIgM expressers (Median EFS 23 months vs 22 months, p=0.98). However, M-MCL patients with low surface IgM expression trended to longer cumulative OS (Median OS High sIgM 30 months vs low sIgM 155 months, p=0.70) and had significantly longer EFS (Median EFS 5 vs 34 months, p=0.04) (**Figure 6.5 A-B**). The WHO clinical counterparts mirrored the mutation status. LNN MCL with low sIgM expression trended to longer OS (Median OS High sIgM 62 months vs low sIgM 155 months, p=0.57) and EFS than their high sIgM expressing counterparts (Median EFS High sIgM 15 vs low sIgM 123 months, p=0.09). Peak anti-IgM induced calcium signalling was significantly lower in the M-IGHV and LNN low sIgM groups (Median 98% vs 74%, p=0.02; Median 99% vs 74%, p=0.01).
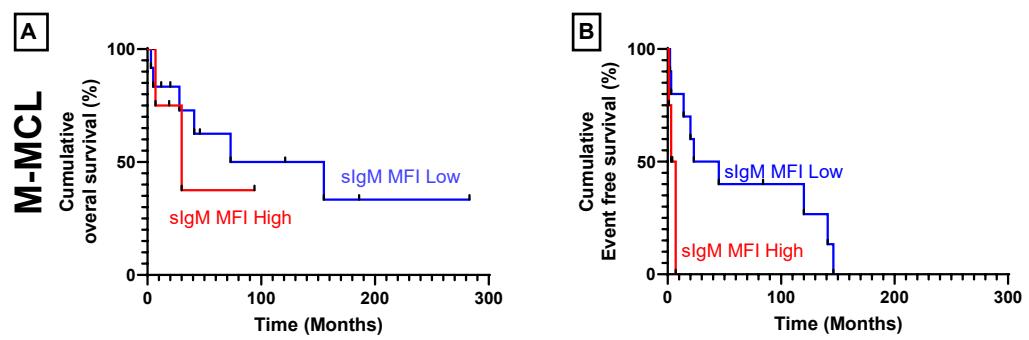
sIgM MFI	Median Months	95% CI of ratio	Events	P value
High	30	0.03906 to 0.9590	2/5	
Low	155	1.043 to 25.60	6/12	0.7020

sIgM MFI	Median Months	95% CI of ratio	Events	P value
High	5	0.04625 to 0.8099	3/4	
Low	34	1.235 to 21.62	9/10	0.0388*

Figure 6.5: Clinical significance of sIgM expression mutated MCL.

The cut off sIgM expression of 946 was used to delineate between high and low expression. This cut-off best represents the upper limit of CLL-like signalling. Overall survival (A) and event free survival (B) were investigated in the mutated subset of peripheral blood MCL cohort samples. Survival analysis was performed by Kaplan-Meier algorithm.

U-MCL, in contrast, behaved differently where cumulative OS and EFS was non-significantly longer in high surface IgM expressing MCL (Median OS 128months vs 39 months, p=0.09; Median EFS 36 vs 14 months, p=0.08).

There was no significant difference in clinical characteristics between the high/low sIgM expression in either U- or M-MCL in terms of age, biological sex, performance status at diagnosis, mIPI, or WHO subgroup clinical diagnosis. Two cases in the high sIgM M-MCL were classed as blastoid histology as opposed to one in the low sIgM M-MCL group. Peak and mean anti-IgM induced calcium signalling was statistically associated with high surface IgM expression in both U-MCL (p<0.02) and M-MCL (p=0.04) subgroups. Surface expression of CD38, CD23 and sIgD was equal across U-MCL subgroups. U-MCL with high sIgM expressed significantly more CD49d than those with low sIgM expression (U-MCL Median CD49d % High sIgM 37 vs Low 10, p<0.01*). M-MCL expressed CD38 and CD49d equally across sIgM high and low groups. There was a non-significant trend for higher sIgD in the high sIgM group and higher CD23 expression in the low sIgM M-MCL subgroup (**Table 6.2**).

Table 6.2: Clinical and phenotypic characteristics of U- and M-MCL by surface IgM expression.

	PB MCL (n=36)		U-MCL (n=17)			M-MCL (n=17)		
PB MCL cohort (n=36)	Frequency n (%)	Median (Range)	**High sIgM (n=11)**	**Low sIgM (n=6)**	**P (High vs Low)**	**High sIgM (n=5)**	**Low sIgM (n=12)**	**P (High vs Low)**
Clinical Characteristics								
Diagnostic Age (Years)	27 (90)	70 (45-93)	75	68	0.48	69	70	0.3626
Male	22 (75)		7 (63)	5 (83)	0.60	4 (80)	10 (83)	>0.99
High MIPI	15 (72)		3 (60)	3 (100)	0.46	1(50)	8 (89)	0.34
Leukaemic Non-nodal	13 (36)		2 (18)	0 (0)	0.51	3 (60)	11 (92)	0.19
Blastoid/Pleomorphic Histology	4 (11)		0	1 (17)	0.35	2 (40)	1 (8)	0.19
Performance Status (0-1)	33(92)		10 (91)	6 (100)	>0.99	4 (80)	11 (92)	0.33
Median OS (months)	36 (100)	73 (1-286)	**128**	**39**	0.09	**3**	**155**	0.31
Median EFS (Months)	32 (88)	23 (1-94)	**36**	**14**	0.08	**5**	**34**	0.03*
Phenotypic Characteristics								
CD38 %	31 (86)	98 (0-100)	**100**	**100**	0.95	**93**	**86**	0.97
CD49 %	30 (83)	38 (0-98)	**37**	**10**	<0.01*	**58**	**48**	0.50
CD23 %	30 (83)	6 (0 – 84)	**2**	**2**	0.8798	**9**	**38**	0.11
sIgD MFI	36 (100)	36 (1-252)	**29**	**29**	0.8467	**91**	**19**	0.08
Calcium mobilisation & Signalling capacity								
Peak sIgM (%)	23 (67)	88 (20-100)	**90**	**60**	0.02*	**99**	**74**	0.02*
Mean sIgM (%)	23 (67)	76 (5-100)	**84**	**32**	0.01*	**95**	**59**	0.04*

Clinical data extracted from electronic records from University Hospitals Southampton 2018 to January 2020. Statistics generated using GraphPad software. Non-parametric statistics were calculated using the Mann-Whitney test and categorical data using Fishers exact test   * denotes significance <0.05. (PB, peripheral blood. U-MCL, unmutated mantle cell lymphoma. M-MCL, mutated MCL. MIPI, Mantle cell International Prognostic Index, High risk>6.1. OS, Overall survival. EFS, event free survival. IGHV, immunoglobulin heavy chain variable. CD, cluster of differentiation. sIg, surface immunoglobulin. MFI, mean fluorescence intensity).

6.6 Summary of main findings

To further investigate the role of the BCR in MCL it was hypothesised that BCR characteristics would have clinically significant correlates, as seen in CLL. Clinical data was analysed in the context of radiological, genetic, imunophenotypic and BCR characteristics. Surface IgM expression was employed as the main determinant of BCR phenotype as it was well characterised and has shown clinical assocaitions in CLL.[340]

In this cohort, the experimental data found:

1. Untreated MCL was principally the LNN subtype with M-*IGHV* but there was no statistical association with BCR characteristics.
2. Chemo-immnotherapy was used in all of the treated MCL groups; 16% of cases received a BTK inhibitor as second line.
3. There was a non-significant trend to improved clinical outcome in patients with M-*IGHV* and a LNN presentation.
4. In patients with LNN- and M-MCL, low sIgM expression further sub defined a group with low signaling capacity, longer event free survival (significantly in M-*IGHV*) and trend to improved overall survival.

Overall, these findings suggest BCR charactersistcs may play a clinically significant role in MCL behaviour. However, the cohort size is underpowered and requires a larger, ideally prospective, study to investigate this relationship further.

6.7 Discussion

In this chapter a small, but well characterised, cohort of mantle cell lymphoma samples were assessed for clinical correlates. Due to limited sample size, caution should be taken when drawing firm conclusions. Additionally, there was an implicit bias amongst this cohort as they represented both patients fit enough to attend hospital clinics and a proportion of samples were from patients referred through to tertiary centres; there is a risk this may not represent typical MCL biology. Moreover, peripheral blood samples were investigated based upon availability and sample viability. Samples with higher cell number and good *in vitro* viability may have been more likely to produce good quality and reproducible results. This may have induced a further selection bias to a more indolent presentation with persisting lymphocytosis. Although the majority of patients received immuno-chemotherapy as first line therapy, variation in treatment types will have introduced differences in clinical outcomes through clonal selection and genetic damage resulting in unknown bias. Treatment ranged from splenectomy to high dose chemo-immunotherapy with stem cell transplantation consolidation through to BTK inhibition. Furthermore, time to first treatment, event free and overall survival would be heavily impacted by physician attitude towards active monitoring, availability of rituximab and, more recently, novel therapies and trials.

In this cohort, the median event free survival was 23 months and median OS was 73 months (5 years OS 70%). In comparison, in a slightly older UK real world population survey, the 5 year OS was 30% and median survival was 29 months, highlighting the indolence of this cohort.[456] The median OS for both M and U-MCL were 6 years (72 and 73 months respectively) with 5 year OS rates of 62% (95% CI, 31–63) and 63% (95% CI, 37–64) respectively. This compares to a large international group dataset where M-MCL (mutated IGHV defined as less than 97% homology to germline) had a 5-year OS of 59% (95% CI, 41–77) and U-MCL a 5-year OS of 40% (95% CI, 28–52).[250] This may suggest a more indolent U-MCL group in this cohort, which will affect interpretation. In contrast to *IGHV* mutation status, WHO MCL subtype has been more regularly associated with improved clinical outcomes.[168 171-173] A comparative dataset of 80 unselected MCL patients demonstrated a median OS of 79 and 30 months in LNN and nodal MCL respectively.[168] In agreement, the data from this study, though not statistically significant, strongly supported improved

outcome with median OS of 155 months in LNN-MCL and 72 months in conventional, nodal MCL. The longer OS for both subtypes again highlights the indolence of this cohort. However, a second published dataset, of 59 MCL European patients from Royo et al. 2012, at least partially mirrors this cohort with a 5 year OS of 86% in low risk (defined by gene expression profile) LNN-MCL (estimated median OS of 140 months) and 5 year OS of 42% in conventional nodal MCL (estimated median OS 36months).[171]

In Chapter 5, a CLL like subgroup of MCL was identified with low anti-IgM signalling and surface IgM expression with *in vitro* evidence of ongoing BCR engagement. It was hypothesised that this group may, like CLL, have a more indolent prognosis. However examining the clinical characteristics in this chapter, this functionally defined group showed no significant clinical correlation when stratified for signalling ability for TTFT, EFS or OS. Nevertheless, the sample size was very small, heterogeneous and genetic factors, such as *TP53* disruption, were not characterised fully in this study making it difficult to draw firm conclusions.

To increase the limited sample size, and due to the absence of complete signalling characterisation, the correlation between signalling capacity and surface IgM expression (r=0.77, p<0.01) was used to extrapolate a sIgM expression cut off. This created a high/low sIgM category to discriminate between proposed 'CLL' and 'non-CLL' like signalling capacity in MCL. Employing this cut off revealed M-MCL with low 'CLL-like' sIgM expression to be associated with longer EFS and OS though without statistical significance in the latter. Though not reaching statistical significance, LNN MCL also mirrored the trend towards better outcomes when divided by sIgM expression. As previously stated, there are reservations regarding the cohort size and no statistical corrections (i.e. false discovery rate correction) were performed to account for multiple testing during subgroup analysis, so there should be caution in drawing conclusions from this result. The high sIgM M-MCL cohort had a higher proportion of blastoid MCL and were more frequently nodal in presentation in comparison to their low sIgM counterparts. Despite these caveats, this interesting finding in M-MCL mirrors the relationship in CLL where the anergic phenotype, characterised by low sIgM and signalling, associates with better prognosis.[340]

In contrast, U- and nodal MCL with high sIgM expression revealed a non-significant association with longer OS and EFS. This unexpected trend may result from chance, due to the small sample size or, if confirmed, it may be that these MCL cases are less influenced by BCR engagement. These cases may have lost, or never have been under, down modulatory pressure, and may be driven by BCR independent or genetic factors.

Indeed, using gene expression profiling, a subset of leukaemic MCL cases have been demonstrated to have germline (*RNF31* and *SHARPIN*, both components of the linear ubiquitin chain assembly complex (LUBAC)) or somatic mutations e.g. *RELA*, resulting in active downstream BCR and NF-κB signalling independent of their microenvironment.[240][457] It could be hypothesised that a complex interplay of BCR engagement and constitutive signalling drive survival and/or proliferation in U-MCL. Therefore, the cases of U-MCL, with relatively low down modulated sIgM, still under putative (super) antigenic influence, are thus driven to proliferation by both BCR engagement and genetic damage producing a more aggressive phenotype. In contrast, cases of U-MCL, with higher sIgM, may have reduced BCR drive from putative (super) antigenic influence and have a less proliferative phenotype driven principally by genetic factors.

Four samples (11%) were taken from patients who had never required treatment; three of which were LNN with a M-*IGHV.* The two LNN M-MCL cases with low sIgM are again consistent with a CLL like anergic phenotype resulting in indolence. Two samples with high surface IgM expression and maximal signalling capacity reveal that, perhaps like U-MCL with high sIgM, some cases are effectively BCR independent having lost, or escaped from, tissue based antigenic influence. These high sIgM untreated cases could therefore, represent the weakest driven MCL cases; minimal BCR engagement driven signalling and weak gentic driven factors. Unfortunately, genetic factors were not assessed.

Biological indicators identifying indolence are complex; a large British Columbia registry data of patients on watch and wait included cases that were *TP53* disrupted (13%) and had a high Ki67% (14%).[282] Interestingly, one of the four untreated samples in the cohort (M-MCL 12 with low sIgM) harboured such a disruption and was untreated at 45 months follow

up. Such cases support the existence of a complex combination of factors that lead to disease progression.

Chapter 7 Final Discussion

7.1 Background

This thesis has focused on MCL BCR biology. MCL is a disease which had historically very poor clinical outcomes and was often treated aggressively.[270] As a relatively rare mature B-cell malignancy, outcomes have greatly improved with recognition of its heterogeneity and the advent of anti CD20 antibodies and small molecule inhibitors of the BCR.   As a key example, the BTK inhibitor ibrutinib, which targets the BCR signalling pathway, is the standard of care for relapsed disease and is available in the front line setting during the COVID-19 pandemic.[155 252 458 459]   Despite these improvements it is still difficult to predict clinical behaviour and treatment is not tailored to the underlying biology of each patient. Clinical improvements, through understanding of the BCR, have led to improved management in other mature B-cell malignancies, principally CLL. This has been driven through the study of immunogenetic, phenotypic and functional characterisation of the CLL BCR and potentially offers a model for MCL.[145 329 340 351 355]

Although sharing many similarities with CLL, the MCL BCR is much less well understood. Like CLL, MCL is a CD5$^+$ mature B-cell malignancy in possession of a functioning BCR but is defined by a t(11;14) translocation resulting in cyclin overexpression. There is immunogenetic bias within the BCR *IGHV* repertoire and, like CLL, the presence of CDR stereotypy.[388] There are two clinically defined subsets with immunogenetic correlates.[158 168 460] The more common, conventional, nodal form of MCL arises from a pre-germinal cell with unmutated *IGHV* and is generally SOX11 positive. The less common, leukaemic, non-nodal form arises from a post germinal centre cell with generally mutated *IGHV* and has a more favourable clinical outcome.[250] Although there is some evidence to support the importance of *IGHV* mutational status in MCL natural history, there is conflicting evidence about its predictive role and it is not routinely tested or used in prognostic indexes.[250 450 451 461]

In contrast, CLL is a disease where *IGHV* mutation status is a key prognostic factor.[328] Mutation status associates with sIgM expression identifying a high and low BCR signalling group that predicts clinical behaviour.[340] CLL surface IgM is generally low but variable having been down modulated through ongoing BCR engagement as a consequence of the interaction with tissue based putative antigen. M-CLL, expressing lower sIgM with better outcomes than U-CLL, are generally regarded as anergised with low signalling capacity and a non-proliferative phenotype.[329][340]

It was not known whether MCL BCR expression and signalling varied by *IGHV* mutation status or clinical subgroup, and whether this might explain some differences in outcome. Moreover, it was not known whether BCR engagement played a prominent role in explaining MCL natural history like in CLL.[340][460] As MCL has generally high sIgM expression it seemed unlikely that it would share the same characteristics or natural history as CLL. However, given the significant heterogeneity in clinical outcome, where a small number of cases do not require treatment for many years, it was possible that a proportion of MCL would share features with CLL.[462] Given this background, the primary hypothesis of the thesis stated that a subgroup of MCL share BCR characteristics with CLL, revealing the influence of BCR engagement on MCL behaviour.

7.2 Key Findings

Within the limitations of a small study, this thesis has identified several findings. The first key finding is:

- The demonstration of a significant association between sIgM expression, *IGHV* mutation status and MCL WHO clinical subtype.

Higher sIgM expression was statistically associated with U-*IGHV* in non-blastoid leukaemic MCL and in its clinical correlate conventional, nodal, MCL. There was no difference in sIgD expression by *IGHV* status or clinical subgroup. Mirroring CLL, sIgM expression varied by cell of origin suggesting BCR-environmental interactions occur through the M isoform. MCL sIgM expression was variable as seen in other studies.[275] Surface IgM was expressed brightly like other mature B cell lymphomas, such as diffuse large B cell lymphomas (DLBCL) and follicular (FL) and weakly, like CLL.[275] As was already known, signalling capacity positively correlated with sIgM expression. Analysis and comparison with the CLL cohort revealed the second key finding of this thesis:

- The identification of a group of MCL with weak, CLL like, signalling and low sIgM expression.

This sub-group was compared to MCL cases with higher signalling capacity and sIgM expression, to asses for evidence of phenotypic and functional differences that may support ongoing receptor engagement. The low sIgM, low signalling group was identified in LNN MCL with M-IGHV and some conventional U-MCL particularly encoding *IGHV*3-21. 18 cases were classified as low sIgM expressers; 11 of which were LNN and both U-*IGHV*3-21s were present in this group. These MCL cases possessed functional BCR characteristics well described in CLL; *in vitro* recovery of sIgM, but not D, expression, evidence of immature glycosylation residues and identification of high basal phosphorylation (high pSYK). Comparison between these two groups thus provided the third key finding:

- The presence of a BCR engaged functional phenotype.

Taken together these findings support the overarching hypothesis – a subgroup in MCL have evidence of ongoing engagement of the BCR (**Figure 7.1**). A significant improvement in EFS was identified in the treated M-MCL group with low sIgM expression. Improved outcomes were associated with an anergic, lower signalling phenotype suggesting clinical relevance of the BCR in at least the LNN, M-MCL subgroup.

'Antigen'   Surface IgM
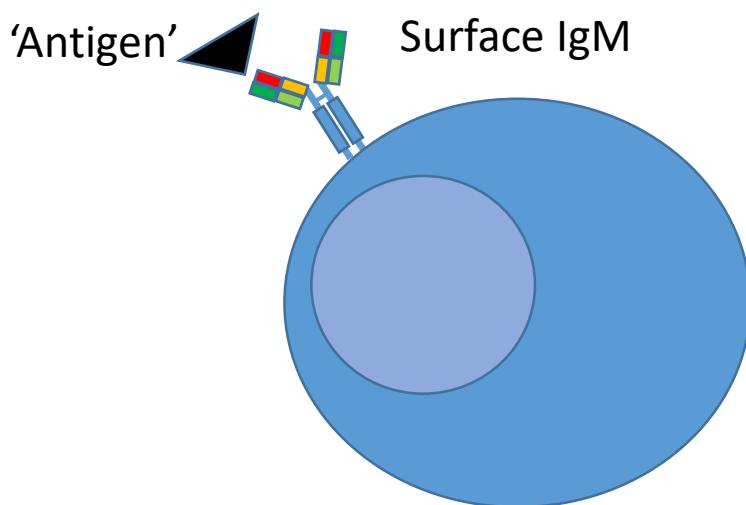
Chronic BCR engagement leading to:

Low, down-modulated, sIgM expression
Basal signalling
Low capacity to respond to re-stimulation of sIgM *in vitro*
Activated sIgM glycosylation pattern

Associated with:

Mutated IGHV and Leukemic, non-nodal disease
Longer event-free survival

Unmutated IGHV (especially IGHV3-21)

Figure 7.1: A proposed model of BCR activation in MCL low signalling subgroups.

7.3 BCR engagement – antigenic drive?

Although the data provides evidence for engagement in a subgroup of MCL it is not known what that the nature of the driver may be. Given the function of normal BCRs and documented similarities with CLL, it is possible that antigen is the BCR driver.   Antigen plays a frequent and significant role across the spectrum of mature B cell lymphomas through the BCR.[415] However, direct evidence in MCL for candidate antigen is generally weak with no described exogenous or endogenous (auto) antigen drivers.

Nevertheless, it is known that a third of MCL derived immunoglobulins are able to bind auto antigen expressed on HEp-2 cells.[395] However, this is similar to healthy naïve (20%) and immature B-lymphocytes (40%) and may not be significant.[42] Mature B-cell malignancies with BCRs encoding *IGHV*4-34, a V gene with intrinsic auto-reactivity to I/i glycoproteins, are consistently over represented in MCL. BCRs encoding *IGHV*4-34 are increased in disease driven by autoantigens; ABC DLBCLs (30%), primary central nervous system lymphoma (55%) and, to a lesser extent, CLL (8.9%).[372 434] *IGHV*4-34 is overrepresented in MCL at 14.6% in one large study suggesting auto-antigenic drive may play a role.[388] Overall, 21% of cases encoded *IGHV*4-34 in this MCL cohort equally represented in U and M-MCL. However, no MCL cases expressing *IGHV*4-34 were present in the BCR engaged low signalling group. Evidence for the nature of auto-antigenic drive in MCL appears theoretical at present.

In contrast to MCL, there is strong evidence for antigenic drivers in CLL. Putative autoantigens include myosin heavy chain IIa, vimentin and the by-products of protein oxidation, 'neoantigens'.[146 342-344] Endogenous antigens may engage with CLL BCRs with low affinity and this auto-, polyspecific, antigen reactivity may be more common in U-CLL.[396] However, specificity may still be retained in CLL. Pathogen derived antigens from cytomegalovirus react in U-CLL with *IGHV*1-69 encoded BCRs and fungal β-(1,6)-glucan react in a proportion of M-CLL with *IGHV*3-7/*IGKV*2-24-encoded BCRs. [440463]

MCL has generally high BCR sIgM expression more closely resembling the germinal centre derived diseases DLBCL and FL where the mechanism of BCR drive is better understood.

Although FL expresses sIgM relatively strongly like MCL, its BCR appears to be under quite different selection pressures. FL remains within the germinal centre, protected by upregulated BCL2 expression and is dependent upon the microenvironment.[362] Its selection and survival is driven through acquired N-glycosylation sites in the immunoglobulin variable regions.[363] Lectins, found on tumour-associated macrophages, have been shown to generate intracellular Ca^{2+} flux in FL, promoting survival and proliferation. In contrast to more typical anti-Ig ligation, endocytosis does not occur; the Ig is thus not down modulated and the anergic phenotype characteristic of CLL is not seen. [364] [42] MCL appears, therefore, to differ from the FL BCR as there is evidence of down modulation and, significantly, no evidence of acquired N-glycosylation sites.[427]

DLBCL is a highly proliferative lymphoma composed of multiple distinct molecular subtypes with differing clinical outcomes.[464] The ABC DLBCL subtype is an aggressive disease with chronic BCR signalling and NF-κB dependence. Due to aberrant Ig class switch recombination, the ABC DLBCL is predominantly sIgM positive with weak sIgD co-expression.[369] Like CLL, BCR signalling in this subset arises, at least in part, from autoantigen activation; ABC cell lines auto react with their own V region glycoproteins, self-antigens in apoptotic debris and their own V region idiotypic epitope.[114] However, ABC DLBCL retain signalling capacity because recurrent mutations in the CD79A and CD79B ITAM regions interfere with receptor endocytosis, preventing sIgM down-modulation and anergy. Moreover, activating mutations are seen in ABC DLBCL such as CARD11-BCL10-MALT1 (CBM) complex (~10%), the linear ubiquitin chain assembly complex (LUBAC) and the Toll like receptor downstream target MYD88 resulting in sustained NF-κB signalling.[435] [436] [465] [466] MCL shares some clinical and genetic features with DLBCL (*MLL* and *CARD11* mutations) but MCL are not known to have aberrant CD79A/B function and thus down modulate their sIgM.[467]

In the absence of clear auto- or pathogenic antigen drive, there is some indirect evidence of super antigen interaction in MCL development. Super antigens (**1.12 Super antigens**) are able to bind to framework regions (FRs) instead of the Ig complementarity determining regions (CDRs). The bacterium *Staphylococcus aureus* is well studied and produces Staphylococal protein A (SpA). SpA binds strongly to the FR of the *IGHV*3 family IgG.[468] *In vitro* SpA stimulation of human B-cells activates *IGHV*3 expressing cells causing proliferation

and an *IGHV*3 biased repetoire.[469] Furthermore, SpA has been shown to activate MCL cell line BCRs resulting in calcium influx.[397] 16 (47%) of the thirty-four cases in this cohort encoded *IGHV*3 in the studied cohort including four (50%) of the eight BCR engaged cases.

7.4 The site of BCR engagement.

If the nature of putative antigen resulting in BCR engagement in MCL remains unknown, the anatomical site of exposure may be clearer. Mimicking the process in CLL, exposure to exogenous or endogenous antigen is likely to occur in the lymphoid tissues.[240 470] This thesis has demonstrated significantly lower sIgM expression and signalling capacity in the lymph node compartment compared to peripheral blood consistent with tissue based BCR down modulation. There were too few splenic samples to make strong inferences regarding the role of the splenic compartment. Saba et al. have previously demonstrated higher BCR and NF-κB signalling in the lymph nodes of paired (peripheral blood) samples. [240] This was accompanied by proliferative gene expression profiles further supporting BCR engagement in the lymph node compartment. In another study, the importance of the lymph node microenvironment was demonstrated by the greater presence of activation-induced cytidine deaminase (AID) transcripts in the lymph node of paired node and peripheral blood MCL samples.[471] The data in this thesis has also shown lower sIgM expression in paired samples further supporting tissue based down modulation secondary to BCR engagement. Moreover, signalling capacity was significantly lower in lymph node tissue samples. Together, these findings support tissue-based stimulation of some MCL BCRs.

This partially mirrors the process in CLL, though MCL does not appear to possess the proliferation centres that are the presumed site of putative antigenic stimulation in CLL.[356 357 472 473] The reason for this difference is unknown but the variation in adhesion molecules may be important. It is possible that the differing outcomes seen in LNN-MCL and conventional nodal MCL may at least in part be due to inherent compartmental differences occurring through the BCR.[168 170-173] Furthermore, this may explain the efficacy of BCR inhibition where the inhibitor induced lymphocytosis releases the MCL BCR from engagement and proliferation.

7.5 BCR signalling – a balance between anergy and proliferation?

Since BCR signalling can result in many different outcomes; proliferation, migration, survival, apoptosis or differentiation, it is interesting to speculate on the consequences of this BCR signal in MCL.[474]

The subset of MCL with evidence of ongoing BCR engagement were identified in LNN MCL with M-IGHV and in conventional U-MCL encoding (though not exclusively) IGHV3-21. There was no statistically significant clinical association with indolence in this group but there were few cases and the genetic features of these cases were not assessed making overall interpretation difficult. However, both LNN-MCL and IGHV3-21 have been shown to be more indolent in other studies and thus may suggest the presence of a weak tumour suppressor function as opposed to proliferation in the nodal forms.[426 475] In normal B-cells chronic engagement results in a state of anergy.[144 146] This appears to be the outcome for most CLL cells too; differential signalling responses leading to a balance between proliferation and indolence.[85] Signalling was generally much higher than CLL. The data does not, therefore, generally support the presence of anergy *per se* in MCL but a spectrum of influence on the BCR where only a few cases may be classed as anergised. In this cohort, the LNN M-MCL cases had the most anergic phenotype: low signalling capacity with an indolent phenotype. CD23 expression was highest in this group and CD23's known inhibitory effect on BCR clustering may contribute to this effect highlighting the importance of co-regulators.[130]

Anergy, and the indolent disease associated with it, may be less prominent in MCL due to known intrinsic genetic changes, such as cyclin overexpression and SOX11 status, and also unknown factors affecting the BCR. This may include the propensity for receptor endocytosis and microenvironmental, extrinsic, factors. CLL cells retain functioning antigen-receptor endocytosis allowing sIgM down modulation and anergy whilst, in contrast, a proportion of ABC DLBCL possess CD79A/B mutations preventing efficient endocytosis.[114 476] Alternatively, the MCL BCR may not become anergised due to the putative antigens themselves, perhaps due to transience or binding affinity weakness. The

relative absence of anergy and proliferation centres in MCL may reflect variability in cellular adhesion molecules or differences in the microenvironmental stromal cells.

It is likely that the biological outcomes of BCR engagement are context dependent and may be distinct compared to CLL. CLL signalling outcomes are affected by cell of origin and genetic alterations where trisomy 12 and del(17p) increase anti-IgM induced calcium signalling.[340] MCL are characterised by the overexpression of cyclins.[220] The effect of cyclinD1 overexpression on BCR signalling capacity is not directly known but it does induce global transcriptional dysregulation leading to genetic instability.[477] Intriguingly, in SOX11 transgenic mice models, the presence of SOX11 has been shown to increase BCR signalling in response to anti-IgM and may induce more proliferation.[478] Not all of the cases in this cohort had SOX11 assessed and it is not known how SOX11 status affects BCR function in a patient population.

7.6 BCR engagement independence

In the MCL cohort studied in this thesis, only eight of thirty-six (22%) were designated as low signallers with clear hallmarks of BCR engagement. Therefore, the majority of MCL in this cohort do not appear to be BCR engaged or under the clear influence of putative antigen drive. This may suggest that the BCR in these cases have never been under an environmental influence through the BCR and the disease is BCR independent. Alternatively, and perhaps more likely, an initial selective BCR driving event has been lost or become weak, and further acquired genetic change has led to disease progression. Supporting this, mutations in the CARD11 scaffold protein that links BCR signalling to mutation to NF-κB signalling, have been identified in 5.5% of cases both at initial presentation and at relapse. [414] Some of these mutations are identical to those found in DLBCL conferring BCR independence and resistance to upstream inhibitors such as ibrutinib.

Antigen independent intrinsic lymphoma drive is seen in germinal centre (GCB) diseases like DLBCL and Burkitt lymphoma (BL).[367 382 383] These two disease have co-opted tonic signalling through the PI3 kinase pathway. GCB DLBCL are dependent upon SYK mediated

induction of PI3K whilst another significant proportion have inactivated PTEN, an inhibitory regulator of PI3K.[479] Signalling magnitude through PI3K dependent process is directly proportional to BCR density and requires CD79A phosphorylation.[376] BL, defined by t(8;14) *MYC* overexpression, are driven by aberrant PI3K tonic signalling through TCF3 dependent pathways and, in a third of patients, cyclin D3 driven cell cycle progression.[383] MCL are genetically dysregulated and a number of studies have identified aberrantly activated PI3K/AKT pathways associated with PTEN inactivation.[398][399] This occurred frequently, though not always, in advanced, blastoid disease suggesting a route to BCR signalling independence in MCL.

Although biologically plausible that more advanced disease has acquired more genetic damage and subsequent antigen independence reflected in high sIgM expression, this cohort did not universally show a relationship between high sIgM and poor outcome. Indeed, in U-MCL high sIgM was non-significantly associated with improved outcomes. Given high Ig expression and the proven absence of functional BCR engaged characteristics, it is unlikely that these BCRs are under the influence of putative antigen. In the absence of classical BCR engagement and down modulation, these cases may persist through a balance of weak tonic BCR signal, non-BCR signalling and survival enhancing genetic damage such as anti-apoptotic mutations.[480] Further disease progression may then occur through either the accumulation of further genetic damage or, theoretically, by the reoccurrence of the original driving antigen. Theoretically, this may result in a spectrum of disease where proliferative BCR engagement is lost and a 'reversed' relationship between sIgM expression and outcome is seen.

The sIg is the key molecule through which malignant B-cells exploit their microenvironment to gain selective advantage.[115] Overall, therefore, it appears there is a spectrum of influence by putative antigen on the MCL BCR that may influence clinical behaviour; CLL like relative anergy through to antigen driven proliferation and finally independence from antigen. Like other mature B-cell malignancies, the clinical significance of this effect may be affected by the cell of origin, microenvironment interactions, co-receptors and epigenetic/genetic damage. Despite this complexity, the BCR's continuing importance is underlined by clinical response to the BCR inhibitors *in vivo*.[156][302]

7.7 Future studies

This thesis has addressed the immunophenotypic, functional and clinical characteristics of the MCL BCR in order to better understand its natural history. MCL is a heterogeneous and rare disease and this is reflected in the relatively low numbers of cases analysed. Therefore, to improve the quality of this study it clearly necessitates an increase in sample size with good quality signalling and genetic characterisation, ideally pre-treatment acquired in a prospective manner. There is a strong relationship between genetic damage and sIgM expression in CLL and correlates should be looked for between these factors. In particular, sequential changes following relapse and BCR inhibitor therapy may reveal further insights into BCR biology.

A major question that arises from this study is the role of cyclin D1 and SOX11 on BCR signalling. CRISPR modified cell lines could be used to investigate the role of these two molecules on BCR signalling strength and outcomes and identify how BCR inhibitors affect this pathway. RNA sequencing of unstimulated and α-IgM stimulated cells would identify translational outcomes of MCL signalling revealing the pathways affecting proliferation and anergy. Ideally this could be linked to a well characterised patient cohort where it could be seen if SOX11 or cyclin D1 expressing tumour cells have a greater activated signalling pathway.

In CLL, cell adhesion markers are prognostic.[440][481] BCR inhibition with ibrutinib results in cell adhesion disruption leading to lymphocytosis.[482] CD49D and the surface marker CD23 are associated with the LNN form and indolence.[130][199][439] CD23 affects the BCR surface structure and negatively regulates signalling.[130] The relationship between CD23 expression, BCR signalling and sIgM expression should be investigated further by knockout models. Larger studies assessing cell adhesion markers and their clinical correlates may help in developing more accurate prognostic scores further stratifying high and low risk MCL.

To build on the findings of this study, clinical trial data could be used to assess the effects of BCR inhibition when stratified by BCR phenotype and genetic aberrations. The hypothesis to be tested would be: BCR inhibition will lead to better PFS in M-MCL with low

signalling MCL, with limited genetic aberrations, than high signalling U-MCL genetically complex MCL. This strategy may identify a phenotypically and genetically stratified approach to therapy with reduced toxicity and improved PFS.

Chapter 8 Appendix

Table 8.1: Equipment used for cell counting and cell culturing.

Item	Product details	
RPMI 1640 w/o L-Glutamine, w HEPES	GE Healthcare	E15-039
Penicillin/ Streptomycin (100x)	Sigma	P4333-100ml
Fetal Bovine Serum	PAA	A15-102
Trypan Blue	Sigma	T8154
10 x PBS	VWR	437117K
Sodium Azide	Fluka BioChemika71289	
0.5M EDTA pH8	Fisher scientific	BP2482-500
Bovine Serum Albumin Fraction V	PAA	K41-001
Lymphoprep reagent	Axis-Shield/Alere (1114547)	
Glutamine	Sigma-Aldrich (56-85-9)	
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (67-68-5)	
Liquid Nitrogen	-	
Waterbath	Clifton	
Falcon Tubes	Sarstedt	
Pasteur pipettes	Sarstedt	
Laminar Flow Class II Cabinet	Hera Safe Fisher Scientific	
Heraeus Multifuge 3SR+ centrifuge	Thermo Scientific	
Incubator Hera Cell	Fisher Scientific	
P1000 Pipetman	Gilson	
P200 Pipetman	Gilson	
P20 Pipetman	Gilson	

Table 8.2: Equipment and reagents for flow cytometry and calcium signalling

Item of equipment	Supplier	Catalogue Number
FACSCalibur Flow cytometer	Becton Dickinson	
BD CANTOII FACS	BD Bioscience	
Flow-Jo Flow Cytometry analysis software	TreeStar Inc	
Round bottomed Flow cytometry (FACS) tubes	Falcon	352052
Pluronic Acid F-127	Sigma Aldrich	9003-11-6
Fluo 3-dye**	ThermoFisher	F1242

*Stored at 4°C; **Stored at -20°C Antibodies

Table 8.3: Antibodies used for PhosphoFlow phenotyping and barcoding

Antibody	Volume per 100 µl Tube	Company Catalogue Number
BD Phosflow™ Alexa Fluor® 488 Mouse anti-Akt (pS473) *	20µl	BD Biosciences 560404
Anti-Btk (pY223)/Itk (pY180) Clone N35-86 (RUO) *	5µl	BD Biosciences 564847
Alexa Fluor® 488 Mouse Anti-ERK1/2 (pT202/pY204) Clone 20A*	20µl	BD Biosciences 612592
Phospho-SYK (Tyr525/526) (C87C1) Rabbit mAb (Alexa Fluor® 488 Conjugate) *	2µl	Cell Signalling Technologies 4349S
Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP Rabbit mAb (Alexa Fluor® 488 Conjugate) *	2µl	Cell Signalling Technologies 4803S
BCL2*	20µl	Thermo Fisher Scientific (MHBCL04)
BD Phosflow™ PerCP-Cy™5.5 Mouse Anti-Human CD20*	20µl	BD Biosciences 558021
APC anti-human CD3 Antibody*	5µl	Biolegend 300412
PE-Cy7 anti-human CD5 Antibody	5µl	

*Stored at 4°C; **Stored at -20°C; Peridinin chlorophyll protein-Cy5.5 (PerCP/Cy5.5);

Allophycocyanin (APC); R-Phycoerythrin (PE); Fluorescein isothiocynate (FITC).

Table 8.4: Surface Phenotype Antibodies used in Phosflow phenotyping

Antibody	Volume	Company Catalogue Number
BD Phosflow™ PerCP-Cy™5.5 Mouse Anti-Human CD20	20µl	BD Biosciences 558021*
APC anti-human CD3 Antibody	5µl	Biolegend 300412*
PE-Cy7 anti-human CD5 Antibody	5µl	
Pacific Blue Dye		Thermo Fisher Scientific (P10163) **
Pacific Orange Dye		Thermo Fisher Scientific (P30253) **

*Stored at 4°C; **Stored at -20°C; Peridinin chlorophyll protein-Cy5.5 (PerCP/Cy5.5); Allophycocyanin (APC); R-Phycoerythrin (PE); Fluorescein isothiocynate (FITC).

Table 8.5: Wessex genetic laboratory FISH probes

Probes	Target
CytoCell – *IGH/CCND1*	t(11;14)
(Vysis) LSI13 and LSID13S319	del13q14 (del(13q))
CEP12	chromosome 12 aneuploidy
LSIp53	del17p13 (del(17p))
LSIATM	del11q22-q23 (del(11q))

.

Table 8.6: Antibodies used for immunoblotting

Antibody	Source	Molecular Weight (kDa)	Dilution	Company
Phospho-AKT (Ser473) (D9E) XP® Antibody**	Rabbit	60	Dilution Factor: 1:1000; Dilution media: 3% Milk-TBS-T	Cell Signalling (#4060S)
AKT Antibody**	Rabbit	60	Dilution Factor: 1:1000; Dilution media: 3% Milk-TBS-T	Cell Signalling (#9272S)
Phospho-SYK (Tyr525/526) (C87C1) Antibody**	Rabbit	72	Dilution Factor: 1:1000; Dilution media: 3% Milk-TBS-T	Cell Signalling (#2710S)
SYK Antibody**	Rabbit	72	Dilution Factor: 1:1000; Dilution media: 3% Milk-TBS-T	Cell Signalling (#2712S)
Phospho-BTK (Tyr223) Antibody**	Rabbit	77	Dilution Factor: 1:1000; Dilution media: 3% Milk-TBS-T	Cell Signalling (#5082)
BTK (D3H5) Antibody**	Rabbit	77	Dilution Factor: 1:1000; Dilution media: 3% Milk-TBS-T	Cell Signalling (#8547)
Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) Antibody**	Rabbit	42,44	Dilution Factor: 1:1000; Dilution media: 3% Milk-TBS-T	Cell Signalling (#9101S)
p44/42 MAPK (ERK1/2) Antibody**	Rabbit	42,44	Dilution Factor: 1:1000; Dilution media: 3% Milk-TBS-T	Cell Signalling (#9102S)

Polyclonal Goat AntiMouse Immunoglobulins/HRP*	Goat	-	Dilution Factor: 1:2000; Dilution media: 3% Milk-TBS-T	Dako (#P0447)
Polyclonal Goat AntiRabbit Immunoglobulins/HRP*	Goat	-	Dilution Factor: 1:2000; Dilution media: 3% Milk-TBS-T	Dako(#P0048)
GAPDH Antibody (6C5) **	Mouse	37	Dilution Factor: 1:60000; Dilution media: 3% Milk-TBS-T	Applies Biosystem (AM4300)
Anti-µ**	Rabbit	84	Dilution Factor: 1:2000; Dilution media: 3% Milk-TBS-T	Jackson Immuno Research

*Stored at 4°C; **Stored at -20°C

Table 8.7: Reagents for Western Blotting and Glycosylation

Procedure	Reagent	Product Details
Protein Extraction	RIPA buffer	0.75M NaCl, 5%NP40, 2.5%DOC, 0.5%SDS, 0.25MTris pH 8.0, in 100ml deionised H_2O
	Protease inhibitor**	Sigma-Aldrich (#P8340)
	Phosphatase Inhibitor Cocktail 2* and 3*	Sigma-Aldrich (#P5726 and #P0044)
Quantification	Pierce BCA Protein Assay Kit	Thermo Scientific (#23227)
Gel Electrophoresis	Ammonium Persulfate (APS)	Melford (#A1512)
	Sodium dodecyl sulfate (SDS)	Fisher Scientific (#10090490)
	Acrylamide/Bis-Acrylamide,30% solution*	Sigma-Aldrich(#A3574)
	TEMED (*N,N,N',N'-Tetramethylethylenediamine)	Sigma-Aldrich (#T9281)
	Red Loading Buffer (Loading dye and DTT**)	Cell Signalling(#7723S)
	PageRuler PlusProtein Ladder**	Thermo Scientific (#26619)
	Running Buffer (10x)	250mM Tris-base, 1.9M Glycine, 35mM SDS
	Transfer Buffer	200ml running buffer (10x), 500ml 100% ethanol , 1300ml deionised H_2O
	Nitrocelulose	Amersham (#10600003)
	2 gel Tetra and Blotting Module	Bio Rad (1660828EDU)
Antibody incubation	Bovine Serum Albumine (BSA)*	Sigma-Aldrich (#A7906)
	Detection Reagent 1 and 2*	Thermo Scientific (#1859701 and #1859698)
	Tween-20 (Polysorbate)	Molecular biology grade (VW R Chemicals;#437032Q)
	Tris-Buffered Saline – Tween-20 (TBS-T	20mM Tris-base, 137mM NaCl, diluted in 1 litre deionosed H2O plus 1ml Tween
Glycosylation	Pierce Cell Surface Protein Isolation Kit	Thermo Scientific (#89881)

*Stored at 4°C; **Stored at -20°C

Chapter 9 Reference List

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