MECHANISM EMPLOYED BY GERMINAL CENTRE B CELLS FOR PROMOTING AFFINITY MATURATION

Isabelle Stewart

Wolfson College

University of Oxford

MRC Weatherall Institute of Molecular Medicine

Supervised by Oliver Bannard, and Richard Cornall









Submitted for the degree of Doctor of Philosophy

Trinity Term 2019

ACKNOWLEDGMENTS

It has been an immense privilege to be supervised by Oliver Bannard. I am sincerely grateful to have been led by someone as creative and insightful. Oliver is energetic and indefatigable, and it has been a great joy to work with him. These past four years have been the most intellectually transformative of my life, and this is largely attributable to Oliver's immense generosity with his time. I really cannot thank him enough.

I have also received fantastic help from my co-supervisor Richard Cornall. Additionally, I am very grateful for the insights from my thesis committee, Simon Davis and Georg Holländer; and my transfer committee, Michael Dustin and Andrew McMichael. I was so fortunate for all of these people to take time out of their very busy schedules to give fresh insights and perspectives into my work.

I also wish to thank past members of the lab. I am especially grateful to Daniel Radkle, who was immeasurably helpful to me with basically anything related to a computer. Bethan Phillips good was incredibly helpful with ELISAs and single cell cultures. Elise French was a fantastic addition to the lab and always had high spirits and humour. I am very grateful to Ziqi Long, who has been very generous with allowing me jump in on some of her exciting experiments.

I am thankful to many people who run fantastic services at the Weatherall Institute of Molecule Medicine. In particular, I have relied upon the staff at the flow cytometry facility, and am especially gratefully to Craig Waugh, and Sally Anne Clarke. Furthermore, Tom Rostron from the WIMM sequencing facility was immensely helpful to me. I am also incredibly Neil Ashley, who does an impeccable job running the WIMM single cell facility. The staff at the John Radcliff Biomedical services, who were immeasurably patient with me and my mouse-anxiety.

I am very grateful to our neighbouring lab, the Drakesmith's. In particular my brother in arms Joseph Frost, to whom I am always so mean, and who is never mean to me.

I am very grateful to those friends and family who performed proof reading for me, Ursula Robinson-Shaw, Robert Freeman, Rebecca Stewart, and Ange Wither.

And of course, my parents, Deborah and Gordon Stewart, two pillars of strength, generosity, good nature, and wisdom, to whom I owe everything.

MECHANISM EMPLOYED BY GERMINAL CENTRE B CELLS FOR PROMOTING AFFINITY MATURATION

Isabelle Stewart

Doctor of Philosophy

Trinity Term, 2019

The adaptive immune system has the extraordinary ability to develop bespoke antibodies against almost any invading agent to which the host may be exposed. These antibodies are produced through affinity maturation, a dynamic process of iterative diversification and refinement. This process occurs in germinal centres (GCs), microanatomic loci physically divided into dark zones (DZs), in which the immunoglobulins are diversified though somatic hypermutation (SHM), and light zone (LZs), in which cells are selected such that the population can be refined. Accurate selection requires that cells fully replace surface B cell receptors (BCRs) following SHM, but whether this happens before LZ entry is not clear. We found that most GC B cells degrade pre-SHM receptors before leaving the DZ, and that B cells acquiring crippling mutations during SHM rarely reached the LZ. Instead, apoptosis was triggered preferentially in late G1, a stage wherein cells with functional BCRs re-entered cell cycle or reduced surface expression of the chemokine receptor CXCR4 to enable LZ migration. Ectopic expression of the anti-apoptotic gene Bcl2 was not sufficient for cells with damaging mutations to reach the LZ, suggesting that BCR-dependent cues may actively facilitate the transition. We postulated that a BCR mediated signal may be required as a “LZ promoting cue”. Thus, BCR replacement and pre-screening in DZs prevents the accumulation of clones with non-functional receptors and facilitates selection in the LZ.

CONTENTS

Chapter One: Introduction 1

1.1 The breadth and depth of the immune response increases over time 1

1.1.1 Activated B cells can seed germinal centres 1

1.1.2 Germinal centres are highly organised microanatomic structures 5

The dark zone is the site of gene diversification and cellular division 6

Germinal Centre B cells encounter antigen in the Light zone 10

1.1.3 Signals received in the light zone drive selection 13

B cell receptor signal attenuated 13

T cell help 14

B cell signal revisited 16

Proteins necessary for selection and maintenance of the dark zone program 18

1.1.4 Negative selection in germinal centres removes cells with autoreactive B cell receptors 22

1.1.5 Permissive selection allows low affinity clones to be maintained in germinal centres 24

1.2 Plasma cells and long-lived memory cells are the products of affinity maturation 28

1.2.1 Plasma cells are terminally differentiated B cells 28

High affinity cells are selected into the plasma cell compartment, without impeding affinity maturation 29

1.2.2 Memory B cells 32

The memory compartment is selected for breadth 32

Memory B cells can sometimes re-enter germinal centres 36

1.3 B cell receptor engages with antigen to trigger signalling 37

1.3.1 B Cell Receptor engagement with antigen triggers a cascade of signalling events 38

1.3.2 B Cell Receptor engagement with antigen triggers massive changes in the molecular architecture of the cell 42

1.3.3 Do mature B cells require “tonic signal”? 44

1.3.4 The B cell receptor also signals independently of antigen 47

1.4 Conceptualisation germinal centre biology requires an understanding of exchange of the B cell receptor 48

Chapter Two: Methods 50

2.1 Tables of key resources 50

2.2 Mouse models 58
2.3 Mouse treatments 59
Immunisation and adoptive transfer 59
Diphtheria toxin 59
Hy10* treatment 60
HeleaGFP 60
Tamoxifen treatment 60
Bone Marrow chimeras 60
Mixed Bone marrow Chimeras 60
Bone marrow chimeras with transduced bone marrow 61
2.4 Genetic Tools 62
Construction of retroviral vectors 62
293T cell transfection 64
2.5 Flow cytometry and single cell sorting 64
2.6 *IgHV* sequencing 65
SW_{HEL} B cell receptor sequence analysis 65
Sanger sequencing 65
Analysis of B cell receptor by next generation sequencing 66
Single cell *Ighv* mRNA sequencing 67
2.7 Quantitative PCR 69
Single cell qPCR 69
Bulk population qPCR 70
2.8 *In vitro* culture germinal centre B cells 71
Single cell culture 71
Short term cultures 72
Internalisation assay 72
ELISA 73
2.9 Quantification and statistical analysis 73
Statistical analysis 73
Calculating frequencies of cells carrying stop codons from sanger sequencing 74

Estimates of expected premature stop codon frequency....75

Chapter Three: Cells appear to exchange their B cell receptors in the Dark zone....79

3.1 The B cell receptor is handled fundamentally differently between Germinal centre B cells and naïve B cells....79

3.2 Dark zones contain populations of cells that have undetectable surface BCR....85

3.3 Generation of the BCR^{low} DZ population depends on the presence of AID yet is not related to the generation of aberrant antibodies....92

3.4 Germinal centre B cells replace their antigen receptors following somatic hypermutation but while still in the dark zone....99

3.5 The B cell receptor is replaced rapidly in the dark zone....103

3.6 Summary....104

Chapter Four: cells transition into the LZ expressing the B cell receptor they encode, but fail the transition if their B cell receptor is destroyed....108

4.1 Evidence of somatic hypermutation at G1 in the dark zone followed by continued initiation of and progression through cell cycle without functional B cell receptor genes....108

4.2 Germinal centre B cells acquiring damaging immunoglobulin gene mutations rarely reach the Light zone phase....112

4.3 GC B cells may undergo cell death as an alternative fate to initiating another cell division or transitioning to the light zone stage....118

4.4 Summary....126

Chapter Five: investigation into a possible Light zone promoting cue....129

5.1 Relief of competition between dark zone cells does not allow cells with disrupted B cell receptors to enter the light zone....129

5.2 Blocking antigen engagement does not prevent cells from entering the light zone....133

5.3 There are cells in the light zone which have lost their ability to interact with antigen....136

5.4 Inducibly ablating B cell receptors from the surface of germinal centre cells leads to an increase in cell death....144

5.5 Summary....147

Chapter Six: Discussion....150

6.1 B cell receptors are mainly exchanged while cells are in the dark zone....152

6.2. Cells with non-functional B cell receptors are deleted in the dark zone....154

6.3. Cells may require active light zone promoting cues to transition from dark zones to light zones....159

6.3.1 Mature B cells may require “tonic” B cell receptor signal, or may “sensitised” to death upon loss of surface BCR....160

6.3.2 Signals downstream of the BCR promote Light zone phenotype....161
6.3.4 A B cell receptor mediated light zone promoting cue may be antigen dependent or independent....166
6.3.5 NF-κB signalling is unlikely the Light zone promoting cue....169
6.4. Summary....170
Chapter Seven: Appendix....171
7.1 Appendix one: efforts to analyse exchange of the B cell receptor in the GC....171
7.1.1 IgM-KIKGR....171
7.1.2 Igβ Fluorescent timer....175
7.1.3 Igκ-SNAP tag....180
7.2 Appendix two: exchange of pre and post SHM BCR mRNA....183
Bibliography....190

LIST OF FIGURES

Figure 1.1| The B cell receptor complex is made up of six chains....4
Figure 1.2| Cells undergo somatic hypermutations as the divide in the dark zone....9
Figure 1.3| affinity mautration occurs in germinal centres....12
Figure 3.1| Differential surface expression and internalisation of B cell receptor in Germinal centre and naïve B cells....84
Figure 3.2| B cell receptor down-regulation by dark zone germinal centre B cells....88
Figure 3.3| Down-regulation of B cell receptor in the germinal Centre is not related to cell cycle....91
Figure 3.4| Generation of BCR^{low} populations is related to Somatic hypermutation, but not dependent on the creation of aberrant antibodies....94
Figure 3.5| Generation of the BCR^{low} population is not related to the damage to the BCR....96
Figure 3.6| BCR^{low} cells can re-express surface B cell receptor *in vitro* after short-term culture....98
Figure 3.7| Dark zone cells acquiring detrimental mutations in their immunoglobulin V region genes accumulate in BCR^{low} gates....102
Figure 4.1| Correlating cell cycle and the acquisition of stop codons....111
Figure 4.2| Cells carrying premature stop codons fail to accumulate in the light zone....116

Figure 4.3| BCR^{low} light zone cells are enriched for pre-plasma cells....117

Figure 4.4| BCR^{low} germinal centre B cells undergo apoptosis in the dark zone....119

Figure 4.5| BCL2 germinal centre phenotype....120

Figure 4.6| delaying apoptosis is not sufficient to drive cells into the LZ....122

Figure 4.7| The temporal relationship between G1 sub-stage and S phase re-entry or CXCR4 down-regulation by Dark zone cells....125

Figure 5.1| relieving competition between B cells in the germinal centre dark zone....132

Figure 5.2| Blocking antigen access in the germinal centre....135

Figure 5.3| presence of non-HEL binding cells....137

Figure 5.4| Cells which have apparently lost capacity to bind HEL fail to present antigen....139

Figure 5.5| Sequence analysis of cells in non-binding gate....141

Figure 5.6| Summary of mutation profile in Hel binding and non-binding cells....142

Figure 5.7| HEL binding assessed *in vitro*....143

Figure 5.8| Induced ablation of surface B cell receptor....147

Figure 6.1| Revisited model of selection in the germinal centre....151

Figure 7.1| labelling B cell receptor component IgM with photoconvertable protein KikGR 174

Figure 7.2| Labelling B cell receptor component Igβ with fluorescent timer proteins....179

Figure 7.3| labelling B cell receptor component Igκ with SNAP tag....182

Figure 7.4| Cells do not appear to be purging pre-SHM BCR mRNA....186

Figure 7.5| Cells containing both pre- and post-SHM mRNA BCR are found in both BCR^{high} and BCR^{low} Dark zone compartments....189

LIST OF TABLES

Table 2.1a Antibodies....50

Table 2.1b Experimental models, mice....51

Table 2.1c Cell strains....51

Table 2.1d Oligonucleotides....51

Table 2.1e Plasmids....53

Table 2.1f Taqman x20 Assays.....53

Table 2.1g Chemical, peptides, and recombinant proteins.....54

Table 2.1h Software and algorithms.....57

Table 2.1i Miscellaneous reagents.....57

LIST OF ABBREVIATIONS

Abbreviation	Explanation
act-Casp3	Active form of caspase3
AID	Activation induced cytidine deaminase
BAFF	B cell activating factor
BAFF-R	BAFF receptor
BCR	B cell receptor
Blnk	B cell linker protein
BM	Bone marrow
bNAbs	Broadly neutralising antibodies
Btk	Bruton's tyrosine kinase
CDR	Complementarity determining region
cSMAC	Central supramolecular activation cluster
dSTORM	Stochastical optical reconstruction microscopy
DT	Diphtheria toxin
DZ	Dark zone
EBV	Epstein-Barr Virus
FDCs	Follicular dendritic cells
FRET	Fluorescence resonance energy transfer
FT	Fluorescent timer proteins
FWR	Framework region
GCs	Germinal centres
HEL	Hen egg lysosyme
HEL-OVA	Hen egg lysosyme conjugated to ovalbumin
HIV	Human immunodeficiency virus
i.p.	Intraperitoneal
indels	Insertions or deletions
IRE1	Ribonuclease inositol-requiring enzyme 1
ITAMs	Immune tyrosine activation motifs
LMP2a	Latent membrane protein 2a

LZ	Light zone
MHC-II	MHC class II
myr-Akt,	Myristoylated Akt (constituitively active)
NP	4-Hydroxy-3-nitrophenyl
NP-OVA	NP conjugation ovalbulin
OVA	Ovalbumin
PE	Phycoerythrin
PI3K	Phosphoinositide3-kinase
PLCγ2	Phospholipase-Cγ2
pSMAC	Peripheral supramolecular activation cluster
s.c.	Subcutaneous
S1PR2	Sphingosine-1-phosphate receptor 2
SHIP	SH2 domain–containing inositol 5 phosphatase
SHM	Somatic hypermutation
SHP-1	SH2 domain–containing phosphatase-1
SHP-1	SH2 domain–containing phosphatase-1
SLAP	Src-like adaptor protein
SRBCS	Sheep red blood cells
SW_{HEL}	Switchable HEL mouse line
T2A	Thoseaasigna virus 2A
T_{FH}	CD4+ T follicular helper
UPR	Unfolded protein response

CHAPTER ONE: INTRODUCTION

1.1 THE BREADTH AND DEPTH OF THE IMMUNE RESPONSE INCREASES OVER TIME

The host and pathogen are locked in an endlessly escalating immunological arms race. Pathogens are under great pressure to prevent recognition of their antigen epitopes, either by diversifying these targets or by masking them[1–5]. At the same time, hosts have an equally pressing need to protect themselves against a constantly diversifying pool of pathogens, and so must produce a dynamic and broad immunological barrier to infection. Antibodies are pathogen-specific soluble effector proteins; they are the foot soldiers of the immune system. At their best, antibodies can elicit sterilising immunity by neutralising toxins, obstructing host cell entry, and by facilitating the opsonisation of pathogens; at their worst, they can trigger massive systemic autoimmune attack.

Not only are antibodies powerful actors against foreign invaders, but, as was demonstrated by Eisen and Siskin's seminal finding, their affinities improve during immune responses[6,7]. This occurs as the result of antibody affinity maturation. Affinity maturation is an iterative process of immunoglobulin gene diversification, or somatic hypermutation (SHM), and selection; processes that occur almost exclusively within germinal centres (GCs)[8,9]. Subsets of selected cells differentiate into long-lived memory and plasma cells that leave GCs and are the effectors of immunity. The highly mutated antibodies that are generated during affinity maturation are critical for the resolution for diseases of great global consequence. Arguably, the ultimate immune

response produced by a vaccine for malaria, human immunodeficiency virus (HIV), or influenza, would be one that produced a universal neutralising antibody[10–14]. While broadly neutralising antibodies (bNAbs) against these pathogens have been identified, these antibodies are often extensively mutated and the challenge going forward is to understand how the maturation of such antibodies might be induced with vaccines[10–13,15–17]

It is presumed that a key requirement of selection is that cells are chosen on the basis of the B cell receptor (BCR) that their immunoglobulin genes encode. This presents a unique pressure for GC B cells, as the time period between mutation and selection is much shorter than any speculated half-life of the BCR. The aim of this DPhil has been to investigate how, and to what extent, BCR complexes are exchanged with freshly mutated versions following SHM. We also aimed to determine the implications of this for selection. The consequences of being inappropriately judged in the GC due to expression of a pre-SHM BCR are dire. It could reduce the efficiency by which affinity maturation occurs. It might also result in the production of a possibly "useless" memory B and/or plasma cells, both of which are cells that have the potential to endure for the entirety of a person's life.

1.1.1 ACTIVATED B CELLS CAN SEED GERMINAL CENTRES

Mature naïve (or follicular) B cells encounter antigen in B cell follicles of secondary lymphoid organs, which they recognise through their unique surface BCR. The BCR complex is made up of six chains of two types, signalling chains and variable chains (immunoglobulins). The variable chains are a membrane associated antibody, differing from serum antibody only in the presence of transmembrane and intracellular regions at the C-terminal. The variable chains are made up of two heavy chains and two light chains, the latter of which have no

transmembrane region, but associate with extracellular regions of the heavy chains. The two signalling chains, Igα and Igβ (or CD79a and CD79b) form a transmembrane heterodimer that associates non-convalently with the variable chains and each other[18] (Figure 1.1).

B cell activation is a two-step process. The first step is the engagement of the BCR and the second step is some sort of confirmation signal, to prevent the generation of autoimmunity. This can take the form of a CD40/CD40L signalling, which confirms that the foreign bodies have also been registered as such by T cells; alternatively, a toll-like receptor signal can prove the foreign origin of the protein[19]. Following antigen engagement, B cells up-regulate CCR7 and EBi2, triggering their relocation to the B cell follicle-T cell zone border[20–22]. Activated B cells will also increase expression of CD86, a co-receptor for CD28 on the T cell surface, enabling B cells to receive a pro-survival signal from T cells, which balances death signals received via FAS ligand. In combination with BCR ligation and CD40L signals, FAS signals contribute to the proliferation and differentiation of B cells[23–25]. However, if cells fail to receive a second signal, they die. Thus, BCR signalling leads to the binary outcomes of either cell death, to prevent the production of autoantibodies; or proliferation, which leads to the eventual generation of plasma cells and long-lived memory cells[26–29].

Approximately three days after antigen exposure, at least in model systems where the kinetics have been carefully mapped, B cells will commit to one of three fates: they will either become unmutated recirculating memory B cells, differentiate into short-lived plasmablasts expressing (mostly) non-class switched unmutated antibodies, or enter GCs. The decision to become a GC B cell, as opposed to a short-lived antibody producing cell, is not determined by previous encounter with endogenous antigen. Cells with identical BCRs can go down any of the fate decision paths[30,31]. Later in the response, GC B cells may differentiate into high affinity plasma cells,

producing an elegantly refined antibody that is specific to the pathogen at hand, or differentiate into long lived memory cells within a broad and diverse fleet, that are prepared to protect the individual against not just identical pathogens but related interlopers.

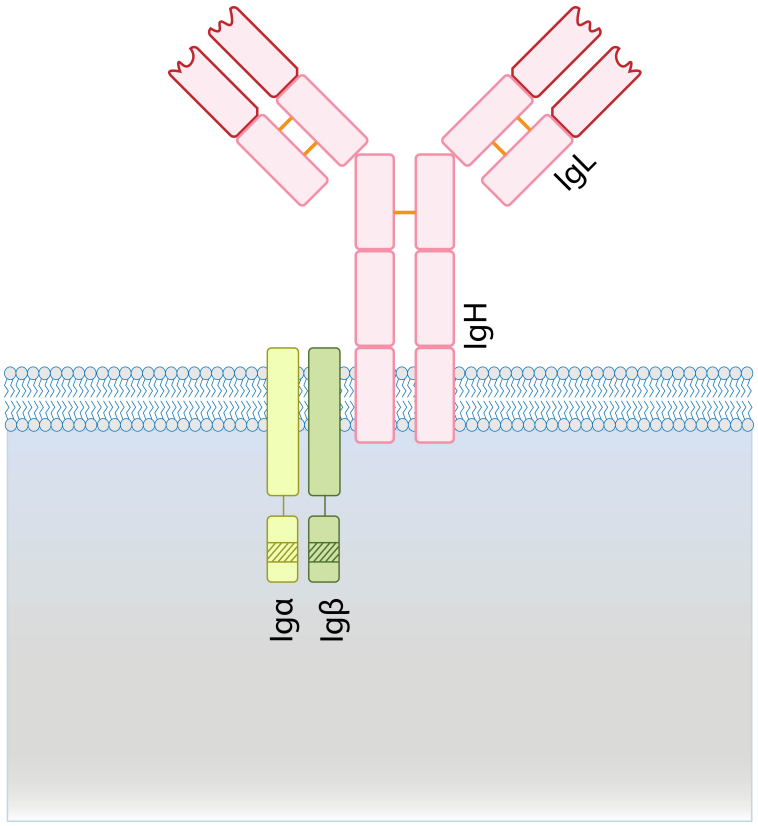


FIGURE 1.1| THE B CELL RECEPTOR COMPLEX IS MADE UP OF SIX CHAINS

The B cell receptor complex is made up of six chains of two different types. The signalling chains Igα and Igβ are depicted in different shades of green, their ITAM motifs (discussed in section 1.3) demarcated with shaded regions in the cytoplasmic domain. The variable chains (IgH and IgL) are shown in pink, the heavy chain spanning the membrane and the light chain associating with the extracellular regions of the heavy chain. The variable regions, which associate with antigen, are outlined in red. The disulphide bonds between immunoglobulin heavy chains, and between the heavy and light chain, are shown in orange.

1.1.2 GERMINAL CENTRES ARE HIGHLY ORGANISED MICROANATOMIC STRUCTURES

GCs are microanatomical loci that form within the B cell follicles of secondary lymphoid organs. GCs are physically separated into two zones known as dark zones (DZs) and light zones (LZs)[32]. The DZ is the site of antibody gene diversification and proliferation[33,34], while the LZ is the site of antigen encounter and selection (expanded on in later sections). A major environmental difference between LZs and DZs is the presence in the former of a specialised stromal cell subset, follicular dendritic cells (FDCs)[35–37], the roles of which include the sequestration and display of intact antigens (immune complexes) through high expression of Fc receptors (CD16/32, CD23) and complement receptors (CR1/2)[38,39]. FDCs also express the critical chemokine ligand for CXCR5, CXCL13, which is essential for B cell follicle structural organisation, and have been predicted to also play other yet-unknown critical functions[40,41]. In addition to responding to CXCL13, GC B cells express another G protein coupled receptor, sphingosine-1-phosphate receptor 2 (S1PR2), which promotes their retention physically within the GC by constraining their movement towards sphingosine-1-phosphate "richer" regions outside of the B cell follicle[42,43].

The physical polarisation of the GC is not critical for the transitioning of cells between the DZ state and the LZ state. When GC LZ/DZ spatial polarisation was experimentally disrupted by deletion of the DZ promoting chemokine receptor CXCR4, cells still iteratively switch between DZ and LZ phenotypes. Therefore, it was proposed that GC B cells have an intrinsic cellular program that contributes to their switching between mutation and selection states[44]. Despite GC B cell behaviour being determined in large part by their intrinsic programs, there is also evidence that the physical polarisation of the GC seems to enhance the efficiency of AM[44]. The reasons for this are not yet clear, but it seems plausible that it may relate to increased concentrations of antigen

being restricted to sites where it is most needed (i.e. where antigen-driven selection will take place); conversely, it may be by restricting antigen from cells that are not yet ready for selection, e.g. because they have not yet replaced pre-SHM BCRs[44].

THE DARK ZONE IS THE SITE OF GENE DIVERSIFICATION AND CELLULAR DIVISION

The DZ is the predominant site of mitosis in the GC[34]. Cells undergo an average of two divisions each time they enter the DZ, the first of which is initiated before cells move there from the LZ[45]. GC B cells are directed to, and held within, DZs by the chemokine CXCL12. The receptor for CXCL12 (CXCR4) is highly expressed in DZ cells and is a useful marker when differentiating these cells from LZ cells[40,46,47]. Having exhausted their proliferative potential in DZs, GC B cells will down-regulate CXCR4 expression and transition to the LZ[40].

Concurrent to division, DZ cells introduce random mutations in the variable regions of their immunoglobulin genes through SHM. The enzyme driving antibody gene diversification is activation induced cytidine deaminase (AID, encoded by *Aicda*)[48]. The DZ is considered to be the primary site of SHM because DZ cells (also called centroblasts) express higher mRNA and protein levels of AID[46]. GC B cells are uniquely prepared for the burden of SHM, presenting a genetic expression profile commensurate with the task of accommodating the genotoxic stress generated by AID activity. BCL6, a critical GC transcription factor, prevents DNA damage from triggering apoptosis or cell cycle arrest by triggering the DNA damage response. In GC B cells, BCL6 dampens the DNA damage response by inhibiting p53[49], p21[50], CHEK1[51], and ATR[52].

AID activity is subject to complex post-translational regulation, which involves being phosphorylated[53,54], dimerising[55], and translocating to the nucleus (associated with replication protein A[56]), where it subsequently deaminates cytosine residues, converting them to uracils[57,58], in the variable regions of actively transcribed immunoglobulin genes[48,59–65]. The deamination of cytidines to uracils produces U/G mismatches, which are subsequently recognised by mismatch repair and base excision repair, thereby triggering repair processing. The U/G mismatch can be repaired by "short patch" excision with error prone polymerases such as polymerases η[66] ξ[67–69] and ι[70,71]. These introduce mutations at surrounding nucleotides. At this stage, SHM can also result in the introduction of insertions or deletions[72,73]. The targeting of AID is tightly controlled, as might be expected of an enzyme with the potential to be so catastrophically damaging[74].

AID is most active at specific sequences, known as "hotspots" that are preferentially mutated both because they are slightly more error prone for polymerases, and because the AID focuses on these sequences[75]. AID prefers to target sites with the sequence WRC (W being an A or a T, R being an A or a G) motifs, and avoids the "coldspots" SYC (S being a G or C, Y being a C or a T) or the third Cs in the trinucleotides TTC, CAC, GGC and GAC[76–78]. While the action of AID, within the restrictions of hotspot targeting, is somewhat random, the variable region sequence has evolved to favour diversity. The sequence of the immunoglobulin variable regions involves the precise placement of nucleotides to prevent the dominance of specific kinds of mutations[79]. Even with the recruitment of error prone polymerases, one would expect certain kinds of amino acid substitutions, directed by the C to T and G to A mutations, to dominate. However, it appears that, within the variable region genes, the placement of C and G nucleotides means that most of these mutations are silent or conservative. Zheng et al. postulated that this positioning increases the

efficiency of affinity maturation by preventing certain replacements from dominating. These biases are further exaggerated by the placement of these codons in hotspots[76,77]

Antibody variable regions can be divided into framework regions (FWRs) and complementarity determining regions (CDRs). The CDRs are the sites of antigen contact between antibody and antigen[80], and account for most of the antigen binding specificity[81]. The FWRs maintain the structural integrity of the variable domains of the antibody. The FWRs encodes nine antiparallel β-strands, arranged in two sheets. These are somewhat invariant and serve as a scaffold for the CDR loops[80]. The antibody variable regions were first divided into FWRs and CDR based on the number of mutations at these sites[82]. In commonly found antibodies, mutations in the FWRs do not accumulate, and those mutations that are tolerated in the FWRs are more likely to be neutral substitutions[83,84].

Hotspots are enriched in CDRs relative to FWRs[79,84]. Therefore, it appears that FWRs have evolved a nucleotide coding sequence that resists mutation, given that invariant beta pleated sheets are necessary to provide a skeleton for the CDR[83–86]. Yeap et al. performed elegant experiments comparing the extent of mutation accumulation in "productive" immunoglobulin genes to those that are "passengers" in the non-productive *Ighv* region (but are still targets for AID), thereby comparing the targeting of AID to identical sequences in the presence or absence of selective pressures[74]. The authors found that identical hotspots were targeted in the CDRs but not in the FRWs, both in the productive and the passenger sequences. Similarly, they compared targeting in non-Ig passenger sequences, and found that some hotspots were highly targeted, while other identical motifs were not. This was consistent with a model in which AID targets DNA in response to the larger context of the surrounding sequence, and it appears that *Ighv* genes may have evolved to direct AID towards the CDRs and away from FWRs[74].

The rate at which SHM occurs in GCs has been estimated to 1 mutation per 10^3 template base pairs per division, with similar numbers proposed through analysis of the clonal tree relationship of sequences from *ex vivo* micro-dissections[79], analysing rates of mutation *in vitro* in hybridomas[87], and computational simulations[88]. Using this information, I have calculated that in the DZ, cells have a ~62% chance of acquiring a SHM in either their heavy or light chain variable region during each division. If cells are in the DZ for an average of two divisions, 86% of cells will have at least one mutation by the end of their DZ cycle (shown in Figure 1.2). These issues/calculations will become highly relevant when I return to the specific questions tackled, and the experimental approaches employed, later in this thesis.

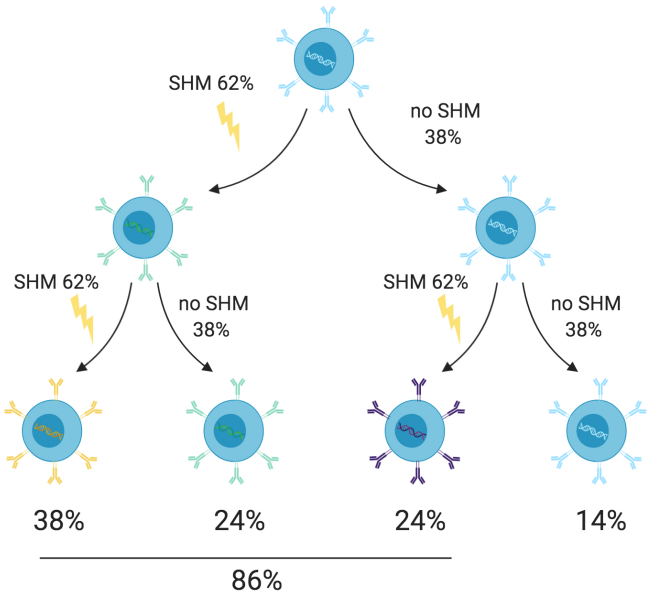


FIGURE 1.2| CELLS UNDERGO SOMATIC HYPERMUTATIONS AS THE DIVIDE IN THE DARK ZONE

Schematic depicting the frequency with which cells incur a SHM per division. SHM events are demonstrated with a lightning bolt cartoon. A change in the DNA sequence is symbolised with a change in the colour of the DNA molecule depicted in the nucleus of the cell, and a corresponding change in the BCR molecules is depicted with an associated change in the surface molecule.

Having diversified their immunoglobulin genes in DZs, B cells transition to LZs where they encounter antigen and undergo selection. The transition to the LZ is associated with a drastic phenotypic and behavioural change that is not related to exposure to local cues but rather controlled by cell stage-specific intrinsic cellular programs[44]. The LZ is orientated towards the afferent lymphatic duct in lymph nodes that is the route of antigen entry, and are distal to the T cell zone; the DZ is oriented proximal to the T cell zone. The LZ is so named because when stained with DAPI to mark nuclei, the extensive cytoplasm of the FDC network demarcates this zone as comparatively clear and unstained[32].

In LZs, antigen is presented in the context of immune complexes on the surface of FDCs[35,89]. Interestingly, FDCs are not passive "felt boards" of the GC; antigen cycles between the surface of the FDCs and non-degradative endocytic compartments. This facilitates long-term retention and display of antigen in the context of immune complexes allows GC B cells to continuously test new variants of their BCR[90]. For example, an influenza infection in mice will result in the formation of GCs within a week of infection, however these GCs will persist for multiple months, despite the fact the virus is cleared within 6-7 days[91,92]. This activity likely supports the long-term survival of intact antigen in the GC and thereby facilitates long lasting GC responses which may be required for generating antibodies against difficult to access epitopes[91,93]. For example, recent work from the Crotty group showed that a long, drawn-out GC response is conducive to the generation of high affinity antibodies against HIV[93].

LZ cells (also called centrocytes) express high levels of various activation associated markers, including CD83[94] and CD86[95,96], and are primed to interact with antigen and present peptides to T cells. Having internalised antigen, LZ GC B cells present the antigenic peptides

on MHC class II (MHC-II) thereby enabling them to receive "help" from follicular helper $CD4^+$ T cells (T_{FH}), which are more abundant in that zone[8,9]. Consequently, the LZ is the site of selection in GCs (described in greater detail below). The selection process is annotated in Figure 1.3.

For selection and productive clonal evolution to occur, B cells must encounter their cognate antigen and efficiently discriminate between high affinity and low affinity BCR-antigen interactions. GC B cells have been shown to internalise antigen with higher discernment of affinity that than naïve B cells[97]. Cells that have acquired mutations that improve their antigen binding ability will preferentially re-enter cell cycle, transition back into the DZ and proliferate there. While the concept that GC B cells move from LZs to DZs had long been predicted based upon suggestive experimental observations[98,99], the definitive proof came when Victora et al.[100] used a photo-activatable GFP to mark cells in one zone and track their subsequent behaviour. Furthermore, this approach allowed the authors to quantify the kinetics of movement, and lead to estimates that only 10-30% of LZ cells are successful in returning to the DZs each cycle[100,101]. Therefore, the LZ to DZ transition represents a selection bottleneck that favours affinity enhancements[100].

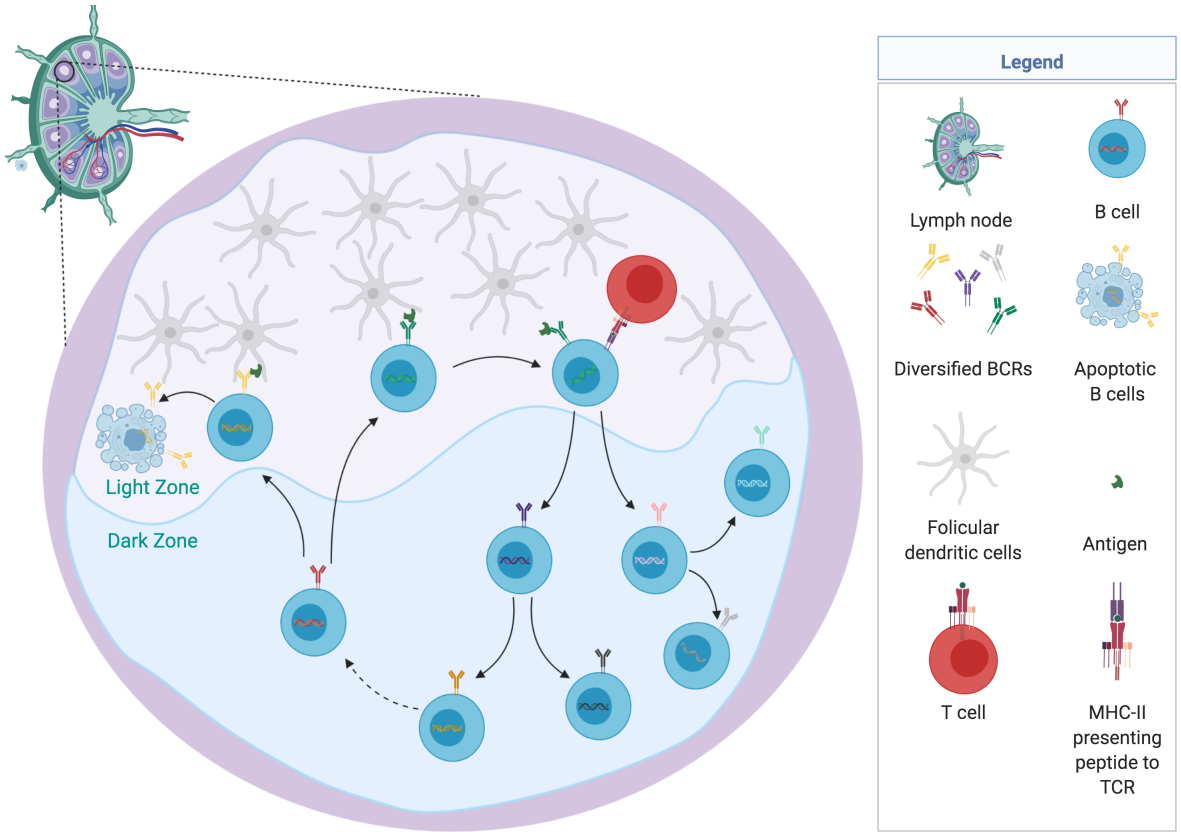


FIGURE 1.3| AFFINITY MAUTRATION OCCURS IN GERMINAL CENTRES

Schematic of selection in the GC. In the DZ cells divide and introduce mutation in the immunoglobulin genes. Cells then transition in the LZ where they encounter antigen on the surface of follicular dendritic cells. Cells which have acquired mutations which interfere with antigen binding die by neglect in the LZ. Cells which have maintained or improved the ability to interact with antigen will internalise antigen, present it to T_{FH} cells, and re-enter the DZ, where they will proceed to proliferate and introduce further mutations in their immunoglobulin genes.

1.1.3 SIGNALS RECEIVED IN THE LIGHT ZONE DRIVE SELECTION

There are two ways in which a LZ cell could learn that it has improved its affinity for antigen. Most intuitively, it could receive stronger signals through the BCR[100]. However, as well as being the ligand-binding domain of a signalling complex, the BCR is also an endocytic receptor which captures antigen and internalises it for degradation and loading into MHC-II complexes. Therefore, it was also recognised that GC B cells could be selected on the basis of T cell help[102–105]. I will discuss in the following sections the evidence regarding how these two pathways may contribute to selection.

B CELL RECEPTOR SIGNAL ATTENUATED

The role of BCR signalling in the GC selection has been controversial in recent years. A high-profile study from the Shlomchik lab suggested that BCR signalling in GC B cells may be "short-circuited" by high phosphatase activity[106]. That is, their findings seemingly showed that BCR signalling was limited in GC B cells, as compared to naïve B cells, as a result of increased phosphatase activity. Both SH2 domain–containing phosphatase-1 (SHP-1) and SH2 domain–containing inositol 5 phosphatase (SHIP) were hyperphosphorylated in GC B cells, and they seemingly remained co-localised with BCR complexes following BCR engagement. This attenuation of BCR signalling seemed critical for normal GC B cell biology, because the induced deletion of SHP-1 *in vivo* caused GCs to collapse[106]. This signalling attenuation was later attributed to a GC B cell-specific AKT-kinase-driven negative feedback loop. In GC B cells Akt signalling activates three upstream negative regulators of BCR signalling (CSK, SHP-1 and HPK1 when) through phosphorylation, and enhances their activity[107]. This forms a BCR negative feedback loop of the GC, in which BCR dependent phosphorylation of SHP-1[106] triggers SHP-1 to

dephosphorylate Syk/Lyn[108,109]. In GC B cells inhibition of Akt enhanced BCR signalling, but not in naïve B cells[107]. The "redirecting" of Akt activation in GC B cells is dependent on PTEN, a negative regulator of PI3 kinase activity, which in GC cells has an additional role in modulating Akt activity[107]. Interestingly, there appeared to be a temporary reprieve in this phosphatase activity specifically when cells reached the G2 late cell cycle stage[106]. However, this observation was puzzling because most cellular division in GCs occurs in DZs rather than in LZs, and it therefore seemed unlikely that the purpose of this brief responsiveness to signalling is related to selection events restricted to the LZ.

For the reasons outlined above, it was argued by some in the field that BCR signalling may not play an important role in the GC at all, and that its dampening may be a critical means for promoting affinity discrimination[110]. In support of this, CD45-deficient B cells, which have defective BCR signalling, can enter GCs and undergo affinity maturation, although it should be noted that their responses do not last beyond 7 days[111]. As a result, it was proposed that the main or only role of the BCR in GC B cells may be to internalise antigen. However, recent evidence suggests a more nuanced story and that BCR and T cell signalling may both be important for GC biology - I will return to this topic shortly.

T CELL HELP

The finding that BCR-signalling is attenuated in GC B cells came at a time that other lines of research pointed towards affinity discrimination occurring as a direct result of interactions between B cells and T cells. During selection, GC B cells would be competing for T cell help on the basis of how effectively they internalised antigen, and this would directly influence the likelihood of a cell receiving T cell help. In the GC, cognate T cells deliver help to B cells through unique interactions leading to CD40-CD40L and ICOS-ICOSL signalling. Engagement

with pMHC-II on B cells induces the co-expression of Ca^{2+} IL-4 and IL-21 in T_{FH} cells[112]. Disruption of CD40-CD40L signalling, either by inducing deletion of CD40[113], or by blocking CD40 with an antibody[114], will lead to collapse of the GC. The interactions between GC B cells and T_{FH} cells are different to those which occur at the T-B border during the initiation of responses. T_{FH} cells in GCs move relatively quickly and form multiple transient contacts with GC B cells, such that they can efficiently find those B cells that are expressing high levels of cognate peptide in the context of MHC-II, and provide help to these cells. T_{FH} cells form contacts with many cells rather than forming a single prolonged contact with a single high affinity cell. However, T_{FH} cells form larger contacts with cells expressing high levels of cognate antigen, and these contacts last for longer[112]. Furthermore, there was evidence that T cell help is limiting in the GC[114–117].

Victora et al. were able to experimentally uncouple antigen-presentation from BCR signalling events in GC B cells, by delivering peptide antigen to the MHC-II targeting compartment of subsets of cells *in vivo* in a BCR-independent manner[100]. To do this, they made use of a system that had been designed to augment antigen presentation by dendritic cells[118]. This system involved using an anti-DEC205 antibody that was coupled to a key antigenic peptide from ovalbumin. DEC205 is an endocytic receptor which carries associated proteins into MHC-II processing compartments, and subsets of cells were targeted by delivering the chimeric antibody to mice containing a mixture of $DEC205^{+/+}$ and $DEC205^{-/-}$ GC B cells. This work showed that augmenting the amounts of T cell help caused GC B cells to accumulate in DZs, consistent with a model in with T cell help directs interzonal migration[22,119].

Gitlin et al. used the same antigen augmentation approach to identify that GC B cells presenting more antigen will also undergo more cell divisions once in the DZ[45]. The authors titrated the T cell help induced, and found that GC B cells presenting more antigen had shorter division times and

increased SHM rates. Mechanistically, they found that stronger T cell help shortens S phase through regulation of replication fork progression. During B cell selection, T cell help induces higher levels of cell cycle regulators like c-Myc and CDK[120], and also induces higher levels of nucleotide metabolism to accelerate DNA synthesis . As such, high affinity B cells receive more T cell help, and so experience a longer DZ dwell time wherein they undergo accelerated cell cycles[45121].

Together, these studies demonstrated that higher affinity GC B cells are better at presenting peptide antigens to T cells, and that this causes them to outcompete their neighbours in receiving help from T cells. In addition, cells receiving stronger help will reside in the DZ for longer, will proceed through cell cycle more quickly, and will complete more cell divisions. The "bottleneck" for DZ re-entry, together with the preferential amplification of higher affinity clones, therefore leads to increases in the affinity of the humoral immune response at the population level.

B CELL SIGNAL REVISITED

Despite the arguments outlined above, the evidence that BCR signalling may indeed be active and play important roles in the GC came relatively thick and fast. Firstly, it is relevant that Nowosad et al found that BCR signalling is necessary for membrane bound antigen extraction by GC B cells *in vitro*[97]. While it had been postulated that for individual receptors these were mutually exclusive fates[122] it was considered relevant for the process in general[123]. Outside of the direct context of antigen internalisation, a study using Nur77-GFP "BCR reporter" mice provided evidence of BCR signalling in both LZ and DZ cells, although predominantly in the LZ[124], arguing that BCR signalling was not totally curtailed in the GC. A

limitation of this system is that T cell engagement and cytokines may also trigger expression of Nur77 in some settings, although the authors did at least demonstrate a partial dependence on Btk (which is a BCR proximal signalling molecule). Furthermore, delivery of an ectopic BCR signal through the Epstein-Barr virus (EBV) derived LMP2a (latent membrane protein 2a) disrupts affinity maturation in the GC[125,126], suggesting that it likely plays a role in selection.

Subsequent follow-up work from the Shlomchik lab directly elucidated an important role for BCR signalling in GC B cells. Luo et al. demonstrated that BCR signalling not only occurs in GCs, but is seemingly required for selection. This study focused on the induction of c-Myc as a surrogate marker for selection. c-Myc is a key cell cycle/metabolic regulator that is associated with LZ cells re-entering cell cycle (in preparation of transitioning into the DZ), and is therefore considered a marker of selection[127–129]. Luo et al. found that, while CD40 ligation and BCR activation were both sufficient to induce c-Myc in naïve B cells, c-Myc induction in GC B cells required both signals. Blocking BCR signalling *in vivo* prevents CD40 ligation from promoting c-Myc expression among GC B cell, but not naïve cells[130]. This model of selection proposed that BCR signal gave a "licensing" signal, in which it synergises with T cell signals to allow DZ promoting selection cues[130].

Luo et al also used an anti-IgM antibody to deliver BCR signals *in vivo* in a manner independent of BCR affinity. They saw that only a small increase in frequencies of cells expressing c-Myc (15.5% compared to 8.2% in controls). Therefore, while inducing BCR signalling did increase selection in the LZ, it likely only did so among cells which had already received T cell help[130]. However, different experimental approaches revealed BCR signalling could promote selection of LZ cells under conditions where T cell help was limiting. More recently, Turner et al. highlighted that the delivery of saturating amounts of T cell help, as has been performed by other groups, by

the administration of high concentration of α-DEC205 antibodies conjugated to cognate antigen creates supra-physiological conditions. The authors found that upon delivery of 10μg of α-DEC205 conjugated to antigen, BCR engagement did not increase GC participation; however, when lower doses of α-DEC205 conjugated to cognate antigen were delivered (0.5μg), BCR engagement did increase selection in the GC. However, they found that while introducing T cell help in the absence of artificially enhanced BCR engagement does promote selection in the GC, triggering BCR signalling in the absence of T cell help does not promote selection. Therefore, while T cell help can clearly deliver cues which promote proliferation in the DZ, under more physiological conditions cells require both T cell help and BCR signal to trigger clonal bursts[131].

PROTEINS NECESSARY FOR SELECTION AND MAINTENANCE OF THE DARK ZONE PROGRAM

While various molecular factors that are relevant for the maintenance of the LZ and DZ program have been identified in recent years, mechanistically what controls the transition from the LZ to DZ, and the timing of the LZ to DZ transition (i.e. downstream of selection), are less clear. If cells are to proliferate in the DZ in a manner that is proportional to the quality/quantity of signal they received in the LZ, then they must proceed into the DZ with memory of their experience in the LZ. Below, I discuss candidates for controlling the DZ program c-Myc, and two downstream proteins AP4 and FOXO1; as well as mTORC.

C-MYC, FOXO1 AND AP4

Induction of c-Myc is a good candidate as a B cell division counter in the DZ as it has been shown to serve as a "division timer" in non-GC B cell responses. c-Myc is a master cell cycle

regulator, and controls the expression of many of the genes that control cell division including cyclin-dependent kinase, cyclins, and E2F transcription factors, CDK activating kinase, and CDC25[132,133]. Recent work by Finkin et al. demonstrated that T_{FH} cells induce the expression of c-Myc in GC B cells in direct proportion to the extent of antigen capture and presentation of MHC-II. Like in previous studies from the same lab, they titrated cognate antigen transfer by delivering different ratios of α-DEC205 conjugated to either cognate or irrelevant antigen[134]. The authors demonstrated that c-Myc expression *in vitro* was proportional to the amounts of T cell help received. Similar dose dependent responses were also observed in LZ cells *in vivo*, but this time using cell size as an indirect indicator of pathway induction. Furthermore, the authors genetically titrated c-Myc expression *in vivo* using conditional c-Myc deletions, haploinsufficiency, and over-expressions to show that that c-Myc proportionately regulates B cell expansion[134]. As a result of these findings, it was proposed that c-Myc expression in selected cells allows these cells to stockpile necessary metabolites for subsequent cell cycles, with more help facilitating a larger number of divisions upon DZ entry. Interestingly, however, increasing the amounts of T cell help did not increase the frequency of LZ cells which were entering S phase, and transitioning to the DZ, which was argued to indicate T cell numbers are limiting[134].

A small subset of c-Myc$^+$ LZ cells (that is, cells which have presumably recently been selected) have active FOXO1 localised in the nucleus[134]. Nuclear translocation of this transcription factor is required for the maintenance and presentation of a DZ phenotype[135,136]. Therefore, it is possible that expression of c-Myc is associated with the induction of the DZ phenotype through activation of FOXO1. It is noteworthy that those functions most obviously associated with the DZ program, proliferation and induction of SHM, were maintained in FOXO1 deficient GC B cells despite them mostly having a LZ phenotype. It is possible that the induction of proliferation, and therefore the

extent of proliferation that is induced by selection-based cues, is controlled by a different or independent molecular pathway. Interestingly, despite them undergoing SHM, FOXO1 deficient GCs have defective AM[137]. Therefore, while some other signal may be triggering proliferation and SHM in the LZ, the full DZ program is critical for the efficiency of clonal selection.

c-Myc induces a proportional dose-dependent increase in expression of genes which dictate cell cycle and which sustain cell division in the DZ, critically including AP4[138]. The transcription factor AP4 is a potential DZ program timer. In contrast to the findings for FOXO1, this transcription factor is required for proliferation of cells in the DZ, (ablating AP4 decreases the ability of cells to undergo additional rounds of mitosis in the DZ)[138]. Consistent with assertions that selection involves both BCR signalling and CD40L engagement, AP4 nuclear translocation is triggered by both[138]. Critically, AP4 reinforces the cell cycle in the absence of c-Myc expression[139]. Therefore, its role could be to amplify the c-Myc signal, or it might act as a separate "cell counter" candidate. Interestingly, AP4 deficient GC cells are able to generate affinity matured antibodies against the model antigen NP-CGG, however neutralising antibodies against LCMV did not develop in this setting, even after three months[138]. Therefore, AP4 is required for complex affinity maturation, like that seen in natural infections, but not against haptens. Haptens have simple, easy to access epitopes, and dramatically increased affinity is achieved with single mutations, whereas neutralising antibodies may require more complex selection events for their development.

MTORC1

A further critical pathway that is induced in GC B cells as a result of selection is mTORC[140]. BCR and CD40L signals also synergistically activate mTORC1, and individually induce weak

smTORC1 signal[140]. mTORC1 activity induction was proportional to amounts of T cell help, as was evidenced by the increased activity in cells which received a higher dose of the α-DEC205-OVA chimeric protein; or by altering the ratio of cells which are capable of responding to the antibody, which alters the degree of competition between B cells[140].

Using the α-DEC205 system to synchronise the GC selection events, and blocking mTORC1 activity at specific stages, Erschling et al. were able to delicately tease apart the stage at which mTORC1 activity is required for amplification of clones that have received more T cell help. mTORC1 activation is required early after T cell engagement to trigger anabolic cell growth. Following T cell help, mTORC1 activation triggers a cascade of events that allows for the build-up of metabolites which prepares the cell for subsequent clonal expansion in the DZ. The promotion of an anabolic phase sustains successive rounds of division upon DZ entry. If mTORC1 activity was inhibited prior to cell growth, the effect of forced selection upon administration of α-DEC205 was severely diminished. However, blocking T cell help after pS6 induction had much less of an effect. Therefore, the authors concluded that the role of mTORC1 is to ensure that cells have a sufficient biomass, and are sufficiently prepared the rapid cell cycling which occurs in the DZ[140]. This is consistent with other studies demonstrating mTORC1 to be involved in anabolism for cell cycle[130].

Erschling et al. postulated the anabolism and cell cycling were physically and temporally segregated in the GC. mTORC1 activity did not appear to be critical for cell cycle progression, with which c-Myc expression is strongly associated[141,142], and treatment with rapamycin, an inhibitor of mTORC1, leads to only a mild decreases in c-Myc signature genes and c-Myc protein. However, that disruption or amplification of either mTORC1 or c-Myc mediated signalling can severely impact selection implied that the pathways are interconnected rather than redundant.

Despite these recent important advances, further work is required to fully discern how exactly all these signals synergise and cross-regulate each other to ensure proper coordination.

1.1.4 NEGATIVE SELECTION IN GERMINAL CENTRES REMOVES CELLS WITH AUTOREACTIVE B CELL RECEPTORS

SHM can "redeem" B cells expressing autoreactive antibodies[143,144]. Naïve B cells with autoreactive antibodies are maintained in circulation in a well described state of anergy, most obviously characterised by low surface IgM and recalcitrance to signalling[145,146]. It is interesting that the GC B cell's recalcitrance to BCR signalling, and the lower surface BCR, have parallels with the state of anergy itself[20,106]. Therefore, it is unclear if recalcitrance to signalling in anergic GC B cells in amplified, (i.e. they are doubly resistant to signalling) or if there is no additive effect, in which case an anergic GC B cell is less distinctive compared to its non-anergic sister cells. The benefit of desensitising autoreactive B cells to BCR signalling has been clear, but the reasons for maintaining autoreactive B cells could be confusing. These are by no means minor populations, with anergic cells making up 2.5% of naïve B cells populations in humans (defined as $CD19^{+}$ IgD^{+} IgM^{low} $CD27^{-}$), and with 75% of these cells encoding antibodies that have measurable autoreactivity against self epitopes[20]. For example, 5% of antibodies cloned from anergic cells are reactive to poly-N-acetyllactosamine carbohydrates (I/i antigen) on erythrocytes[147].

Recent experimental evidence has demonstrated that, under certain immunisation conditions, anergic B cells can enter GCs and have their specificity actively diverted away from self. Having lost affinity for self-antigen through SHM, the previously autoreactive B cells outcompete clones who have maintained affinity for the self-antigen, even when the self-

reactive clones have better affinity for cognate antigen[143]. Burnett et al. compared the mutation trajectory of SW_{HEL} B cells (cells with a knock-in BCR directed against hen egg lysosyme, or HEL) following immunisation with duck egg lysosyme. The authors generated mixed bone marrow chimeric mice, with SW_{HEL} bone marrow combined with bone marrow that did or did not express HEL3X (a variant of HEL against which the SW_{HEL} BCR has limited affinity). This allowed the authors to compare identical cells in autoreactive and non-autoreactive settings. They found that anergy was a reversible state, and asserted that self-reactivity was not a barrier to B cell differentiation but actually (in this setting) guided cells down an alternative fate, which ultimately generated cells of higher affinity[144]. It is thought that this helps the immune system avoid repertoire holes, for example for foreign antigens that have significant similarity to self-proteins.

The benefit of "redemption" is therefore clear, but the mechanism within the current paradigm of GC selection is not. Most simply, decreasing self-recognition can increase the ability of these cells to respond to foreign antigens even if the affinity to said foreign antigen is decreased. It is possible that the GC state allows for relief of the anergic phenotype, or that GC cells are not relieved from anergy until they acquire BCR mutations which deviate the receptors specificity away from the autoantigen – although how they would initially enter GCs in this scenario is not clear. Burnett et al. noted that higher levels of antigen were required to trigger anergic cells to participate in GC responses[144]. If the GC state involves a relief of anergy, then auto-reactive B cells would be receiving high levels of BCR signal from auto-antigen; the cells would be continuously interacting with cognate antigen. The newly mutated BCR could face less distraction from self-antigen in the GC, that is, having lost the constant signal from the ubiquitously expressed self-peptide allowing it to receive better signalling through the BCR. Conversely, the recalcitrance to signalling and low surface BCR levels characteristic of anergy may not be relieved until the cell

mutates away from autoreactivity. In this model, cells would not be able to signal in the LZ sufficiently to internalise and present antigen derived peptides until they the antibody have been "redeemed" and autoreactivity has been lost.

This process was described by the authors as "complementary" to other earlier processes of removing self-reactive B cells, i.e. the deleting of self-reactive cells following VDJ recombination and receptor editing[148,149]. The redemption of autoantibodies in B cell is complementary to these systems because it allows them to be applied more permissively. Both systems, if they were to be applied too stringently, would generate "holes in the repertoire". In the absence of auto-antibody redemption, these holes in the repertoire could be exploited, i.e. microbes could evolve to mask themselves in self-antigens. And to a certain extent this does happen, for example HIV "masks" itself from the immune system using host cell glycans[1].

1.1.5 PERMISSIVE SELECTION ALLOWS LOW AFFINITY CLONES TO BE MAINTAINED IN GERMINAL CENTRES

The adage "don't let the perfect be the enemy of the good" is definitely applicable to GC B cells. The GC has conflicting evolutionary pressures to amplify high affinity clones and maintain breadth of the humoral response. The maintenance of low affinity clones in GCs is critical for the development of antibodies against difficult to access epitopes, and to produce a humoral response that is more prepared to deal with related variants of the invader. The germline reverted versions of bNAbs sometimes appear to have almost no affinity to the cognate antigen, or be autoreactive[150]. In 2016, two studies elegantly demonstrated that during complex immunological challenges lower affinity clones, which in classical models of selection would be rapidly outcompeted, are actively maintained in GCs.

Tas et al. utilised a "brainbow" method for clonal lineage visualisation. It was found that, in some GCs, individual clones would come to dominate the response; however, in others, diversity was maintained. Individual GCs lost diversity at wildly disparate rates. The "clonal bursts" observed were most likely the result of T cell interactions triggering explosive B cell proliferation[45,100,120]. The authors hypothesised that there was a slow and steady out-competing of low affinity clones, and that this was occasionally punctuated by clonal bursts of high affinity clones. As discussed earlier, higher affinity cells are more likely to receive T cell help and expand[151]. However, that any given GC B cell might encounter a particularly large piece of antigen, or interact with T_{FH} cells, may be subject to a degree of randomness. Therefore, while on the level of the organism the affinity of the antibodies increases over time, the unpredictability of a natural system allows lower affinity cells to persist. In support of stochasticity playing a role, the authors also noted evidence of clonal domination by cells that cannot perform affinity maturation[152].

Similar to Tas et al., Kuraoka et al. also followed the maturation of GC responses against complex protein antigens[153]. While in this study the clonal dynamics within individual structures were not assessed, additional power was gained by the development of an approach for studying the affinity of antibodies from hundreds or thousands of individual B cells. This was achieved by optimising an *ex vivo* culture method where single B cells were expanded several thousand-fold, and differentiated into antibody secreting cells. When day 8 and day 16 responses were compared, the expected affinity enhancements were evident, yet the later time point populations still maintained a substantial population of low affinity cells. Thus, while affinity enhancements did occur with time, GCs also permitted the maintenance of cells with lower affinity BCRs. They pointed to this breadth of affinity as evidence for a possible lack of a "systemic selection threshold", and proposed that when GC affinity exceeds some yet quantified threshold,

proliferation is guaranteed. Regardless of the exact mechanism, this study demonstrated that that intense GC selection occurred alongside a biological process that enables lower affinity B cells to participate in GCs.

Previous models of selection presumed the sole purpose of the GC was to amplify high affinity clones, which would ultimately result in a narrowing of the antibody repertoire as B cells focus in on a restricted set of epitopes[121,154–156]. However, the necessity of increasing the diversity of antibodies is becoming increasingly more apparent. Baumgarth went so far as to stipulate that the primary function of the GC reaction may be to increase the diversity of the response[157]. This model is based on the fact that, while the affinity of antibodies produced during an immune response generally increases, it does not always do so, for example, in VSV infections wherein the initial germline encoded BCR has high affinity[158]. Baumgarth speculates that often, although clearly not always, antibodies produced early in infections can have a high enough affinity to effectively neutralise the invading pathogen. However, these antibodies would be of a restricted spectrum, and easy for pathogen to evade. Without a diversifying and dynamic humoral response, the pathogen would easily be able to overcome the host. Therefore, the affinity maturation process is less a response to the evolutionary pressure of being re-infected with the same subtype of the virus, but a response to the risk of a heterotypic infection. This perspective is somewhat extreme, but serves as an interesting contrast to the long-standing dogma that role of the GC is to increase affinity.

The GC has been called a microevolutionary environment, and obviously the generation of stronger better antibodies through repetitive mutation and selection in the GC is reminiscent of evolution. However, the GC is the product of evolution, not an allegory thereof. A critical distinction in discussion of the GC is the evolutionary pressures that produced the GC reaction,

and the microevolutionary pressures that are placed on the cells within the GC. The selective pressure on the individual organism may be towards a humoral immune system that maintains breadth, but the selective pressure on the individual B cell may still be to interact with and internalise antigen. It is evident from evolution on the scale of organisms that strong selective pressures do breed diversity. Selection in the GC could be conceptualised as a "fight" or a "race". In the "fight" model, cells are directly competing with one another for the limited resources of antigen or T cell help, or both. However, in a "race" perspective, higher affinity clones come to predominate the GC by switching between zones, and dividing more rapidly, yet lower affinity cells are still allowed to tick along in the background, and are maintained even in a competitive environment.

When animals are competing, both the "fittest" and the "different" survive; there is selective pressure to be the most effective within a niche. Therefore, those cells, which have apparently lost affinity for antigen but are being selected in what seems to be some kind of GC welfare state, may simply be interacting with antigen in a different way, which allows for better interaction with antigen. The lower affinity clones observed may still be competing within the original more simple selection model if the lower affinity variant actually improves presentation of antigen, by preserving an epitope that would is better presented on MHC-II by shielding it from proteases[152]. Additionally, as GC reactions progress and generate plasma cells, antibodies made by those plasma cells can proceed to block antigen access and encourage B cells to mutate away from previously targeted epitopes[8].

1.2 PLASMA CELLS AND LONG-LIVED MEMORY CELLS ARE THE PRODUCTS OF AFFINITY MATURATION

Memory B cells and long-lived plasma cells are the crown jewels of the humoral immune system. Memory B cells are primed to quickly produce potentially affinity matured antibodies upon re-exposure to antigen[15,159]. Long-lived plasma cells continue to produce high affinity antibodies long after an infection has been cleared[160].These cells can last a lifetime, and are the products of an elegantly evolved system which actively selects the cells most appropriate for their respective roles based on the properties of their BCRs.

1.2.1 PLASMA CELLS ARE TERMINALLY DIFFERENTIATED B CELLS

Plasma cells are terminally differentiated B cells that produce thousands of antibody molecules per second. Long-lived GC-derived plasma cells have basically no surface BCR, and can persist without re-exposure to the antigen for decades[161–164]. Having transitioned from a state of immune surveillance to large-scale protein production, their phenotype differs vastly from their predecessors, and they ultimately have little in common with their precursors transcriptionally.

The "master" transcriptional director of plasma cell differentiation is Blimp-1 (encoded by *Prdm1* in mice)[165]. A key function of Blimp-1 is to repress key B cell and GC-specific transcriptional programs, for example a key target is the GC master transcriptional regulator BCL6. Blimp-1 also represses other genes required for the individual LZ and DZ programs. For example, Blimp-1 represses MHC-II presentation, characteristic of the LZ phenotype, by repressing the class II transactivator (*Ciita*)[166–168]. It also represses proliferation, a characteristic feature of the DZ phenotype, by blocking proliferation through repression of *c-Myc*[169]. Other key B cell regulatory genes repressed by Blimp-1 include *SpiB,* and *Id3*[170]. The identity of

plasma cells deviates so significantly from their precursors that even factors that define the B cell lineage, such as *Pax5*, are repressed[171–173].

Plasma cells synthesise and secrete antibodies at enormously high rates, producing thousands of molecules per second[174–176], and as such are particularly sensitive to ER stress. Blimp-1 triggers the induction of genes which make it possible to tolerate the ER stress[173]. ER stress is induced by the accumulation of unfolded or misfolded proteins, which in turn induces the unfolded protein response (UPR), for example the mediator of UPR XBP1[177]. In mature naïve B cells, the transcription factor PAX5 represses expression of *Xbp1*[178]. XBP1 is necessary for normal serum antibody levels[179], for *Igh* mRNA processing, immunoglobulin secretion, and ER remodelling required for plasma cell function[180]. Other ER remodelling pathways operate to maintain the plasma cell phenotype, such as the transcription elongation factor ELL2[181–183], and the ribonuclease inositol-requiring enzyme 1 (IRE1)[184–186]. Plasma cells also accommodate for ER stress though autophagosomal activity; disruptions to autophagy are detrimental to immunoglobulin production[187].

HIGH AFFINITY CELLS ARE SELECTED INTO THE PLASMA CELL COMPARTMENT, WITHOUT IMPEDING AFFINITY MATURATION

Long-lived plasma cells seemingly are generated in GCs[188]. Selection into the plasma cell compartment is thought to be more stringent than selection into memory cell subsets; affinity-conferring mutations are enriched in post GC plasma cells, even at points in time when these mutations are scarce in the GC itself[104]. Most models of plasma cell differentiation involve LZ cells receiving a “strong” differentiation inducing signal. Based upon the nature/strength of the signal received, LZ cells may either transition back into the DZ, or differentiate into plasma cells. Consistent with this model, pre-plasma cells have been observed in LZs[30,104,189]. Ise et al. saw

evidence consistent with this model, observing a subset of GC cells in the LZ which were BCL6^{low}, CD69^{high}- and IRF4^{+}; IRF4 is a transcription factor which is necessary for Blimp-1 expression. These cells were seemingly primed for plasma cell differentiation, and their appearance was dependent on, and proportional to, T cell help[27,190,191].

Cells were presumed to be selected to enter plasma cell compartments because they had received strong T cell signals, given that the GC derived plasma cells were selected for high affinity clones relative to the memory cell compartment[27]; and while augmentation of T-B cell interactions by increasing presentation of antigen to T cells through the α-DEC205 system increased plasma cell differentiation[104,192,193], simply decreasing death with the anti-apoptotic Bcl2 transgene did not[100,189]. In the GC, T cell help in the form of CD40 engagement triggers the activation of the canonical NFκB pathways, two subunits of which- RelA and c-Rel- are important for plasma cell differentiation and GC maintenance respectively[130]. Having received a strong T cell signal in the LZ, it seems possible that GC cells differentiate into plasma cells in the LZ.

A paradoxical feature of GCs and plasma cell development is that, while high affinity cells are actively selected into the plasma cell compartment, if these cells are removed from the GC via this route they cannot continue to participate in the proliferative response. If DZ re-entry and plasma cell differentiation represent a fate decision bifurcation, and the cells which receive the strongest BCR-mediated signal are channelled into the plasma cell compartment, then this constant skimming off the top of higher affinity cells could prevent the accumulation of high affinity cells in the GC. Initial indications that clones were conserved in the GC before selection into the plasma cell compartment came from *in vitro* evidence that signals which encourage proliferation in the DZ also encourage plasma cell differentiation (IL-21, CD40

ligation)[27,103,194], with differentiation occurring only after a number of cell division were completed[195].

Pre-plasma cells can be identified in GCs by their expression of low levels of Blimp-1. While Blimp-1^{low} cells have been observed in LZs, a higher proportion of Blimp-1^{low} cells that were not yet expressing the "later" CD138 for plasma cells, were found in DZs. Despite not yet being bona fide plasma cells, they were seemingly primed for differentiation down that route[190]. Consequently, it was hypothesised that, having received strong signals in the LZ, cells may transition into DZs, divide such that a successful iteration of the antibody remains in the GC, and concurrently upregulate BLIMP-1, sensitising them for plasma cell differentiation[196].

While plasma cells are clearly a selected population, the nature of the plasma cell promoting cues is not clear. Considering the evidence noted above regarding T cell help, it is interesting that T cell signals are not acutely required for Blimp-1 expression in pre-plasma cells. Radtke et al. and Krautler et al. found that T cell help was not required for the generation of pre-plasma cells, which they determined by genetically deleting and depleting T cells, respectively[190,196]. However, disruption of T cell help did lead to seem to perturb later differentiation stages. Krautler et al. found that, following T cell depletion, plasma cell differentiation in the GC was halted at an immature Blimp-1^{low} stage. Pre-plasma cells were sequestered in the LZ upon antibody mediated ablation of T cells, but not upon antibody mediated blocking of CD40-CD40L interactions, implying that a different T cell mediated signal drives LZ egress[190,196]. Radtke et al. found that those cells which fell into the plasma cell gate were less developed in terms of the antibody-producing cell transcriptional profile[196]. However, Ise et al. observed that a T cell signal was required for the generation of IRF4^{+} BCL6^{low} CD69^{high} cells[197]. Notably, blocking BCR-antigen engagement prevented plasma cell differentiation, although it is not clear if this disruption is due

to changes in BCR signalling or changes in complement or FDC derived factors[27]. One possibility suggested for reconciling these observations is that high affinity cells might preferentially receive cues from T cells that drive high IRF4 expression and that subsequently drive cells to proliferate rapidly upon returning to DZs. During this phase, some daughter cells will undergo programming changes that mean they acquire competence for plasma cell differentiation while other daughter cells will help maintain that clone within the GC[196].

1.2.2 MEMORY B CELLS

Memory B cells are long-lived cells which are reactivated to rapidly produce antibody upon re-exposure to antigen[27]. A subset of memory B cells may also re-enter GCs and continue to diversify after similar or related infections, as I will discuss below. A significant proportion of memory B cells are GC derived, but memory B cells with no evidence of history in the GC are also easily identified[198,199]. Furthermore, memory B cells can form in mice which lack BCL6 and are unable to form GCs[200–204]. However, for reasons described in the following section, the antibodies produced by GC derived memory B cells are often much more efficacious than those from non-GC derived memory B cells.

THE MEMORY COMPARTMENT IS SELECTED FOR BREADTH

In the GC, cells are shuttled into the memory B cell population with the selective pressures preferring a population of increased breadth[205,206]. The role of memory B cells is to prepare the host for subsequent infections, which may be identical to the initial immune challenge but often will deviate slightly. Efforts on the part of the pathogen to obfuscate the immune memory of the host are met by the host creating a diverse memory pool. Therefore, memory B cell pools are selected for diversity, and lower affinity cells are over-represented in the memory

compartment relative to the GC as a whole[207]. Shinnakasu et al. found that LZ cells expressing lower affinity BCRs had higher expression of the transcriptional repressor Bach2 than those in the high affinity compartment, and that these cells were more likely to enter the memory compartment. By analysing the induction of the Bach2 expression *in vitro* following antibody mediated stimulation of CD40L, the expression of Bach2 was found to be inversely correlated with the strength of T cell help received. Furthermore, induced Bach2 haploinsufficiency resulted in defects in the generation of memory B cells *in vivo*. As such, the authors proposed that low BCR affinity results in low presentation of pMHC-II to T cells in the LZ, which then leads to higher levels of Bach2, which predisposes these low affinity B cells to memory cell differentiation. The authors postulated that Bach2 expression induced anti-proliferation pro-survival effects that were necessary for GC B cells to transition into memory cell phenotype[193].

There is evidence of memory B cells lasting an entire lifetime in both humans and mice. In mice, memory B cells generated against HEL or NP show no reduction in numbers over the animal's lifetime[208,209]. In humans, memory B cells against smallpox have been found 50 years after the exposure that elicited their generation[210209,211]. Conversely, there are different immune responses wherein this is not the case. In mice, memory B cells against malaria merozoite surface protein 1 decline after immunisation[212]. In humans, memory B cells against the conserved stem region of haemagglutinin from influenza A virus decline over a period of 70 weeks, in contrast to those generated against other components of the protein[213]. Therefore, the memory pool is deeply heterogenous in terms of the longevity of the cells.

It appears that a high germline BCR-antigen affinity is associated with the generation of shorter-lived memory B cells. One interesting context for this heterogeneity is memory B cells generated against the algal protein phycoerythrin (PE). C57BL/6 and C.B-17 mouse strains share an *Ighv*

genetic profile, and both form memory B cells against PE with half-lives of only ~80 days[214]. However, BALB/c mice, which have a different naïve pool in terms of *Ighv* sequences, form a stable population of memory cells. Pape et al saw that the memory B cells generated in response to PE in the B6 and C.B17 mice used the same very high affinity germline encoded V_H region. Memory B cells which used this region did have evidence of somatic mutations, but had fewer replacement mutations than memory B cells which used different VH regions[215]. These cells had evidence of SHM and so were most likely GC derived. Gitlin et al. showed that these short-lived memory cells were associated with cross-reactivity to self-antigens in insulin and DNA, and attributed their short life spans to active deletion of these potentially dangerous cells[216]. Pape et al. correlated the generation of short-lived memory B cells with high germline affinity for antigen, specifically attributed to the use of the VH1-81 region. Similarly, using model antigens, it has been shown that BCR-antigen interactions that are too high can favour the development of more transient memory B cells[103,104], and short lived plasma cells[215]. Weisel et al. performed Bromodeoxyuridine / 5-bromo-2'-deoxyuridine (BRDU) pulse chase labelling to address the time point for a generation of long-lived plasma cells and memory B cells, and confirmed these results using an anti-CD40L antibody to destroy the GCs at key time points. They found that un-switched memory B cells were produced earlier in a response, followed by class switched memory B cells and later by long-lived plasma cells. They concluded that less committed humoral effector cells are derived from pre- and early-GC responses, and that more committed cells are from later, more refined GC stages[31,215]. Thus, GC cells may be "programmed" to differentiate into longer lasting memory B cells if they were more effortful for the host to generate.

MEMORY B CELLS CAN SOMETIMES RE-ENTER GERMINAL CENTRES

There is evidence that certain subsets of memory B cells are capable of re-entering GCs upon re-activation. Theoretically, it would be valuable to allow memory B cells that have acquired high affinity for a specific epitope on a pathogen be refined to a related epitope on a related pathogen. However, GC re-entry is in some ways antithetical to the key role of the memory compartment. If the role of memory is to preserve a population of rapidly responding B cells to a specific epitope, then shuttling preserved populations into a new pathway would not only pervert their delicately refined BCR, but also take over a week to yield antibody producing cells. There is also the theoretical risk of successive infections "burning out" the memory pools, if the memory cells are repeatedly shuttled in the GC to produce effector memory cells which are terminal effector cells[217]- although it is important to note that another of the significant outputs of the GC is memory cells[218]. The solution to this paradox lies in the heterogeneity of the memory pool.

While some studies examining transferred memory B cells in irradiated hosts failed to deliver evidence of memory cells re-entering GCs[217], other studies observed an accumulation of mutations in the immunoglobulin V region genes, which was consistent with GC re-entry in tertiary immune responses in mice one year after two earlier immunisations[219]. Dogan et al. lineage marked GC derived cells, using mice a tamoxifen inducible Cre (CreERT2) to indelibly mark cells expressing AID at defined time points. The authors found memory B cell derived cells in the GC, and so concluded memory cells do re-enter the GC[220].

Large scale analysis of gene expression (through microarray) demonstrated the heterogeneity of memory cells, which could be differentiated on the basis of the particular markers CD80 and PD-L2[204]. Memory B cells which expressed both CD80 and PD-L2 carried more extensive V region mutations, and were more likely to have undergone class switch recombination than cells which expressed neither of these markers[204]. That the induction of SHM and class-switching are

irreversible and acute events implies that there is not plasticity in these cell types - that is, they are committed to either profile, and cannot transition between phenotypes within the memory pool.

Dogan et al. observed the engagement of these cells in different compartments and demonstrated that some memory B cells will enter GCs upon re-challenge. Notably, while IgG memory B cells were more likely to differentiate into plasmablasts, IgM^+ memory B cells were more likely to participate in GCs[220]. However, there was greater heterogeneity still within the un-switched population. When IgM^+ $CD80^+$ $PD\text{-}L2^+$ memory B cells were transferred and reactivated by immunisation, there was a strong plasmablast response but no demonstrable participation in the GC[204]. Moreover, IgM^+ $CD80^-$ $PD\text{-}L2^-$ cells could participate in the GC on a timescale similar to naïve B cells. Therefore, it is not exclusively B cell isotype which dictates re-seeding in the GC, but rather previous signals and the history of the B cell[204].

The underappreciated heterogeneity of the memory B cell pool and the difficult-to-reconcile maintenance of low affinity clones in the GC are rarely combined, but are likely critical for contextualising one another. The results described above suggest that the memory cell compartments contain cells which have spent less time GCs, but have acquired some mutations and undergone some selection. These cells may not have acquired the perfecting mutations categorical of the theoretical ideal of a memory B cells we hold but are more likely to be re-engaged in the GC upon re-infection.

The capacity of memory B cells to re-enter GCs and continue to diversify their immunoglobulin genes has been demonstrated as valuable in humans. Memory B cells from a previous dengue virus infections can be reactivated after Zika virus infections, and affinity

mature towards a shared epitope that generates potent bNAbs[218]. Evaluations of humoral responses to drifted influenza viruses showed that, while the plasmablasts had likely derived from the memory B cells, these cells also appeared to have undergone an additional round of SHM. Wrammert et al. found that, following the 2009 H1N1 influenza pandemic, the antibodies generated tended to differ significantly from those generated by seasonal vaccination. The dominant trend in neutralising antibodies was that they were highly cross-reactive and had an unusually high number of SHMs. The authors took these features as evidence that antibodies were from memory B cells that had re-entered GCs and had undergone significant further SHM[221]. It is noteworthy, however, that AID activity and V region mutations are not necessarily restricted to GCs[222,223].

1.3 B CELL RECEPTOR ENGAGES WITH ANTIGEN TO TRIGGER SIGNALLING

BCR engagement by antigen causes a cascade of signalling events that result in changes in the transcription profile and activation status of the cell[224,225]. As discussed above, BCR signalling may be attenuated in GCs, but it is required for a number of processes: for antigen internalisation[106,107], to "license" CD40L signals during selection[97], and to improve selection under conditions where T cell help is limiting[130]. Additionally, gene expression analysis of LZ cells showed evidence of ongoing BCR signalling[131]. It is also noteworthy that GC B cells internalise antigen with greater affinity discrimination than naïve B cells, and form immunological synapses with deviated architecture than in naïve B cells[122]. Therefore, the basic nature of BCR signal warrants some discussion.

1.3.1 B CELL RECEPTOR ENGAGEMENT WITH ANTIGEN TRIGGERS A CASCADE OF SIGNALLING EVENTS

Several mutually exclusive models have been proposed to account for the manner in which this extracellular event is communicated across the cell membrane[226,227]. The cytoplasmic domains of Igα and Igβ contain immune tyrosine activation motifs (ITAMS). All models of antigen-mediated BCR signalling involve phosphorylation of the ITAMs as a key event. Phosphorylation is known to be mediated by the immune tyrosine kinases Lyn and Syk. Lyn phosphorylates the first tyrosine[228,229], and Syk phosphorylates the second tyrosine, which in turn permits Syk to bind to the immunoreceptors, which triggers signal amplification[230–232]. The phosphorylation of the ITAMs initiates a cascade of sequential activation events which results in the formation of the signalosome. The signalosome is an assembly of signalling molecules that includes Syk, phospholipase-Cγ2 (PLCγ2), phosphoinositide3-kinase (PIK), Bruton's tyrosine kinase (Btk), Vav, and adaptor proteins such as B cell linker protein (Blnk)[233].

There is consensus that engaged BCRs must differ fundamentally from those on the surface of a resting state. However, there is opposing evidence suggesting that BCR engagement by soluble antigen causes complexes to aggregate[234], and that antigen engagement may disrupt BCR multimers [235].

The evidence that the BCR complexes on resting B cells exist largely in oligomers first came from biomechanical methods, in which BCRs isolated from detergent solubilised B cells were found mostly to be in large aggregates[236]. Later, Kläsener et al. used a modified proximity ligation assay to analyse how the association of BCR molecules with each other changes after B cell activation. The results indicated that individual IgM molecules on resting B cells were closely associated, between 10-20nm apart, whereas the distances between molecules increased to >20nm (but below 40nM) after activation[237].

The alternate model involves BCRs existing as monomers on the surface of B cells, and that the formation of the signalosome depends upon the close proximity and restriction of diffusion in the BCR within clusters. STED super resolution microscopy[238] and fluorescence resonance energy transfer (FRET)[239] implied that, in resting B cells, the BCR is monomeric. Furthermore, using FRET, Tolar et al. showed that BCRs were not in immediate proximity to each other in resting cells but that they became closer upon BCR engagement. FRET was detected following BCR engagement, indicating that the BCR were clustering, however signals were short-lived and thus suggested associations were most likely transient. In these assays, loss of FRET signal depended on phosphorylation of Igα and Igβ by Src family kinase Lyn. The authors therefore proposed a model in which BCR antigen engagement brought the molecules into contact, wherein the signalling chains were phosphorylated and the molecules subsequently opened. As such, they proposed that the initial oligomerisation was required for the formation of the signalling complex[239]. Consistent with this model, a separate study by Sohn et al. observed that, as BCR clusters grew and more molecules were acquired at the synapse, the FRET between BCRs did not continue to increase. Therefore, this stage must have occurred while cells were still in the "open" confirmation[240]. In this model, close proximity was only required at the initiation of the signalling events.

Other groups have used super-resolution microscopy for determining the organisation of molecules on the surface of resting B cells, and the ways in which these change following activation. The results regarding exact nature of the resting BCR and activated BCR differ between groups depending on their BCR isotypes, the cells being observed (human, mouse, transduced cell line) and the methodology of the analysis. However, all groups found that, in resting B cells, BCRs were in low order, and that the activation event triggered the BCRs to come into closer contact.

Lee et al. utilised direct stochastical optical reconstruction microscopy (dSTORM) to visualise BCR complexes on the surface on healthy human B cells. They saw that the BCRs exist as monomers, which may form signalling active oligomers containing as few as five BCRs. As these come into close contact, they switch into the "open" confirmation, allowing other molecules to join the expanding oligomers[240]. Other groups used similar techniques to find that the BCR on resting B cells was more likely to be found in nanoclusters, and that BCR engagement is associated with the formation of oligomers and decreased BCR motility within those oligomers[241–243]. These data lead to a model in which, in resting B cells, the BCRs are in large structures which allow them to freely diffuse, forming larger structures in which they are immobilised upon antigen engagement[241].

Furthermore, there are mutually exclusive models of how alterations in BCR configuration allows for the ITAMs to be phosphorylated. Firstly, it is possible that the opening of BCR oligomers may allow kinases such as Syk to access to ITAMs that were previously buried within tight oligomers. Syk then phosphorylated the ITAMs and associated with the phosphorylated ITAMs, which subsequently induces further opening of the BCR molecules- in an "inside out" model of BCR signalling[237]. This model involves BCRs making a transition from tightly packed oligomers in resting B cells to loosely packed oligomers on activated cells.

Conversely, the kinetic segregation model holds that, following engagement with antigen, the BCR multimerises, and that this leads to the exclusion of the bulky transmembrane phosphatases CD45 and CD148 that otherwise prevent receptor phosphorylation. Constitutively active Src kinases Lyn phosphorylate the ITAMs on the Igα and Igβ signalling domains, and because the phosphatases are physically excluded after receptor ligation this goes unchecked,

resulting in the ITAMs remaining phosphorylated. This model is evidenced by the fact that that B cells lacking both CD45 and CD148 have impaired BCR signalling[244].

In contrast, the conformational change model postulates that the binding of the extracellular domains of the BCR to antigen confers a conformational change in the intracellular component, allowing access to the ITAMs and leading to their phosphorylation. This model requires a degree of flexibility on the part of the BCR, which has neither been proven nor disproven (these models are reviewed in greater detail in[245]). Ultimately this is an area of much debate, but all theories converge on the phosphorylation of the ITAMs being a critical event.

The cytoplasmic domains of the BCR also maintain a negative regulatory function. While the ITAMs are critical for BCR signalling and Syk recruitment, they also have a negative regulatory effect, which are specifically attributed to the ITAMs of the Igα[246–248]. There are also negative regulators of BCR signalling, preventing an inappropriate escalation of the cascade. Negative regulation of BCR signalling by CD22 is connected to BCR activation though Lyn kinase, which is phosphorylated as part of the signalling cascade initiated by ITAM phosphorylation[249]. CD22, having been phosphorylated by Lyn, leads to the recruitment of SHP-1 tyrosine phosphatases to the BCR[250–253]. However, specifically disrupting the ITAMs of the Igβ chain does not disrupt CD22 or SHP-1 phosphorylation. Cbl-b and c-Cbl also negatively regulate BCR signalling; as ubiquitin ligases[254], they target Syk for degradation. In their absence, Syk is not targeted for degradation[255,256], and with BCR signalling remaining unresolved, the BCR is not internalised properly.

1.3.2 B CELL RECEPTOR ENGAGEMENT WITH ANTIGEN TRIGGERS MASSIVE CHANGES IN THE MOLECULAR ARCHITECTURE OF THE CELL

In vivo, B cells mainly encounter antigen presented on cell membranes. In the B cell follicle, there is evidence of antigen being predominantly associated with FDC plasma membranes[256,257]. The molecular changes associated with BCR signalling can be divided into three stages: the formation of microclusters, membrane spreading, and the contraction and formation of the immunological synapse. As described above, immediately following contact with membrane associated antigen, the initial BCRs aggregate into microclusters. Whether the role of the microclusters is to hold the BCR molecules more tightly or more loosely, it is a key inciting incident in the BCR signalling cascade. The formation of these microclusters, however, does not require ITAM phosphorylation[98,258].

Following the initial antigen engagement, B cells undergo morphological changes which allow the membrane to spread across the antigen-presenting surface[259]. This represents a form of antigen affinity discrimination, as the extent of the spread relates not only to the density of the antigen but also to the affinity of the BCR-antigen interaction. A higher affinity interaction leads to a greater spread, which leads to more antigen being collected. Thus, the extent of antigen collection is related to the affinity of the BCR-antigen interaction. Cell membrane spreading requires B cell signalling at the edge of the membrane and profound cytoskeletal reorganisation. Fleire et al. demonstrated this dependence by demonstrating that BCRs in which signalling was ablated by disruption of the Igβ ITAMs showed a complete lack of ability to spread[260].

After a rapid burst of spreading of BCR molecules across the surface of the B cell, a contraction phase begins. The contraction phase results in the formation of a mature immunological synapse[260]. The immunological synapse, first described in T cells, is the prototypical immunoglobulin signalling complex, and consists of a centre encircled by a

peripheral supramolecular activation cluster (cSMAC and pSMAC respectively). The cSMAC is made up of the immunoreceptors, and the pSMAC is made up of a ring of adhesion molecules, including LFA-1 and VLA-4[260]. Given that, in T cells, calcium flux occurs before the formation of a mature immunological synapse[259], the structure may be more important for internalisation of antigen and the resolution of the signal. This is consistent with notions that BCR signalling and internalisation are separate events[261].

Nowosad et al found that the synapses formed by GC B cells differ from the canonical structures described above. Unlike naïve and memory cells, GC B cells form synapses with small antigen clusters around the periphery of the synapse, as opposed to the antigen being gathered in the centre of the synapse. The different nature of the BCR synapse is dictated by the effects of the GC differentiation program on the B cell cytoskeleton. While naïve cells internalise antigen by pinching the antigen presenting membrane, GC B cells physically separate the antigen from the FDCs before endocytosis. The authors postulated that this allowed GCs to compare the affinity of its own BCR and that of the antibody associated with the antigen in immune complex, and as such GC B cell would need to out-compete existing antibody specificities[97].

Following antigen engagement, the internalisation of the BCR is critical both for antigen presentation and for resolving signal[262]. Having been internalised, the Igβ signalling chain is ubiquitinated, targeting the BCR-antigen complex for antigen processing[263,264]. Following BCR engagement, the src-like adaptor protein SLAP) associates with the BCR through its SH2 domains, allowing c-cbl to ubiquitinate the lysines within the ubiquitin domains of Igβ molecule such that it is targeted for degradation[265,266]. While *ex vivo* high resolution microscopy of BCR-antigen interactions showed that proximal signalling was required for internalisation of antigen in GC B cells[267,268], comparison of the phosphorylation state of BCR which are internalised or maintained

on the surface of B cells has indicated that signalling and internalisation are 'mutually exclusive' fates[97]. Therefore, the exact relationship between BCR signalling and internalisation is somewhat muddied.

1.3.3 DO MATURE B CELLS REQUIRE "TONIC SIGNAL"?

Early studies from the Rajewsky lab showed that inducibly deleting the BCR heavy chain from naïve B cells causes cell death[269], and reconstituting the PI3 kinase signalling can rescue $BCR^{negative}$ cells from apoptosis[270]. These findings led to the concept that B cells require a form of tonic i.e. antigen-independent) signalling for their maintenance. While this remains the most popular view within the field, it is important to note that a more contemporary study suggested that the loss of $BCR^{negative}$ may in fact relate to ER-stress arising from the build-up of truncated and unpaired chains, rather than due to a loss of signals[271]. As discussed above, the BCR is in a fundamentally different form depending on whether it is resting or activated, and the postulated tonic signal has always been somewhat nebulous. It has always been unclear whether any tonic signal requires low level interactions with self-antigens, or a basal signal delivered in the absence of stimulation.

Mature B cells can be rescued after losing their surface BCR by either transgenic expression of constitutively active downstream components of the BCR signalling cascade, like PI3K; or by ablation of PTEN – a key negative regulator of PI3K-AKT signalling. Induction of constitutively active PI3K (p100δ), and ablation of its repression, have somewhat equal effects on mature B cells having lost endogenous BCR. Given that ablation of the negative regulator of PI3K will rescue cells which have lost surface BCR, Srinivasan et al. postulated that mature B cells display some basal PI3K activity, and signal through the BCR simply modulates this[270].

Both the constitutively active PI3K and the deletion of PTEN caused Akt phosphorylation to levels similar to (or slightly higher than) that seen in resting mature B cells[270]. Therefore in naïve mature B cells phosphorylation of Akt depends on BCR signalling through the PI3K pathway[270]. An important Akt target is FOXO1, which it phosphorylates and thereby causes its nuclear exclusion. Mature B cells that have lost surface BCR can also be rescued by ablation of FOXO1. However, unlike cells that have been rescued by constitutively active PI3K or ablation of PTEN, cells that have been rescued by ablation of FOXO1 have some aberrant properties (e.g. defects formation of marginal zone cells; defects in expression of CD62L and CD23; and also the cells were very small)[270].

It is important to note that the activation of PI3K – apparently needed to rescue cells that have lost surface BCRs – may or may not require phosphorylation of BCR ITAMs. PI3K may be activated through the phosphorylation of the adaptor protein BCAP and the CD19 co-receptor by protein tyrosine kinases, in particular Syk[272]. It is also possible that the BCR mediated signal in resting B cells does not involve engagement with external ligands. In the absence of cognate antigen mediated phosphorylation of ITAMS, BCR is connected to PI3K signalling through the GTPase TC21, which directly recruits the p110δ catalytic subunit of PI3K to non-phosphorylated ITAMs[273].

Following work by Lam et al., the general consensus became that cells required a signal to survive[274,275]. The nature of the "tonic" or "maintenance" signal required was, however, somewhat fluid. The term "tonic" is also used to describe the ligand-independent signalling that occurs in pre-B cells through auto-aggregation of the pre-BCR[276] and also in BCR signalling CLL B cells, in which pathological auto-aggregation of the BCR triggers the positive feedback loop of signalling described[277,278]. All models of BCR signalling involve a difference in the BCR state

between engaged and resting cells, whether involving BCR engagement (crosslinking)[230,279] leading to kinetic segregation or conformational change; or if they involve BCR engagement resulting in disruption of BCR oligomers[237,280,281]. Whether the tonic BCR signal seemingly required by mature B cells was more similar to these signals, or was related to low affinity interactions with self-antigens, was unclear.

Conversely, alternate models to assess the necessity of a "tonic" BCR signal in mature B cells showed the opposite effect. While antigen dependent BCR signalling requires that the BCR interact with Syk, as does the signal through the pre-BCR and the BCR signal in CLL[276,278,282], inducibly ablating Syk in mature B does not appear to disrupt resting B cell survival[283]. If activated, engaged BCR differs fundamentally from the BCR at rest, either in terms of its structure or in terms of its functional association with other BCR and co-receptors, then what "tonic" signalling means became murky.

Furthermore, when the surface BCR is ablated by inducible deletion of the Igα signalling chain, rather than deletion of the heavy chain, the results are very different. While genetic deletion of the Igα chain prevents surface expression of the BCR complex, just as deletion of the heavy chain does, cells can survive *in vivo* for ~20 days[271]. Loss of Igα seems to be tolerated in a way that loss of heavy chain is not, regardless of the fact that both phenotypes should have the same profile in terms of loss of "tonic" BCR signal. When cells that have lost heavy chain or signalling chain were studied in parallel, differences in unfolded protein response (UPR) emerged. Levit-Zerdoun et al. related this to the failure in survival of these cells. It seemed possible that the build-up of unpaired light chain triggered the UPR, in a way that ablation of a signalling chain would not, although the reasons that this might happen are not clear[271]. There are components of the UPR which interlace with relevant downstream signalling components.

For example, Akt can directly phosphorylate and inhibit UPR factor PERK. Activation of the PI3K signalling pathway may simply promote the longevity of cells that have lost endogenous BCR by dampening the UPR and desensitising them to death through the UPR. Conversely, inhibition of Akt has been shown to sensitise cells to ER stress induced apoptosis[284,285].

Loss of the ITAM motif of Igα, unlike loss of the whole Igα protein, leads to loss of mature B cells similar to the loss seen upon heavy chain ablation[286].That cells which have lost Igα last longer than cells which have induced expression of a truncated Igα molecule lacking ITAMs indicates that the Igα ITAMs may have a specific role, which is only required when the BCR is on the surface (but not required when it is not on the surface), it must therefore be supplemental to transducing signal. It is feasible that inside the resting BCR the Igα and Igβ inhibit one another, and in the absence of the Igα tail the uncontrolled signalling results in cell death.

1.3.4 THE B CELL RECEPTOR ALSO SIGNALS INDEPENDENTLY OF ANTIGEN

One instance in which non-antigen mediated signalling has been demonstrated is in the pre-B cell through the pre-BCR. The tonic signalling that occurs in pre-B cells following recombination is interesting, as the exchange of the pre-BCR for the mature BCR has obvious conceptual parallels with the exchange of the pre-SHM BCR for the post-SHM BCR. The pre-BCR is constructed of a recombined heavy chain and a surrogate light chain. Its function is to ensure that heavy chain recombination occurs successfully before the cell proceeds with light chain recombination. The surrogate light chain is composed of a PreVB and a λ5 components. The negatively charged alanine residues in the λ5 component of the surrogate light chain are required for signalling through the BCR[287]. There is evidence of signalling through the pre-BCR *in vitro* in the absence of stromal cells, and as such it is most likely that this signalling is not antigen mediated[287]. However, the tonic

signalling which occurs in the pre-BCR does not bear resemblance to the proposed tonic signalling in resting mature B cells. Analysis of the crystal structure of the pre-BCR revealed that the surrogate light chains caused the pre-BCR to auto-aggregate, triggering signalling[288]. Additionally, it does requires functional signalling chains[289]; it is Syk dependent; and most significantly, it is a restricted acute event.

1.4 CONCEPTUALISATION GERMINAL CENTRE BIOLOGY REQUIRES AN UNDERSTANDING OF EXCHANGE OF THE B CELL RECEPTOR

A critical question in GC biology is whether the BCR is exchanged following SHM in DZs before GC B cells enter LZs. This question has implications for selection in the GC. It would appear to be very inefficient if cells were regularly selected in the LZ on the basis of a BCR they no longer encode. However, this could possibly represent a mechanistic explanation for the maintenance of low affinity cells under the selective pressures of the GC.

Having completed SHM, it is possible that cells transition into the LZ before fully replacing their pre-SHM BCR, without this disrupting positive selection. If selection only involves positively amplifying higher affinity clones, then maintaining copies of the pre-SHM BCR on the surface would not pervert selection. However, if GC B cell selection involves an element of negative selection, wherein cells with profoundly damaged BCRs are removed, then it would be required that cells transition into the LZ only expressing BCRs that they genetically encode. In earlier sections, discussed that the GC is probably a clonal diversifier as well as an affinity enhancer – diversification here meaning that it generates variants and disposes of the junk. Therefore, an analysis of the exchange of the BCR following SHM is critical for a full understanding of selection.

Beyond GCs, there are two other contexts in B cell biology where BCR complexes must be actively replaced: in positively selected pre-B cells when the pre-BCRs (with surrogate light chains) are replaced with mature BCRs[290], and in B cells which are undergoing receptor editing, in which the kappa light chain of an autoreactive B cell is replaced with a new lambda chain to avoid autoreactivity[291]. The exchange event represents an effort on behalf of cells to ensure that their receptor identities match that of their genes. However, these contexts do not share the immediacy of the issue of BCR exchange in GCs. ~50% of DZ cells transition into the LZ every six hours[100]. Therefore, the time period between mutation and selection is presumably very short. Moreover, the estimated half-life of the BCR on the surface of mature naïve (follicular) B cells has been estimated to be in the rage of 20-40 hours, which would be equivalent to several average DZ to LZ cycles. Therefore, if the regulation of BCR protein turnover was the same in GC B cells as in follicular B cells, the former would usually undergo "selection" while still expressing a protein that is not reflective of the antibody genes that they carry.

CHAPTER TWO: METHODS

2.1 TABLES OF KEY RESOURCES

TABLE 2.1A ANTIBODIES

Fluorophore, target and clone	Source	Identifier
APC Cy7 B220, clone Ra3 6b2	Biolegend	Cat# 103224
BV785 B220, clone Ra3 6b2	Biolegend	Cat# 103245
FITC B220, clone Ra3 6b2	Biolegend	Cat# 103206
FITC CD19, clone 6d5	Biolegend	Cat# 115506
BV421 CD138, clone 2812	Biolegend	Cat# 356516
BUV 395 IgD, clone 11-26c-2a	BD bioscience	Cat# 564274
PerCP Cy5.5 IgD, clone 11-26c-2a	Biolegend	Cat# 405710
BUV 395 Ig, clone 11-26c-2a	BD bioscience	Cat# 565988
PE Cy7 CD95, clone Jo2	BD bioscience	Cat# 557653
APC Cy7 CD38, clone 90	Biolegend	Cat# 102728
PB T/B cell activation marker, clone Gl7	Biolegend	Cat# 144614
PE CD79b, clone HM79 12	Biolegend	Cat# 132804
FITC CD79b, clone HM79 12	Biolegend	Cat# 132806
PerCP Cy5.5 CD79b, clone HM79 12	Biolegend	Cat# 132810
Purified CD79b, clone HM79 12	Biolegend	Cat# 132802
PE IgG1, clone Rmg1-1	Biolegend	Cat# 406608
PE IgMa, clone MA-69	Biolegend	Cat# 408602
FITC IgMa, clone MA-69	Biolegend	Cat# 408606
Biotin CXCR4 (CD184), clone L276F12	Biolegend	Cat# 146516
AF647 CD83, clone Michel-19	Biolegend	Cat# 121514
PE Cy7 CD83, clone Michel-19	Biolegend	Cat# 121518
PE Ly5.2 (CD45.1), clone A20	Biolegend	Cat# 110708
FITC Ly5.2 (CD45.1), clone A20	Biolegend	Cat# 110706
PerCP Cy5.5 Ly5.2 (CD45.1), clone A20	Biolegend	Cat# 110728
FITC Ly5.1 (CD45.2), clone 104	Biolegend	Cat# 109806
PerCP Cy5.55 Ly5.1 (CD45.2), clone 104	Biolegend	Cat# 109828
BV785 Ly5.1 (CD45.2), clone 104	Biolegend	Cat# 109839
APC Cy7 Igκ, clone RMK-45	Biolegend	Cat# 409504
Active Caspase 3, clone C92-605	BD Pharmingen	Cat# 559565
BCL6, clone K112-91	BD biosciences	Cat# 561525
PB BRDU antibody, clone MoBu	Invitrogen	Cat# B35129
Purified Igk, polyclonal	Southern biotech	Cat# 1050-01
HRP anti-mouse IgG, polyclonal	Southern biotech	Cat# 1033-05
HPR anti-human IgG, polyclonal	Dako	Cat# P0214
Goat F(ab')2 Anti-Human IgG-UNLB, polyclonal	Southern biotech	Cat# 2042-01

Alexa Fluor® 488 AffiniPure Fragment Donkey Anti-Mouse IgG (H+L), polyclonal	Jackson Immunoresearch	Cat# 715-547-003
Biotin Ea52-68 peptide bound to I-Ab Monoclonal Antibody (eBioY-Ae)	eBioscience™, Thermo fisher scientific	Cat# 13-5741-82
IgG $F_{(ab)}$ Fragment	Jackson Immunoresearch	Cat# 115-067-020

TABLE 2.1B EXPERIMENTAL MODELS, MICE

Mouse name	Source	Identifier
SWHEL mice	Phan et al. 2003	N/A
MD4 transgenic mice	Goodnow et al. 1988	N/A
OT-II transgenic mice	Barnden et al. 1998	N/A
R26p-Fucci2 mice Abe et al., 2013,	R26p-Fucci2 mice Abe et al., 2013,	
Bcl-2tg transgenic mice	Strasser et al. 1991	N/A
AIDKO *Aicda*-/- mice	Muramatsu et al. 2000	N/A
B1-8f mice	Lam et al 1997	N/A
AID-Cre	Robbiani 2008	N/A
Rosa26-flox-DTR	Buch et al 2005	N/A
C57BL/6 mice	Oxford University core breeding facility	N/A
B6SJLCD45.1 mice	Oxford University core breeding facility	N/A

TABLE 2.1C CELL STRAINS

Cell line name	Source	Identifier
NB-21.2D9 feeder cells	Kuraoka, M. et al. Immunity 44, 542–552 (2016).	N/A
293T cells	Gift from Cyster Lab	Also available from Merk 85120602
PlatE cells	Gift from Cyster lab	Also available cell biolabs RV-101

TABLE 2.1D OLIGONUCLEOTIDES

Name	Source	Identifier
1st Step SWHEL forward (GTTGTAGCCTAAAAGATGATGGTG)	Integrated DNA Technologies	N/A

1st Step SWHEL reverse (GATAATCTGTCCTAAAGGCTCTGAG)	Integrated DNA Technologies	N/A
2nd Step SWHEL forward (TTGTAGCCTAAAAGATGATGGTGTTAAGTC)	Integrated DNA Technologies	N/A
2nd Step SWHEL forward (CAACTTCTCTCAGCCGGCTC)	Integrated DNA Technologies	N/A
SWHEL for cloning forward (AgeI cut site) (CGATACCGGTGTACATTCTGACGTGCAGCTTCAGGAGTCAGGAC)	Integrated DNA Technologies	N/A
SWHEL for cloning reverse (SalI cut site) (GCATGTCGACGCTGCAGAGACAGTGACCAGAGTCCC)	Integrated DNA Technologies	N/A
SNAP reverse ATTCTCGAGCATCATACCCAGCCCAGGCTTGC ATTCTCGAGCATCATACCCAGCCCAGGCTTGC	Integrated DNA Technologies	N/A
SNAP forward linker 1 GCTTCAACAGGAATGAGTGTGGTGGAGGTGCAGCTTCAATGGACAAAGACTGCGAAATGAAGC	Integrated DNA Technologies	N/A
SNAP forward linker 2 GCTTCAACAGGAATGAGTGTGGATCTGGTTCCGGAATGGACAAAGACTGCGAAATGAAGC	Integrated DNA Technologies	N/A
SNAP forward linker 3 GCTTCAACAGGAATGAGTGTGGAGGTGGCGGTTCTGGCGGTGGAGGCTCAATGGACAAAGACTGCGAAATGAAGC	Integrated DNA Technologies	N/A
Igκ forward ATTGCGGCCGCGCCACCATGGATATTGTGCTAACTCAGTCTCCA	Integrated DNA Technologies	N/A
Igκ reverse CACTCATTCCTGTTGAAGCTCT	Integrated DNA Technologies	N/A
IgM forward (ACACTGACGACATGGTTCTACAGAGGTGCAGCTGCAGGAGTCTGG)	Integrated DNA Technologies	N/A

IgM Reverse 1st step (TGGGAAGGTTCTGATACCCTGGATG)	Integrated DNA Technologies	N/A
IgM Reverse 2nd step (TACGGTAGCAGAGACTTGGTCTTAGTTCCAGGTGAAGGAAATGGTGC)	Integrated DNA Technologies	N/A
IgG forward (ACACTGACGACATGGTTCTACAGAGGTGCAGCTGCAGGAGTCTGG)	Integrated DNA Technologies	N/A
IgG reverse 1st step (AGAAGGTGTGCACACCGCTGGAC)	Integrated DNA Technologies	N/A
IgG reverse 2nd step (TACGGTAGCAGAGACTTGGTCTGCTCAGGGAARTAGCCCTTGAC)	Integrated DNA Technologies	N/A

TABLE 2.1E PLASMIDS

Name	Source	Identifier
AbVec2.0-IGHG1	Tiller 2008	available at Addgene #80795
Igκ apovec	Tiller 2008	available at Addgene #80795
MSCV IRES Thy1.1	Wu, 2006	available at addgene #24828
MSCV Loxp GFP	Green 2011	N/A
PQY-CD90.1	Yang 2016	N/A
Igβ-Fluorescent Timer-Fast	Addgene	N/A
Igβ- Fluorescent Timer -Medium	Addgene	N/A
SNAP tag		Gift from David Lab
Mouse Hy10 variable region		Gift from Davis Lab

TABLE 2.1F TAQMAN X20 ASSAYS

Name	Source	Identifier
Pax5	Applied Biosystems	Mm00435501_m1
Prdm1	Applied Biosystems	Mm00476128_m1
Hprt	Applied Biosystems	Mm03024075_m1
Gapdh	Applied Biosystems	Mm99999915_g1
Ighm	Applied Biosystems	Mm01718955_g1

Cd79a	Applied Biosystems	Mm00432423_m1
Cd79b	Applied Biosystems	Mm00434143_m1
SW_{HEL} (probe 1) Forward TTCTGTACCTGTTGACAGCC Reverse GAGAAGGTTTCACGAGGCTAG Probe AAGCTGCACGTCTGACAGGATACC	Applied Biosystems	Custom (AII1OEO)
SW_{HEL} (probe 2) Forward CACGGTGGACAAAAAACTTGAG Reverse TTTGGAGGGAAGATGAAGACG Probe AGGTTAGGAGCTGGGCATTTGTGA	Applied Biosystems	Custom (AIHSP8G)
Igkc Forward CAACTGTATCCATCTTCCCACC Reverse CCATTTTGTCGTTCACTGCC Probe AGGTGCCTCAGTCGTGTGCTT	Applied Biosystems	Custom (AILJKQ4)

TABLE 2.1G CHEMICAL, PEPTIDES, AND RECOMBINANT PROTEINS

Name	Source	Identifier
Lysosyme from chicken egg white (Hen Egg Lysosyme, HEL)	Sigma Aldrich	Cat# L6876-5g
Albumin from chicken egg white	Sigma Aldrich	Cat# A5503-5g
Sigma Adjuvant system	Sigma Aldrich	Cat# S6322-1VL
Baytril	Bayer	N/A
Fisher BioReagents™ Proteinase K	Fisher Scientific	Cat #10172903
Q5 High Fidelity DNA polymerase	New England Biolabs	Cat #M0491L
KAPA HiFi HotStart Ready Mix PCR Kit	Roche	Cat #07958854001
Phusion® High-Fidelity PCR Master Mix with HF Buffer	New England Biolabs	Cat# M0531L
Nuclease free water	Ambion	Cat# AM9937
Random Hexamer Primer	Thermo	Cat# SO142
RNasin® Ribonuclease	Promega	Cat# 9PIN211
RNaseOUT™ Recombinant Ribonuclease Inhibitor(Invitrogen)	Invitrogen	Cat #10777019
SuperScript™ III RT	Invitrogen	Cat# 18080-093
SuperScript™ II Reverse Transcriptase	Thermo Fisher Scientific	Cat #18064014
DTT (1,4-Dithiothreitol)	Sigma Aldrich	Cat #3483-12-3

Betaine (5M)	Sigma Aldrich	Cat #107-43-7
MgCl2 (1M)	Thermo fisher	Cat #AM9530G
AFIIII	New England Biolabs	Cat #R0541S
NsiI-HF	New England Biolabs	Cat #R3127S
AgeI-HF	New England Biolabs	Cat# R0552L
SalI-HF	New England Biolabs	Cat# R3138M
EcoRV-HF	New England Biolabs	Cat #R3195M
NotI-HF	New England Biolabs	Cat# R3189M
BglII-HF	New England Biolabs	Cat #R0144M
Antarctic Phosphatase	New England Biolabs	Cat# M0289L
T4 ligase	New England Biolabs	Cat# M0202M
Recombinant murine IL-4	Peprotech	Cat# 214-14
Recombinant murine Il-3	Biolegend	Cat# 579702
Recombinant murine Il-6	Biolegend	Cat# 575702
Recombinant murine SCF	Biolegend	Cat# 573902
NP-OVAL	Santa Cruz Biotech	Cat# sc-396355
Alum (Aluminum potassium sulfate dodecahydrate)	Sigma Aldrich	Cat# A6435-100G
Tamoxifen	Sigma Aldrich	Cat# T5648-1G
5-fluorouracil	Sigma aldrich	Cat #F6627
Diphtheria toxin	Merk Millipore	Cat# 322326
Critical commercial assays		
Streptavidin Qdot 605	Thermo fisher scientific	Cat# A63881
Streptavidin BUV 395	BD Biosciences	Cat# 564176
AF467, Alexa Fluor™ 647 Protein Labelling Kit	Thermo fisher scientific	Cat# A20173
TMB Substrate Set	Biolegend	Cat #421101
Stop solution for TMB substrate	Biolegend	Cat #423001
eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set	Invitrogen	Cat #00-5523-00

Fixation/Permeabilisation Solution Kit	BD Biosciences	Cat #554714
Permeabilisation Buffer Plus	BD Biosciences	Cat #561651
NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®	New Enlgand Biolabs	Cat #E7645S
NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 1)	New England Biolabs	Cat #E7600S
USER® Enzyme	New England Biolabs	Cat #M5505S
Access array primers	Fluidigm	Cat# PN 100-4161 D1
Adaptor for Illumina	New England Biolabs	Cat #E7337G
MiSeq Reagent Kit v2 (500-cycles)	Illumina	Cat# MS-102-2003
5-Bromo-2′-deoxyuridine	Sigma Aldrich	Cat# B5002
Qubit™ dsDNA HS Assay Kit	Invitrogen	Cat #Q32854
QIAquick Gel Extraction Kit	Qiagen	Cat No./ID: 28704
QIAquick PCR Purification Kit	Qiagen	Cat No./ID: 28104
QIAprep Spin Miniprep Kit (250	Qiagen	Cat No./ID: 27106
HiSpeed Plasmid Midi Kit (25)	Qiagen	Cat #12643
NEB® 5-α Competent E. coli (High Efficiency)	New England biolabs	Cat# C2987U
CellDirect One-Step qRT-PCR Kits	Invitrogen	11753-100
SUPERase-In RNase Inhibitor	Ambion	AM2694
Low EDTA 1 xTE buffer	Thermofisher scientific	12090-015
96.96 Gene Expression CHIP	Fluidigm	# BMK-M-96.96
2 x Assay loading reagent	Fluidigm	# 100-7611
TaqMan Universal PCR MasterMix (2x)	Thermofisher scienific	4304437
20 x GE sample loading reagent	Fluidigm	# 100-7610

TABLE 2.1H SOFTWARE AND ALGORITHMS

Name	Source	Identifier
FlowJo 10.1r5 (Treestar inc)	https://www.flowjo.com/	RRID:SCR_008520

Graphpad prism version 7	https://www.graphpad.com/scientific-software/prism/	RRID:SCR_002798
IgBlast tool (NCBI)	https://www.ncbi.nlm.nih.gov/igblast/	RRID:SCR_002873
flowCore, R package version 1.44.0	http://bioconductor.org/packages/release/bioc/html/flowCore.html	RRID:SCR_002205
Adobe Illustrator CC 2018	https://www.adobe.com/uk/products/illustrator.html	RRID:SCR_014198
PANDAseq	https://doi.org/10.1186/1471-2105-13-31	RRID:SCR_002705
Needle	https://www.ebi.ac.uk/Tools/psa/emboss_needle/	N/A
Lymanalyzer	https://omictools.com/lymanalyzer-tool	OMICS_10193
Custom perl scripts (find_stop_codons.pl)	http://sara.molbiol.ox.ac.uk/public/obannard/scripts/find_stop_codons_pl.txt	N/A
Custom perl scripts (make_summary.pl).	http://sara.molbiol.ox.ac.uk/public/obannard/scripts/make_summary_pl.txt	N/A

TABLE 2.1I MISCELLANEOUS REAGENTS

Name	**Source**	**Identifier**
Agencourt AMPure XP PCR purification beads	Beckman coulter	Cat #A63881
Tween20	Sigma Aldrich	Cat #9005-64-5
Triton-x	Sigma Aldrich	Cat #9002-93-1
Fetal Bovine Serum, qualified, heat inactivated	Gibco	Cat #10500 056
Fetal bovine serum	Gibco	Cat #10500064
Hepes	Gibco	Cat #15630-054
Glutamine	Thermo fisher scientific	Cat #21051040
Sodium Pyruvate	Gibco	Cat #11360-039
Penicillin and Streptomycin	Thermo fisher scientific	Cat# 15140122
Lipofectamine 2000	(Invitrogen)	Cat# 11668030
Optimem media	Thermofisher Scientific	Cat #31985062
Polybrene	Sigma Aldrich	Cat# TR-1003-G
MEM Non-essential amino acids solution (x100)	Thermo fisher scientific	Cat #11140050

Normal mouse serum	Thermo fisher scientific	Cat #24-5544-94
tRNA	Thermo fisher scientific	Cat #AM7119
EDTA	Invitrogen	Cat #55687
Corn oil	Fisher scientific	Cat #8001-30-7
Sheep red blood cells	Scientific laboratory supplies	Cat #SR0051B

2.2 MOUSE MODELS

SwHEL[292], MD4[293], OT-II[294], R26p-Fucci2[295], Emu-Bcl-2tg[296], *Aicda*$^{-/-}$[297], B1-8f[269], AID-Cre[298], Rosa-flox-Cre[298], mice have been described previously. IgM-KikGR mice were made by Cyagen using Crisp/Cas9 to target the endogenous allele in zygotes. The KikGR was associating with the IgM exon with a flexible linker. Positive founders were identified by PCR and correct gene insertion was assessed by southern blot analysis (performed by the vendors). C57BL/6 and B6SJLCD45.1 mice were purchased from the University of Oxford core breeding facility.

Animals were housed in specific pathogen–free enclosures at the University of Oxford Biomedical Sciences facility. All experiments were approved by a project license granted by the UK Home Office and were also approved by the Institutional Animal Ethics Committee Review Board at the University of Oxford. All mice were over 6 weeks of age and a mixture of male and female mice were used. The mouse lines obtained from other laboratories are described above and may require a Material Transfer Agreement (MTA) with the providing scientists.

2.3 MOUSE TREATMENTS

IMMUNISATION AND ADOPTIVE TRANSFER

For HEL-specific GC responses, $5x10^4$ to $1x10^5$ MD4, SW_{HEL} or SW_{HEL}x R26p-Fucci2 cells were co-transferred with an equal number of OT-II cells into C57BL/6 or B6SJLCD45.1 hosts by intravenous injection. Mice were immunised one to three days later with 50µg of HEL conjugated to ovalbumin (HEL-OVA) with 100µL of Sigma adjuvant system (Ribi), either i.p. or subcutaneously at the flank as previously described[101].

For NP specific GC responses $5x10^4$ to $1x10^5$ B1-8fx R26p-Fucci2 cells were co-transferred with an equal number of OT-II cells into C57BL/6 or B6SJLCD45.1 hosts by intravenous injection. Mice were immunised i.p. with NP-OVA (Santa Cruz biotech) in alum (Sigma Aldrich).

For polyclonal responses, mice were immunised with 2 x 10^8 defribinated SRBCs (Fisher Scientific) by i.p. injection. For *in vivo* BrdU labelling experiments, mice received single i.p. injections of 2mg BrdU in saline.

DIPHTHERIA TOXIN

Mice were treated at indicated time points with 0.75µg of diphtheria toxin (Merk Millipore) in 300µL of PBS, or saline i.p.

HY10* TREATMENT

SW_{HEL} GC were produced through i.p. immunisation described above. Hy10* antibodies were prepared as previously described[190]. 10µg of Hy10* antibody, or a PBS control was delivered to the mice 12 hours before culling.

HEL-EA-GFP

SW_{HEL} GC were generated in the skin draining lymph nodes through sub cutaneous immunisation in the flank, as described above. HEL-Ea-GFP was generated as described previously[299] HEL-BSA was conjugated similarly but not purified as has been performed previously[300]. 5µg HEL-Ea-GFP was delivered to each lymph node by subcutaneous injection 5 hours before culling.

TAMOXIFEN TREATMENT

Mice were treated with 2mg tamoxifen per mouse by i.p. injection in 100ul. Carrier was 10% ethanol 90% corn oil. Tamoxifen (Sigma Aldrich) was added to ethanol, vortexed for one minute, then sterile corn oil was added. This solution was sonicated in a water bath on 90% power for 8 minutes at 37ºC. Solution was then vortexed for a further 1 minute.

BONE MARROW CHIMERAS

MIXED BONE MARROW CHIMERAS

To generate mixed bone marrow chimeric mice, Ly5.2 host mice were lethally irradiated at 4.5 Gy for 300 seconds, followed by a 3 hour rest, and a subsequent 4.5 Gy dose for 300 second. Mice were injected i.v. with a 2:1 mixture Ly5.2 WT and Ly5.1 AID-/- or +/+ bone marrow; or a 1:1 mixture of Ly5.2 WT and Ly5.1 AID-Cre Rosa-flox-DTR mice. The recipient mice were given drinking water containing antibiotics (0.16mg/mL Enrofloxacin (Baytril), Bayer Corporation). Mice then rested for a minimum of 8 weeks.

BONE MARROW CHIMERAS WITH TRANSDUCED BONE MARROW

Three days before harvesting bone marrow, mice were treated with 300µL of 10mg/ml stock (in PBS) in 5-fluorouracil (Sigma), which was dissolved in a 37ºC water bath for 2-3 hours. Bone marrow was harvested with sterile tools and cultured in bone marrow media (DMEM 15% FBS (Gibco), 20ng/mL IL-3, 50ng/mL IL-6, 100ng/mL SCF (all from Biolegend), penicillin and streptomycin (Thermofisher scientific), 10mM HEPES (Gibco), Glutamine). The bone marrow was cultured in 24 well plates, the bone marrow from each mouse using ~3 wells, 2mL per well. The following day spinfection was performed to infect bone marrow cells with virus. Bone marrow cells were spun a for 5 minutes at 2000rpm, no brake, at room temperature. During this time, virus (i.e. supernatant from PlatE, transduced as described below) was harvested and passed through 0.45uM filter syringe to remove cells. Polybrene (4µg /ml, Sigma) and Hepes(1/100, Gibco) are added to virus. Once bone marrow cells had finished spinning, media was removed and saved, and 1 ml of virus per well was added to the cells, which are then spun at 2100 rpm at room temp for 2hrs, no brake. After spinfection, virus was discarded and cytokines replaced. Spinofection was repeated the following day. After an additional 24 hours of culturing, bone marrow was transferred i.v. to mice irradiated as above. The recipient mice were given drinking water containing antibiotics as above, and rested for a minimum 8 weeks.

2.4 GENETIC TOOLS

CONSTRUCTION OF RETROVIRAL VECTORS

AbVec2.0-IGHG1[301], and Igκ apovec [301] were a gifts from Townsend lab; MSCV IRES Thy1.1[302], and MSCV Loxp GFP[43] were gifts from Cyster lab; PQY-CD90.1[303] was a gift from Chris Allen lab. For the construction of the Hy10 variants, for the SNAP labelled Igκ light chain, and for the Igβ labelled with fluorescent timer protein, the same cloning protocol was performed. For the Igβ molecules labelled with fluorescent timer proteins, constructs were bought from Addgene. Insertions were produced flanked with desired cut sites. For the Hy10* variants, variable regions were amplified from previously amplified DNA used for sequencing of binding and non-binding cells. PCR was performed with Q5 High Fidelity DNA polymerase (New England Biolabs), and dNTPs (Thermo Fisher Scientific). Primary PCR used primers "SW_{HEL} for cloning forward (AgeI cut site)" and "SW_{HEL} for cloning reverse (SalI cut site)". Conditions were 98°C for 3 minutes; then 98°C for 15 seconds, 50°C for 1 minute, and 72°C for 1 minute, for 35 cycles; and finally, 3 minutes at 72°C. For the SNAP labelled Igκ, the SNAP gene and Igκ gene were joined by PCR, with three different protein linkers: Linker 1, GGGAAS; Linker 2, GSGSG; Linker 3, GGGGSGGGGS. The Igκ and SNAP protein were amplified with by PCR from plasmids which were gifts from the Davis lab. PCR was performed with Q5 High Fidelity DNA polymerase (New England Biolabs), and dNTPs (Thermo Fisher Scientific). SNAP was amplified using the SNAP reverse primer, and in three separate reaction the SNAP forward linker 1, SNAP forward linker 2, and SNAP forward linker 3 primer. The Igκ was amplified using Igκ forward and Igκ reverse. The products were run on an agarose gel, excised and purified, then combined and added to 1X of Phusion Flash Master Mix were held at 98 °C for 1 minute; then five cycles of 98 °C for 1 minute, 60 °C for 1 minute, followed 72 °C for 2 minutes; then five cycles of 98 °C for 1 minute, 60 °C for 30 second, then 72 °C for 2 minutes; then 72 °C for 3 minutes. Subsequently, forward and reverse primers are added, and

samples were held at 98ºC for 1 minute, followed by five cycles of 98ºC for 1 minute, 60 ºC for 1 minute, followed 72 ºC for 5 minutes. For the SNAP labelled Igκ and SW_{HEL} variable regions inserts were run on agarose gel and excised and purified.

Purified inserts and corresponding vectors were treated with restriction enzymes overnight. The SNAP labelled Igκ and MSCV were cut with EcoRV and NotI; the Igβ labelled with fluorescent timer protein and pQY-CD90.1 were cut with BglII and NotI; the variable regions and IgG ApoVEC were cut with AgeI and SalI. Cut inserts and vectors are run on an agarose gel, excised and purified. The vectors were phosphatase treated with Antarctic phosphatase according to manufactures' instruction. The DNA was subsequently purified, then vector DNA and corresponding insert DNA and added, and ligation was performed using T4 ligase, according to manufacturers' instruction, overnight. The following day DNA was transformed into 5-α Competent *E. coli* (High Efficiency) (New England Biolabs) according to the manufacturers' instruction. The following day individual cells are selected and grown in 5mL of Luria both overnight. The following day minipreps are performed to extract DNA from cultures, and diagnostic digests are performed on cells using the enzyme with which the inserts and vectors were originally cut. Positive clones are sent for sanger sequencing for confirmation. If genuine, the remaining culture was used to seed a 50mL culture, which was used to produce plasmid stock using a QIAprep Spin Midiprep Kit.

293T CELL TRANSFECTION

The same transfection protocol was used to produce virus in PlatE cells for bone marrow transductions, and to express Hy10 antibody variants in 293T cells. Both cell types are cultured in DMEM with 10% FBS (Gibco), 10mM HEPES (Gibco), and 100 units/mL penicillin and streptomycin (Thermofisher scientific).

The day before transfection, cells were split and plated at 3.5-4millions cells in 10mL of media. On the day of transfection, media was placed with pen-strep free media. Transfection mixes were prepared by placing placing 800µL of Optimem (Thermofisher Scientific) per tube in 2 polypropylene facs tubes per sample, then adding 20µL lipofectamine 2000 (Invitrogen) to one tube, add 10µg plasmid to the other. This must rest for 5 minutes at room temperature. The contents of the two tubes were subsequently mixed, and this must then rest for 30 minutes. These mixtures are added to the cultured cells. For the Hy10 variants; cell were cultured for three days after transfection. For bone marrow transductions, media was harvested on day two and three after transfection.

2.5 FLOW CYTOMETRY AND SINGLE CELL SORTING

Single cell suspensions were prepared and stained for with the relevant antibodies. BRDU staining was performed following the fixation/permeabilisation reagents from the BD BrdU flow kit according to manufacturer's instructions but using the Mobu-1 antibody clone (Thermo Fisher Scientific). Intranuclear BCL6 staining was performed on cells permeabilised using the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Invitrogen). Active caspase 3 staining was performed on cells permeabilised using the Fixation/Permeabilisation Solution Kit (BD bioscience). Samples were analysed using BD LSR Fortessa, LSR Fortessa x20, LSR Fortessa x50, LSR II, or Attune NxT machines. Analysis was performed with Flowjo (Treestar

Inc.). Single cell index sorting for sequencing and single cell cultures were performed using the BD FACs Aria II, FACs Aria II SORP, or Fusion II machines.

2.6 *IGHV* SEQUENCING

SW_{HEL} B CELL RECEPTOR SEQUENCE ANALYSIS

SANGER SEQUENCING

Single cells were sorted into 3.8μL of 0.2% Triton-x (Sigma Aldrich) in water. Proteinase K treatment was performed in 1X Q5 reaction buffer, with 1mg/mL tRNA; 1mg/mL proteinase K, and 0.05mM EDTA in a total volume of 10μL by heating to 56°C for 40 minutes followed by 95°C for 8 minutes. PCR was performed with Q5 High Fidelity DNA polymerase (New England Biolabs), and dNTPs (Thermo Fisher Scientific). Primary PCR used primers "1st Step SW_{HEL} forward" and "1st Step SW_{HEL} reverse". Conditions were 98°C for 3 minutes; then 98°C for 15 seconds, 62°C for 1 minute, and 72°C for 1 minute, for 35 cycles; and finally 3 minutes at 72°C. A 1:50 dilution of the initial product was used as template for the second step PCR. The 2nd step PCR was performed using primers "2nd Step SW_{HEL} forward" and "2nd Step SW_{HEL} reverse". Conditions were 98 °C for 3 minutes; then 35 cycles of 98°C for 15 seconds, 65°C for 30 seconds, then 72°C for 1 minute; and finally 3 minutes at 72°C. The PCR had an approximately 80% efficiency. PCR was checked by running 1μL of product on a 96 well agarose gel. PCR product purification was performed using Agencourt AMPure XP PCR purification beads (Beckman coulter). The concentration of DNA was measured by nanodrop. Sanger sequencing was performed by the Weatherall Institute of Molecular Medicine's sequencing unit.

Sanger sequences were converted to FASTA files and analysed using NCBI IgBlast tool to search for premature stop codons. Sequencing data was combined with Indexed FACS data, and converted to an FCS file using, R package version 1.44.0.[304]. Original Fcs files and sequence Fcs files were superimposed using Adobe Illustrator

ANALYSIS OF B CELL RECEPTOR BY NEXT GENERATION SEQUENCING

Bulk populations were sorted in 7.6μL of 0.2% Triton-x (Sigma Aldrich) in water. The PCR was performed as above with the exception that the initial PCR involved only 20 cycles. DNA was purified using Agencourt AMPure XP PCR purification beads (Beckman coulter), and the DNA products were sequentially cut with NsiI-HF and AFlIIII to produce a 435bp product. The DNA was subsequently purified, end prep and adaptor ligation was performed using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New Enlgand Biolabs, E7645S) according to the manufacturer's instructions and NEBNext® Multiplex Oligos for Illumina® (New England Biolabs, E7600S) were added according to the manufacturers' instructions. The DNA was again purified, quantified using Qubit™ dsDNA HS Assay Kit (Invitrogen, Q32854). The DNA was sequenced on an Illumina MiSeq using MiSeq Reagent Kit v2 (500-cycles) (Illumina, MS-102-2003)

PANDAseq was used to assemble paired-end FASTQ reads resulting from sequence analysis[305]. The assembled sequences were compared to the known SW_{HEL} *IgHv* genomic template sequence by pairwise sequence alignment using needle[306]. Following translation of the DNA sequence, STOP codons were identified in the relevant forward frame. Custom perl scripts were used to automate an analysis pipeline (find_stop_codons.pl) and summarise the subsequent data generated (make_summary.pl).

The number of reads of a given unique sample should be proportional to the number of times the read was identified in a sample. If 100 cells were sorted, then one would expect a sequence which occurred in only one cell, to be present in 1% of the reads; if 500 cells were sorted, one would expect a sequence which occurred in only one cells to be present in 0.2% of reads. Therefore, we selected only reads which occurred in more than (100/n)% of reads for that given sample. With this threshold, an average of 49.7% of reads appeared to have a somatic hypermutation of any kind in the AIDWT samples, and an average of 1.3% of reads from the AIDKO appeared to have a SHM of any kind. The frequency of reads carrying a premature stop codon was the combined number of reads carrying a premature stop codon from sequences passing this threshold, divided by the total number of reads passing threshold, multiplied by 100 to give a percentage value.

SINGLE CELL *IGHV* MRNA SEQUENCING

Single cell mRNA sequencing of the BCR was performed according to an amended protocol from the Wardemann lab[307]. Single germinal centre cells (IgG+ and BCR- cells in WT mice, and total GC in *Aicda*$^{-/-}$ mice) were sorted into 96 well PCR plates, with 3.8 µL of lysis buffer (2µL 0,4% Triton X + Rnase Inhibitor mix, 1µL dNTPs (10mM), 0.8µL Water)

Reverse transcription was performed by adding 6.2 reverse transcription master mix per well (2µL SuperScript II first strand buffer (5x), 0.5µL (Sigma Aldrich), 2µL Betaine (Sigma Aldrich), 0.1µL MgCl2 (Thermofisher), 0.25µL RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen), 0.25µL Superscript II RT, 1µL Ultrapure water, and 0.1µL of IgM or IgG reverse 1st step primer (100uM), for *Aicda*$^{-/-}$or WT cells respectively). Plates were transferred to

thermocyclers and held at 42°C for 90 minutes followed by 10 cycles of 50 °C for 2 minutes and 42 °C for2 minutes, and finally held at 70 °C for 15 minutes

The cDNA from this reverse transcription was used to perform a nested PCR. These PCRs were performed using KAPA Hifi HS Ready Mix (2x) (Roche). The first step PCR involved IgG forward primer and IgG reverse 1st step primer for the WT samples, and IgM forward and IgM reverse 1st step primer for the AIDKO. The program was as follows, 95°C for 3 minutes; followed by ten cycles of 98 °C for 20 seconds, then 62°C for 15 seconds then 72°C for 30 seconds; followed by 72°C for 1 minute. 2µL of sample was diluted in 40µL of sterile water. The second step PCR involved IgG forward primer and IgG reverse 2nd step primer for the WT samples, and IgM forward and IgM reverse 2nd step primer for the AIDKO. The program was as follows, 95°C for 3 minutes; followed by 35 cycles of 98 °C for 20 seconds, then 57 °C for 15 seconds, then 72°C for 30 seconds; followed by 72°C for 1 minute. 1µL of PCR product was run on an agarose gel to assess the success of the protocol, this protocol had a 30% success rate. If positive, DNA was purified using Agencourt AMPure XP PCR purification beads (Beckman coulter).

Access array primers were used to add barcodes and adaptors by PCR. These were also added using the KAPA Hifi HS Ready Mix (2x) (Roche). 2.8µL of diluted PCR product was added to 1.2µL of Access Array primer, and 4µL of Kappa HiFi. This was held at 98 °C for 5 min; followed by 15 cycles of 98 °C for 15 seconds, then 60 °C for 30 seconds, then 72 °C for 1 minutes; followed by 72 °C for 3 minutes. The DNA was again purified, quantified using Qubit™ dsDNA HS Assay Kit (Invitrogen, Q32854). The DNA was sequenced on an Illumina MiSeq using MiSeq Reagent Kit v2 (500-cycles) (Illumina, MS-102-2003).

As with the SW_{HEL} sequences PANDAseq was used to assemble paired-end FASTQ reads resulting from sequence analysis[305]. The assembled sequences were compared to the known *Ighv* V, D and J regions using Lymanalyzer[308]. Custom perl scripts were used to summarise the subsequent data generated (make_summary.pl). The "second read" was the second most common read per sample, which used the same V, D, and J region as the first. The "frequency of second read" was calculated as the number of reads for the "second read" divided by the cumulative number of reads for the most common read and "second read".

2.7 QUANTITATIVE PCR

SINGLE CELL QPCR

Cells were sorted directly into chilled reverse transcription and pre-amplification mix in a 96 well plate. Each well contained: 5µl Celldirect 2x Reaction mix and 1.2µl Celldirect RT/Taq mix (Invitrogen); 0.1µl SUPERase-In RNase Inhibitor (Ambion); 1.2µl TE buffer (Invitrogen); 2.5µl pre-diluted (in TE buffer) 0.2x Assay mix. Immediately following sort plates were spun down at 4600rpm for 30 seconds, and placed into thermocycler for pre-amplification. Thermocyler program was follows: 50ºC for 15 minutes for reverse transcription; followed by 95 ºC for 15 seconds then 60ºC for 4 minutes for 16 cycles. After pre-amplification, the pre-amplified cDNA was diluted with 4x volume TE (i.e. 40 µl TE buffer was added to each 10 µl sample). Plates were vortexed, and briefly spun down and store at -20°C. The 96.96 Gene Expression CHIP (Fluidgm) was set up by making an "assay mix" for each assay and a "sample mix" for each sample, and loading these separately into the chip. Assay mix for each Taqman assay by combining 3µL of sample loading reagent to 3µL of each individual 20x Taqman assay (Applied biosystems) assay.

A sample master mix for each sample was made by combing 0.3µL sample loading reagent and 3µL TaqMan Universal PCR MasterMix (2x) (Applied biosystem), and 2.7µL diluted pre-amplified DNA. The sample mix and assay mix are loaded into the Fluidgm chip according to the manufacturers' instructions. This chip was run on the Fluidgm HD according to the manufacturers' instruction.

BULK POPULATION QPCR

Bulk populations were sorted into Eppendorf tubes of media. Cells were pelleted and media removed. Pellets were snap frozen in ethanol baths.

The following components was combined in RNAse free minieppendorf: lysed cells 1µl of Random Hexamer Primer (Thermo scientific) 1µl 10mM dNTPs (Thermo Fisher Scientific), RNAse free sterile water to 13µl. This was heated to 65°C for 5 minutes and incubated on ice for at least 1 minute. The contents of the tube are collected by brief centrifugation and add mix that contains the following: 4 µl 5X First-Strand Buffer, 1 µl 0.1 M DTT (Sigma Aldrich), 1 µl RNasin® Ribonuclease Inhibitor at least 1 u/µl (20-40u/µl) (Promega, Part) 1 µl of SuperScript™ III RT (200 units/µl) (Invitrogen). This was mixed by pipetting then incubated at 25°C for 5 minutes, followed by 30–60 minutes, the reaction was inactivated by heating at 70°C for 15 minutes. 80µL pyrogen free water to the mix and transfer all 100µL into clean 1.5ml tubes.

This DNA was used for qPCR, using Taqman 20X assays TaqMan Universal PCR MasterMix (2x) (Applied biosystems) according to the manufacturers' instructions, on a Quantstudio7 qPCR machine.

2.8 *IN VITRO* CULTURE GERMINAL CENTRE B CELLS

SINGLE CELL CULTURE

Single GC B cells were expanded for 8 days using the NB21 feeder line as previously described[309], with minor adjustments. On day 0 NB21.2D9 cells were seeded into 96-well plates at 1,000 cells/well in 200µL of B cell media (RPMI-1640 (Invitrogen), (Thermo Scientific), 10% FBS (Gibco, Cat #10500 056) $5.5x10^{-5}$M 2-mercaptoethanol (Sigma Aldrich), 10mM HEPES (Gibco), 1mM sodium pyruvate, (Gibco) 100 units/mL penicillin, (Thermo Fisher) 100 mg/mL streptomycin, and MEM nonessential amino acid (Thermo fisher)). On day 1 single cells were sorted into 200µL of media, and recombinant mouse IL-4 (Peprotech; 2 ng/mL) was added. Then, 100µL of media was replaced with 150µL of media without IL-4 on day 3. 150µL media was subsequently discarded and replaced on days 5, 6 and 7. One row on each plate was left blank to control for cross well contamination. Cell growth was confirmed by flow cytometry, detecting either endogenous mCherry florescence (R26p-Fucci2) or after staining with CD19/CD138 antibodies.

SHORT TERM CULTURES

For 8 hour cultures, 1000 cells were sorted into 200μL of B cell medium as described above. 1000 cells were sorted directly into B cell medium. For 48 hour cultures, sorted B cells were cultured in the presence of NB21 feeder cells as described above. 1000 cells were sorted either straight onto NB21 feeder line cells or into media and then transferred into wells containing the cells, and cultured in the presence of IL-4. For both 8 hour and 48 hour cultures, three technical replicates were taken for each sample.

INTERNALISATION ASSAY

Internalisation assay was performed as previously described[267]. Spleens of immunised mice were harvested and single cell suspension was produced. Cells were washed twice in 10mL of RPMI + 1% FBS + P/S . Cells were resuspended at $3x10^7$ per mL. 1mL of cell suspension was stained with 10μg/mL of goat anti-mouse $F_{(ab)}$ fragment (Jackson Immunoresearch) for 25 minutes. Cells were washed twice, in 10mL of RPMI + 1% FBS + P/S. Cells were then resuspended in 6mL of pre-warmed internalisation media (RPMI 15% FBS+ PenStrep + 1/100 Hepes+ Glutamine, +10uM beta mercaptoethanol). Cells were divided in separate FACs tubes for each time point, and placed in an incubator at 37ºC. Tubes were removed from the incubator place on ice, and 3mL of cold RPMI was added at indicated time points. Cells were washed twice and stained for GC markers, and with Streptavidin Qdot.

ELISA

The capacity of cells to produce productive antibodies was assessed by ELISA. Plates were coated O/N at 4°C with anti-Igκ (2μg /ml (1050-01) (Southern biotech) (for ELISAs on supernatant from cultured mouse cells); or with HEL (sigma Aldridge) or anti-IgG Fab (Jackson immunoresearch, **715-547-003**) (form humanised Hy10* variants). Plates were then washed 3x in PBS/0.05% Tween20, blocked for 1hr at RT with 0.5% BSA in PBS before washing as before. Samples were diluted in PBS with 0.05% Tween20 before incubation for 2hrs at RT or O/N at 4°C. Plates are washed as before, and secondary antibody (HRP anti-mouse IgG, Southern Biotech, 1033-05, for mouse ELISAs, or HPRT anti-human IgG Fab, Jackson Immunoresearch,115-067-020) was added at 1:2000 in 1x PBS with 0.05% Tween20 0.5% BSA. Cells washed as before, and plates are developed with TMB (Biolegend). Reactions were stopped with 50μL of Biolegend stop reagent and read on CLARIOstar at 450nm against a mouse or human IgG standard curve, depending on the assay. For the samples from cultured mouse cells, samples were considered positive if they were over 4 standard deviations above the average of the negative controls. For the humanised Hy10* assays an affinity index was calculated as HEL binding relative to the IgG binding; and this was normalised to the unmutated Hy10 antibody, as had previously been performed[310].

2.9 QUANTIFICATION AND STATISTICAL ANALYSIS

STATISTICAL ANALYSIS

Statistical parameters including number of mice, number of replicates, and meaning of error bars are described in figure legends. Centre bars always show mean. Statistics were calculated

using Prism 7 software (Graphpad). Two-tailed unpaired t tests were used. ***p < 0.001, **p<0.01, *p<0.1.

CALCULATING FREQUENCIES OF CELLS CARRYING STOP CODONS FROM SANGER SEQUENCING

During sorting for *Ighv* sequence analysis, minority populations and/or populations of interest were often enriched, for example BCR^{low} cells that are a small fraction of total LZ/DZ subsets. Consequently, in calculating frequencies of stop codons in parental (e.g. DZ) populations, frequencies were normalised to the abundance of their parental populations.

Frequencies of stop codons in BCR^{high} and BCR^{low} LZs and DZs were calculated as follows (example from BCR^{low} DZ BCR^{low}):

$$\boldsymbol{frequency\ of\ stop\ codons\ in\ BCR^{low}\ DZ}$$
$$= \boldsymbol{frequency\ of\ stop\ codons\ in\ mCherry^{low}\ Venus^{low}\ BCR^{low}DZ}$$
$$\times \frac{\boldsymbol{\%mCherry^{low}\ Venus^{low}\ in\ BCR^{low}\ DZ}}{\boldsymbol{100}}$$
$$+ \boldsymbol{frequency\ of\ stop\ codons\ in\ mCherry^{high}\ Venus^{low}\ BCR^{low}DZ}$$
$$\times \frac{\boldsymbol{\%mCherry^{low}\ Venus^{high}\ in\ BCR^{low}\ DZ}}{\boldsymbol{100}}$$
$$+ \boldsymbol{frequency\ of\ stop\ codons\ in\ mCherry^{low}\ Venus^{high}\ BCR^{low}DZ} \frac{\times\ \boldsymbol{\%mCherry^{low}\ Venus^{high}\ in\ BCR^{low}\ DZ}}{\boldsymbol{100}}$$
$$+ \boldsymbol{frequency\ of\ stop\ codons\ in\ mCherry^{high}\ Venus^{high}\ BCR^{low}DZ} \frac{\times\ \boldsymbol{\%mCherry^{low}\ Venus^{high}\ in\ BCR^{low}\ DZ}}{\boldsymbol{100}}$$

Frequencies of stop codons in the total DZ and LZs were calculated similarly, for example frequency of stop codons in total DZ were calculated as such:

$$\boldsymbol{frequency\ of\ stop\ codons\ in\ DZ}$$

$$= \boldsymbol{f}requency\ of\ stop\ codons\ in\ BCR^{high}DZ \times \frac{\%BCR^{high}\ in\ DZ}{100}$$

$$+ frequency\ of\ stop\ codons\ in\ BCR^{low}DZ \times \frac{\%BCR^{low}\ in\ DZ}{100}$$

And frequency stop codons in the whole GC was therefore calculated:

$$frequency\ of\ stop\ codons\ in\ whole\ GC$$

$$= frequency\ of\ stop\ codon\ in\ DZ \times \frac{\%\ DZ\ in\ whole\ GC}{100}$$

$$+ frequency\ of\ stop\ codon\ in\ LZ \times \frac{\%\ LZ\ in\ whole\ GC}{100}$$

$$+ frequency\ of\ stop\ codon\ in\ CXCR4^{low}CD83^{low}$$

$$\times \frac{\%\ CXCR4^{low}CD83^{low}in\ whole\ GC}{100}$$

ESTIMATES OF EXPECTED PREMATURE STOP CODON FREQUENCY

At risk codons. We first consider single nucleotide substitutions. The SW_{HEL} *Ighv* region gene was annotated to determine which codons were at risk of generating stop codons by single point changes at any position. Codons were classified as to whether one point mutation, or two different singular point mutations could terminate the gene. For the former, there is a 1 in 3 chance that a mutation will occur in the stop codon causing position, and a 1 in 3 chance that the mutation will yield a premature stop codon (1 in 9 chance total). The "risk" is doubled (2 in 9) where substitutions in either of 2 different positions can cause stops, or if substitutions of two different

nucleotides in the same position could cause stops. For any given gene sequence, the likelihood of a mutation leading to a stop codon is equal to the frequency of "at risk" codons multiplied by 1/9, plus the frequency of codons which are "doubly at risk" multiplied by 2/9.

Enrichment at specific sites. SHM occurs preferentially in complementarity determining regions (CDRs) and following specific motifs. To account for this in a crude manner, we worked with an assumption that mutations were 5 times more likely to occur in CDRs, and 2 times more likely to occur in motif associated regions. We demarked the SW_{HEL} *Ighv* region gene CDRs; and motif containing regions, both within the CDRs and outside the CDRs. The frequency of point mutations expected to occur in a region is equal to the size of that region, multiplied by the enrichment (2 for 2 times, 5 for 5 times) for that region, divided by the sum of sizes of all other regions of the gene, each multiplied by their respective enrichments.

The frequency of SHMs which will cause a premature stop codon in a given region is equal to the likelihood of a point mutation causing a premature stop codon for that region, multiplied by the percentage of SHM that should occur in that region, divided by 100 to give a percentage. The frequency of SHMs expected to cause a stop codon the entire *Ighv* is equal to the sum of the frequencies of SHMs which will cause stop codons in all the constitutive regions of the gene. Using these calculations, the frequency of point mutations which would cause premature stop codons is 6.4%. N.B. the enrichment had a negligible effect, with no enrichment the frequency is 5.8%.

Accounting for established SHM rates per division. SHM is reported to occur at approximately 10^{-3} mutations per division. The SW_{HEL} *Ighv* gene is 393 bases long, and so a

cell has a 39.3% chance of acquiring a mutation per division. Therefore, the likelihood of the gene acquiring a premature stop codon by point mutation per division is 6.4 x 39.9/100 = 2.5%.

Risk of insertions and deletions (indels) causing stop codons. All indels that are not units of three (i.e. 2/3s of indels) are considered to cause eventual stop codons in the gene. It is difficult to estimate the rate of indels, but there is correlative data of numbers of mutations and frequency of deletions[74]. By correlating point mutation frequency and deletion frequency, we can estimate the likelihood of a deletion per division. If a Ig gene has a mutational load of X, we assume it has undergone X/0.393 divisions. E.g., if a cell has 20 mutation it is assumed to have completed 51 divisions. Yeap et al. correlated frequencies of point mutations and deletions in vivo. If the 40% of cells with 20 SHMs have a deletion, then after 51 divisions 60% of cells do not have a deletion; therefore (likelihood of not acquiring a deletion per division)^{51}=0.6. In this case, the likelihood of not acquiring a deletion per division= 0.99, or 99%. Medium mutation frequency cells, with about 15 mutations, have a 15% frequency of deletion. Low mutation frequency cells, with 5 point mutations, have a frequency of deletion of only 5%. We averaged the likelihood of not acquiring a deletion, and found the chance of not acquiring a deletion per division is 0.994. Therefore, the chance of acquiring a deletion is 0.6%. If one third of those are multiples of three and so do not cause a frameshift mutation, then a cell has a 0.4% chance of acquiring a premature stop codon by deletion per cell division.

Combined risk of induction of premature stop codons by point mutations and indels. The total frequency of SHM causing stop codon is equal to the sum of the likelihood of acquiring a stop codon by point mutation and the likelihood of acquiring a stop codon by deletion. This is 2.5% + 0.4%, so the probability is 2.9%

Frequency of cells with premature stop codons in the dark zone. If cells complete on average two divisions in the DZ, then at a given period 14% will have undergone zero divisions, 29% will have completed one division and 57% will have completed two. Those numbers change to 7% (zero), 14% (one), 27% (two) and 54% (three) where the average number of DZ divisions is three. We assume cells with zero divisions to have a have a 0% chance of having acquired a premature stop codon, cells with one division a chance of 2.9% (and as such a 97.1% chance of having not acquired a stop codon per division). The chance of a cell not acquiring a premature stop codon after n divisions is equal to the 0.971^n. Therefore, the chance of acquiring a premature stop codon after n divisions is equal to $1-0.971^n$. The frequency of cells in the dark zone that carry a premature stop codon is equal to the sum of the proportion of cells which have undergone a given number of divisions multiplied by the likelihood of having acquired a premature stop codon in that number of divisions. For two divisions in the DZ this is 4.1%; for three divisions in the DZ this is 6.4%.

CHAPTER THREE: CELLS APPEAR TO EXCHANGE THEIR B CELL RECEPTORS IN THE DARK ZONE

The GC is a site of diversification and refinement of immunoglobulin genes. Cells rapidly and dynamically transition between DZs and LZs, and so the time period between mutation and selection is presumably very short. Earlier estimates of the half-life of the BCR on mature B cells was 20-40 hours, however if the BCR were exchanged this slowly within GCs then cells would not have time to exchange their BCR before selection, and would be at risk of being selected on the basis of a BCR they no longer encode. Therefore, in this work we set out to address the handling of the BCR complex in the GC DZ in terms of surface protein expression, mRNA levels, and internalisation.

3.1 THE B CELL RECEPTOR IS HANDLED FUNDAMENTALLY DIFFERENTLY BETWEEN GERMINAL CENTRE B CELLS AND NAÏVE B CELLS

Naïve B cells and memory B cells require the BCR to simply survey the immune environment of the individual, and so, for these cells the stability of the BCR is critical. In contrast, GC B cells are iteratively testing newly mutated BCRs, putatively placing a unique pressure on GC B cells to exchange their BCR rapidly. This implies that there may be evolutionary pressures on GC B cells and naïve B cells which result in their BCR complexes having different half-lives. We therefore wished to address if there are differences in expression and degradation of the BCR between GC B cells and naïve B cells. This is not a self-evident hypothesis. In fact, there would be conceptual benefits to the old BCR being simply diluted off the surface of the GC B cell as a result of extensive

division in the DZ. If this were the case, cells which have acquired a detrimental mutation in the DZ could proceed into the LZ while still expressing their pre-SHM BCR. In this case, they may be selected on the basis of their pre-SHM BCR, and as such may re-enter the DZ and possibly acquire redemptive mutations. The persistence of pre-SHM BCR on the surface of GC B cells would allow for the maintenance of low affinity and non-binding cells in the GC, while maintaining technically competitive environment, by providing a post mutation "grace period". A problem with this model of selection is that it would allow an equal "grace period" for cells which have acquired a SHM which decreases antigen binding, or the antigen to interact with the antibody in a different orientation, which could possibly be redeemed by subsequent rounds of selection; and a mutation which structurally destroys the antibody, which is unlikely to be redeemed.

Thus, this investigation begins with the broadest and most basic of questions about differences in BCR levels in naïve versus GC B cells. We quantified surface BCR levels by flow cytometry by staining for the Igβ signalling chain. We analysed the Igβ signalling chain as a surrogate for the antibody protein, because Igβ is a component of the BCR complex that correlates linearly to that Igκ light chain in a BCR knock-in model wherein all antigen-specific BCRs use the Igκ light chain (SW_{HEL}) (Figure 3.1a). The advantage of staining for Igβ is unlike the heavy chain, which is subject to class switch recombination, and the light chain, which is subject to receptor editing, the signalling chains always present in the BCR complex at a 1:1 molar ratio with the other components[18]. We immunised mice with the T-dependent antigen, sheep red blood cells (SRBCS), and compared the expression of surface BCR on splenic GC B cells ($B220^+$ IgD^- $CD95^+$ $GL7^+$) and follicular B cells ($B220^+$ IgD^+ CD95-) (Gating strategy

shown in Figure 3.1b). Consistent with earlier reports[20,106], surface BCR levels were lower on GC B cells than on naïve B cells (Figure 3.1c). This was consistent with early immunohistology characterisations of the levels of surface immunoglobulin between DZ cells and LZ cells, which found DZ cells to have very low levels of surface immunoglobulin[20], and has also been shown by FACs analysis more recently[106].

Subsequently, we investigated if the lower levels of BCR in the GC as compared to the naïve compartment were attributable to changes in transcription of one or all BCR components. We immunised mice with SRBCs, and FACS sorted bulk populations of naïve B cells and IgM^+ GC B cell on day 7. We assessed the relative transcript abundance for each BCR component (*CD79a, CD79b, Ighm, Igk*) in the respectively sorted cells by qPCR by comparing to the *Gapdh* and *Hprt* housekeeping genes, and observed lower expression of all BCR components in GC B cells relative to naïve B cells (Figure 3.1d). While all components of the BCR showed higher expression in the naïve B cells, the changes in expression did vary between cells. The variable chains showed approximately two- and four-fold increased expression for *Ighm* and *Igk* respectively; but *CD79a* and *CD79b* showed closer to an eight-fold change in expression. The relative expressions of heavy and light chains informative than the signalling chains. In considering the analysis of the heavy chains, it is noteworthy that the naïve B cell express both IgM and IgD, whereas the GC B cells are IgD^-. Therefore, the qPCR analysis of IgM would underestimate the difference in surface BCR between GC B cells and naïve B cells. These differences may also help partly explain the greater difference in terms of CD79a and CD79b abundance relative to *Ighm*, because these would be needed for surface expression of both antibody classes. In the case of the light chain, it is noteworthy that a small percentage (1-5%) of cells in both compartments are $Ig\lambda^+$, rather than $Ig\kappa^+$.

However, this small contribution is unlikely to have a dramatic effect on the readout. Despite these caveats, the qPCR data demonstrates with the GC B cells carrying slightly lower levels of transcripts encoding for the BCR complex.

While the reduced immunoglobulin and *Cd79a*/*Cd79b* transcript abundance in GC B cells relative to naïve B cells may be a contributing factor to the lower surface BCR levels, we hypothesised that additional post-translational mechanisms might also play important roles. Specifically, given the conceptual differences noted above in terms of how naïve and GC B cells "use" their BCRs, we thought it possible that the two cell types may internalise and degrade their immunoglobulin proteins at different rates. The alternative, non-mutually exclusive hypothesis was that GC B cells simply down-regulate the BCR upon entry into the GC, either by decreasing new protein synthesis, or by degrading protein more rapidly.

Internalisation of surface BCR complexes by naïve and GC B cells was measured *ex vivo* by staining splenocytes from SRBC immunised mice with a non-activating biotinylated $F_{(ab)}$ fragment that is specific for mouse IgM. Cells were stained on ice and washed prior to their being moved to 37^{o}C and incubated for controlled periods of time. We then analysed the extent of BCR internalisation for each condition by further staining cells after the incubation step with a fluorophore-streptavidin conjugate to provide a relative quantification for the amount of pre-incubation BCR remaining on cell surfaces of IgM^{+} cells[267]. A $F_{(ab)}$ fragment is used instead of an antibody to avoid crosslinking and activation of the BCR. GCs are notoriously difficult to culture, in part because they are predisposed to apoptosis when removed from their niche *in*

vivo, therefore mice carrying a B cell specific Bcl2 transgene (Bcl2-tg) were used in these experiments[296]. Delaying apoptosis in this way allowed for the short-term culture of GC B cells.

GC B cells internalised their BCRs more rapidly than naïve B cells, after two hours of culturing the GC B cells had internalised more than 50% of the initial surface BCR, whereas the naïve cells had only internalised just over 20% (Figure 3.1e). Given the noted difficulties in culturing GC B cells, and in faithfully maintaining all their functional properties *ex vivo*, it is necessary to be conservative with the conclusions from this model. In particular, no clear conclusions can be made regarding absolute protein turnover rates. However, these results are strongly suggestive that the BCR may be internalised more rapidly in GC B cells than in naïve B cells, and provide further evidence that these cell types "handle" their BCR complexes differently. To properly address the question of absolute turnover rates in GC B cells, it will be necessary to measure BCR turnover rates *in vivo,* where cells behave "normally". We designed various unsuccessful approaches to analyse turnover of BCR complexes *in vivo,* which are described in Appendix 1.

Despite the caveats highlighted, the differences observed in these preliminary studies spoke to a global change in the handling of the BCR in the GC, wherein there are lower levels of BCR mRNA and protein present which could possibly allow for more effective clearance of pre-SHM BCRs, and an apparently higher BCR turnover than naïve cells. These differences might reflect adaptations to the unique pressure of GC B cells. This data is consistent with models in which GC B cells are more likely to actively replace their surface BCR than allow it to be simply diluted from the surface of the cell through subsequent divisions.

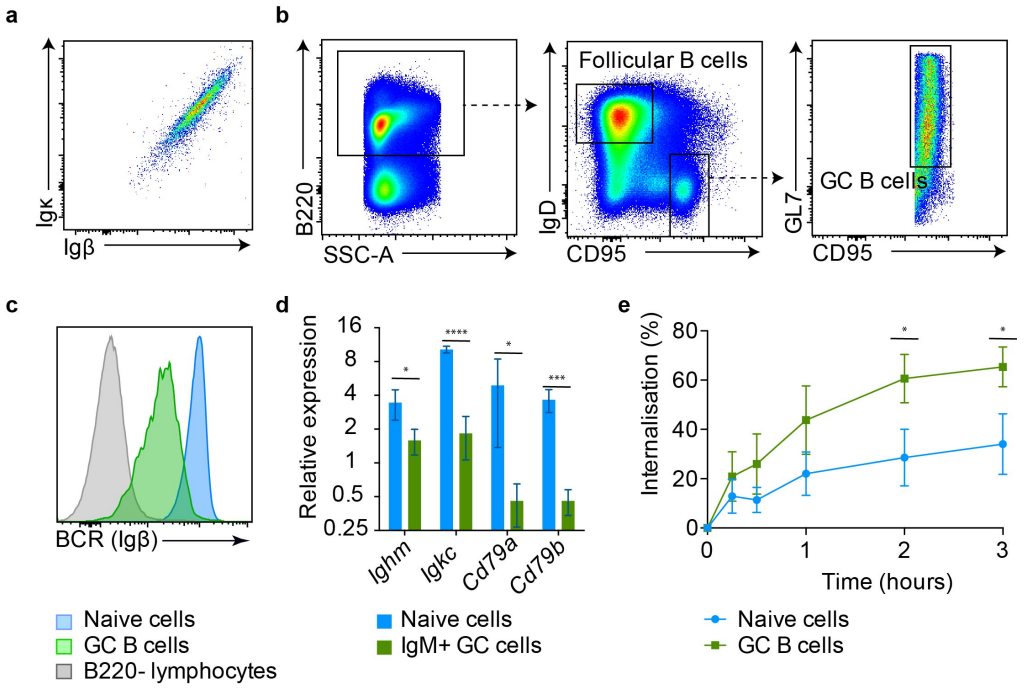


FIGURE 3.1| DIFFERENTIAL SURFACE EXPRESSION AND INTERNALISATION OF B CELL RECEPTOR IN GERMINAL CENTRE AND NAÏVE B CELLS.

(a) Comparison co-expression of Igκ light chain and Igβ signalling chain on SW_{HEL} GC B cells. SW_{HEL} GCs generated by HEL specific SW_{HEL} B cells and OTII T cells being transferred to allotypically marked mice, and immunised with HEL conjugated to Ovalbumin (HEL-OVA) the following day, mice analysed seven days after immunisation. GC B cells identified as IgD^{low} $CD95^{+}$ $GL7^{+}$, and SW_{HEL} cells differentiated from host cells as being $Ly5.2^{+}$. (b-e) Mice were immunised with SRBCs splenic IgD^{high} $CD95^{low}$ naïve follicular B cells and IgD^{low} $CD95^{+}$ $GL7^{+}$ GC B cells were analysed on day 7 (b) Gating strategy for follicular B cells GC B cells in a polyclonal response. (c) Surface BCR levels of GC and naïve cells are compared. (d) Relative expression of BCR heavy chain, light chain, and signalling chains mRNA in naïve cells and IgM^{+} GC cells. Relative expression is normalised to *Hprt* and *Gapdh*, bar represents mean of six mice from two experiments, error bars show SEM. (e)Internalisation of the BCR was analysed in vitro, surface IgM was labelled with a biotinylated $F_{(ab)}$ specific for IgM. After washing, the cells were incubated at 37°C for 0, 0.25, 0.5, 1, 2, or 3 hours, and immediately fixed, IgM remaining on the surface was detected by using streptavidin Qdot605. The data is then expressed as the percentage of surface BCR lost, compared to 0 hours incubation, data points are the average of three mice across two experiments (± SEM). Analysis was performed using an unpaired two-tailed student's t-test, ***, P <0.001; **, P<0.01, *, P<0.1.

3.2 DARK ZONES CONTAIN POPULATIONS OF CELLS THAT HAVE UNDETECTABLE SURFACE BCR

The most relevant question regarding BCR exchange in the GC, in terms of implications for selection, is whether full replacement occurs in DZs before cells enter LZs, that is before new BCRs should be "tested" through selection. The question of whether the BCR is fully exchanged in the DZ, before LZ entry, could be rephrased as a question of whether BCR exchange is an "active" or "passive" process. Passive exchange of the BCR would be characterised by pre-SHM BCR being simply diluted out as a result of cell division and "general" protein turnover, eventually leading to replacement with the post-SMH version. In contrast, if there were a concerted process during which BCR replacement is induced before LZ entry, then this exchange of the BCR could be considered active. Active replacement of the BCR could involve an increased speed of turnover of the BCR in the DZ relative to the LZ, in which there is a period of time where cells express both pre- and post-SHM BCR on their surface; or it could involve a purging of the BCR from the surface of the cell, wherein there would be a brief period in which the GC B cell is 'bald' or negative for surface BCR. In an active replacement model, we hypothesised there would be evidence of differential handling of the BCR in the LZ and DZ. Ultimately, the phenotype we will discover is a map of the evolutionary pressure placed on GC B cells; if there is evidence of an adaptation of GC B cells to actively replace their BCR before LZ entry, this would prove that affinity maturation requires that cells transition into the LZ for selection expressing the BCR they genetically encode. Conversely, a lack of evidence of an active exchange process in the DZ would serve as evidence that continued expression of pre-SHM BCR during selection improves, or at least does not impede, affinity maturation, possibly by allowing cells which have acquired BCR which have lost affinity time to acquire redemptive mutations.

Therefore, we sought to compare the relative abundance of membrane BCR proteins on DZ and LZ GC B cells to look for indications that the complexes may be handled differently at the two stages. We immunised mice with SRBCs, and analysed the expression of surface B cell receptor by flow cytometry, staining for the Igβ signalling chain as described previously. We differentiated between LZ cells and DZ cells using CD83 and CXCR4, LZ cells being $CD83^{high}$ and $CXCR4^{low}$, and the DZ being $CD83^{low}$ and $CXCR4^{high}$. The majority of LZ cells expressed relatively high BCR levels (albeit lower than follicular B cells), however the DZ population contained cells that spanned in surface BCR abundance from similar to levels in the LZ to levels indistinguishable from $B220^{negative(neg)}$ non-B cells, indicating very little or no protein was present (Figure 3.2a, b). Intracellular BCL6 staining confirmed $CXCR4^{high}$ BCR^{low} cells as being bona fide GC B cells (Figure 3.2c). Early microscopy studies found DZ cells to have no surface BCR[20], we saw this only to be true for a subset of DZ cells.

It was possible that the low levels of surface BCR seen in the DZ was the result of BCR proteins being internalised but not necessarily degraded, for example because BCR recycling could occur from early endosomal compartments. It was not clear what might induce recycling, but it seemed plausible it could occur either as a result of BCR signalling or for other unknown reasons. Therefore, we investigated whether intracellular BCR was present within these cells, by saturating surface epitopes with a FITC conjugated antibody prior to fixation, permeabilisation and further staining with a different antibody-conjugate to detect the intracellular protein. Although the Igβ signalling chain is trafficked with and degraded with the heavy chain in the context of BCR mediated antigen internalisation[268], we sought to directly

label the heavy chain of the BCR to address this question. To avoid the problem of class switch recombination, we utilised the MD4 system, in which cells carry a transgenic BCR specific for hen egg lysozyme (HEL), which does not undergo class switch recombination and so all B cells are IgM^{+293}. We adoptively transferred 10^5 HEL-binding B cells, along with 10^5 ovalbumin (ova)-specific OT-II T cells, then the following day we immunised with HEL conjugated to OVA (HEL-OVA). Spleens were harvested on day seven post-immunisation (Protocol summarised in Figure 3.2d). Intracellular staining for IgM revealed the presence of a subpopulation of $CXCR4^{high}$ cells that lacked detectable levels of both surface and intracellular BCRs (Figure 3.2e). Therefore, the lack of surface protein on GC B cells was not caused only by endocytic recycling but presumably reflects that BCR complexes have been degraded in these cells.

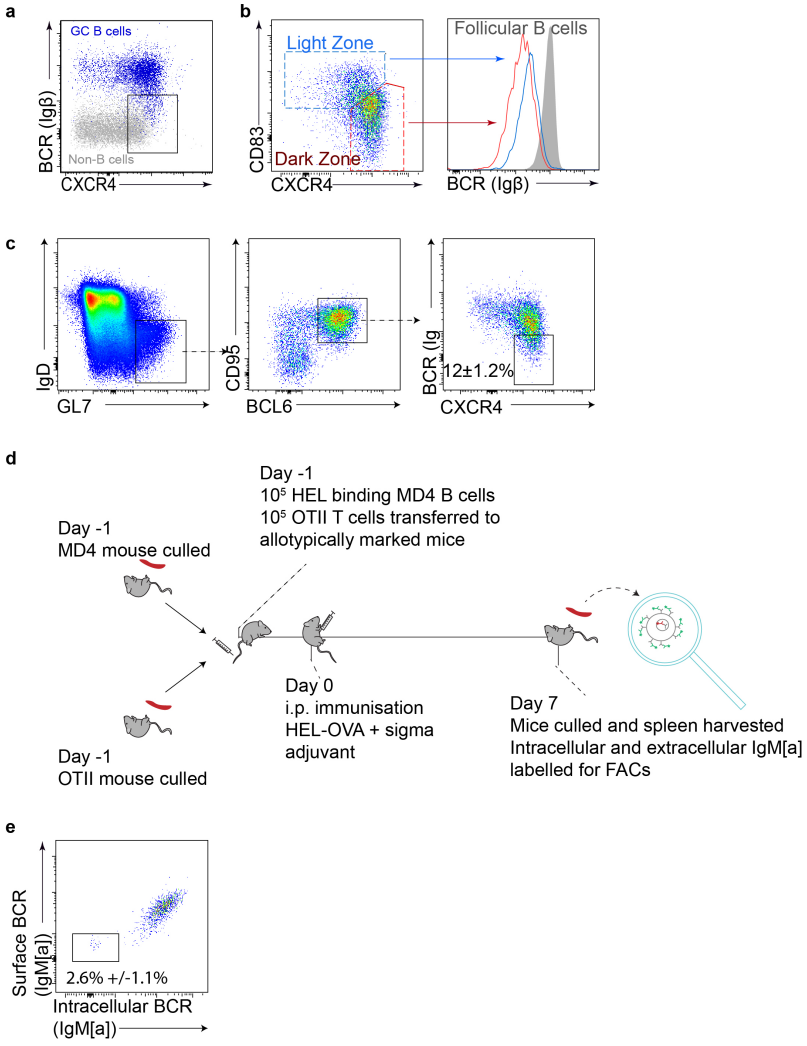


FIGURE 3.2| B CELL RECEPTOR DOWN-REGULATION BY DARK ZONE GERMINAL CENTRE B CELLS

(a and b) Mice were immunised with SRBCs and splenic IgD^{low} $CD95^{+}$ $GL7^{+}$ GC B cells were analysed on day 7. (a) Plot of surface BCR (Igβ) against DZ homing chemokine receptor CXCR4 to compare the levels of BCR in GC cells (blue) and $B220^{neg}$ non-B cells (grey). The gate indicates a BCR^{low} DZ population. (b) DZ and LZ cells are gated on the basis of CXCR4 and CD83 as shown and BCR levels overlaid with IgD^{high} $CD95^{low}$ follicular B cells. (c) Following staining for cell surface GC markers and BCR, cells were fixed, permeabilised and stained for intracellular BCL6, GC transcription factor, to ensure the identity of cells as GC cells. Plot shows the CXCR4/BCR staining pattern on $BCL6^{+}$ GC B cells, gated as shown. (n=6, over two experiments) (d) Schematic showing protocol, HEL-specific MD4 B cells were adoptively co-transferred with OT-II $CD4^{+}$ T cells and mice were immunised with HEL-OVA/sigma adjuvant. Splenic IgD^{low} $CD95^{+}$ $GL7^{+}$ $CD45.1^{+}$ GC B cells were analysed on day 7. (e) Surface and intracellular BCR (IgMa) levels were co-stained in fixed/permeabilised MD4 GC B cells revealing a subset of cells that is negative for both (n=7, over three experiments). Numbers represent mean ± SEM.

Given that the DZ is the site of extensive cellular division, it seemed possible that the BCR^{low} cells were generated through asymmetric cell division; i.e. that BCR proteins were inherited all by one daughter cell during cytokinesis, leaving the one "bald". Asymmetric cell division of internalised antigen associated with BCR has been demonstrated *in vitro* for early activated cells[311]; furthermore asymmetric division has been suggested in the GC for the GC transcriptional regulator BCL6 and the receptor for IL-21[312]. This seemed like an interesting possibility to explore because it could, in principle, play an important role in determining the downstream fates of the daughter cells.

To address the putative role of asymmetric division in the generation of BCR^{low} DZ cells, we analysed the cell cycle distribution of BCR^{low} cells using R26-Fucci2 mice[295]. R26-Fucci2 mice carry a bicistronic Rosa26 driven transgene consisting of mVenus and mCherry fused to the ubiquitinylation domains of Geminin (hGem(1/110)) and Cdt1(hCdt1(30/120)) fused by the Thoseaasigna virus 2A (T2A) self-cleaving peptide[295]. mVenus protein begins to accumulate as cells enter S phase and fluorescence intensity increases with time while mCherry is concurrently degraded. Then, at the completion of mitosis, cells begin to accumulate mCherry again. As such, mVenus fluorescence marks actively dividing cells and the relative abundance of mCherry in $mVenus^{neg}$ cells provides an indication of time since completion of mitosis; it is therefore possible to determine not only the cell's cell cycle stage but also the relative time it has been in that phase. Mice were immunised with SRBCs, then culled and their spleens analysed by FACs on day 7. GCs cells, and the LZ and DZ gates therein, were as described previously in Figure 3.1. The accuracy

of the R26-Fucci2 reporter in GC B cells was validated by co-staining for DNA content (Figure 3.3a) and by confirming that mCherry^{low} mVenus^{neg} post-mitotic "early G1" cells do not incorporate BrdU when the pulse is very short (30mins) but do if assessed 2hr later (Figure 3.3b). We observed that BCR^{low} DZ cells were not restricted within early G1 cells, as might be expected if this phenotype occurred only as a direct result of asymmetrical division during recent mitosis. Instead, such cells were present at all cell cycle stages in the DZ. These findings suggest that BCR loss by some DZ cells is not caused solely by asymmetrical distribution of the receptor complex and is more likely instead to occur as a result of a degradation process (Figures 3.3c-e).

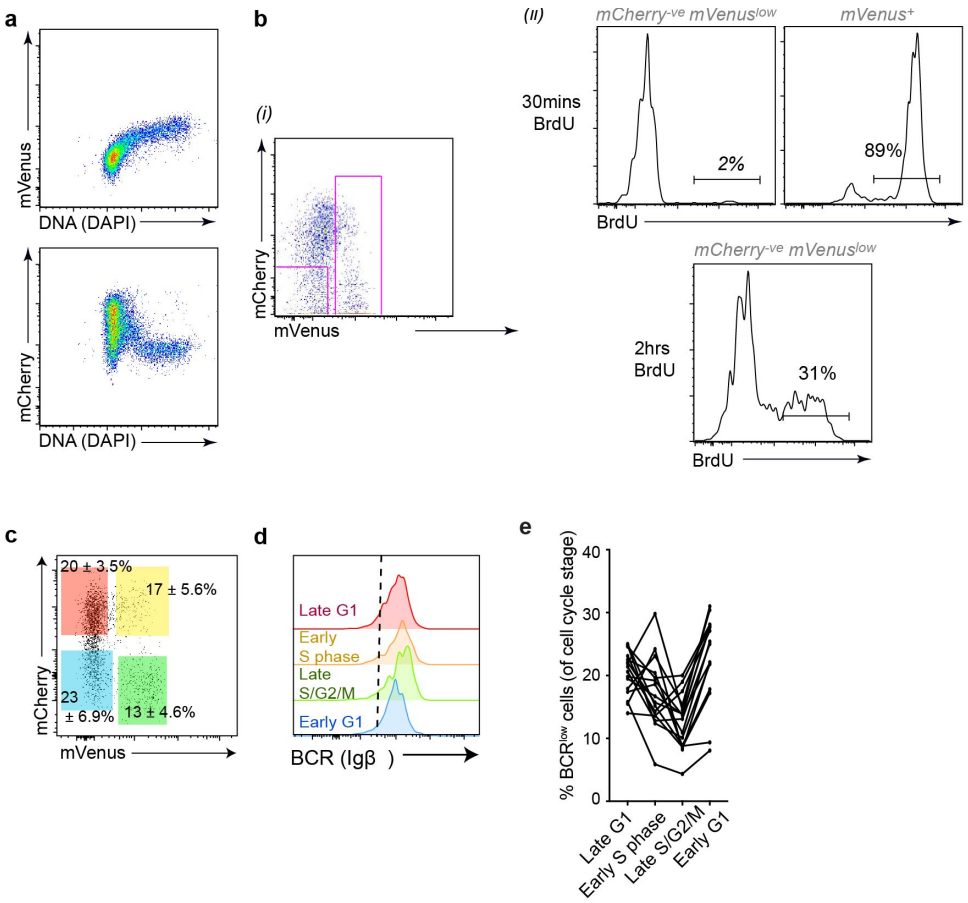


FIGURE 3.3| DOWN-REGULATION OF B CELL RECEPTOR IN THE GERMINAL CENTRE IS NOT RELATED TO CELL CYCLE

(a, b) Verification of utility of R26p-Fucci2 reporter mice for tracking cell cycle in IgD^{low} $CD95^{+}$ $GL7^{+}$ GC B cells on day 10 following SRBC immunisation. mVenus accumulates during S phase as is evidenced by its correlation with DNA content; mCherry is degraded during S phase but accumulates during G1. (a) Plot DAPI, staining for DNA content, vs Venus or mCherry in GC B cells (b) S/G2/M ($mVenus^{+}$) and early G1 ($mCherry^{-}$ $mVenus^{low}$) $CXCR4^{high}$ $CD83^{low}$ DZ cells were gated (i) and assessed for BrdU incorporation 30 minutes and 2 hours after a single injection (ii). S phase cells label at 30 minutes while labelling of early G1 cells require completion of mitosis, which does not occur until 2 hours after labelling. Plots in (a) are representative of 8 mice from 2 experiments, (b) of 4 mice from 2 experiments, numbers represent means. (c-e) SW_{HEL}xR26p-Fucci2 DZ ($CXCR4^{high}$ $CD83^{low}$) GC B cells (IgD^{low} $GL7^{+}$ CD45.1 or 2^{+} or $CD95^{+}$ $GL7^{+}$ CD45.1 or 2^{+}) were generated in the same manner as the adoptively transferred MD4 GC in figure 1.2, and 8 days after immunisation with HEL-OVA/adjuvant were gated for their cell cycle status (c) Frequency of BCR^{low} cells in each population was determined (d) Representative histograms of levels of BCR^{low} cells in each cell cycle stage Dashed line marks BCR^{low} gating. (e) Summary of results from multiple mice with each line of connected dots representing results from a single animal. (n=15, numbers represent mean ± SEM)

3.3 GENERATION OF THE BCR^{LOW} DZ POPULATION DEPENDS ON THE PRESENCE OF AID YET IS NOT RELATED TO THE GENERATION OF ABERRANT ANTIBODIES

Given the described findings that subsets the DZ have seemingly degraded most or all their surface and intracellular BCR stores, we were interested to know what cues might induce this. Our starting hypothesis in this thesis was that BCR turnover may be uniquely "handled" in GC B cells as a consequence of the environment in which they must mutate and then select their BCR genes within quite short time periods. We therefore explored the possibility that BCR degradation was directly associated with the process of SHM. We assessed the expression of the BCR complex in SRBC induced GCs by flow cytometry, staining for Igβ in *Aicda*$^{-/-}$ GC B cells in an *Aicda*$^{-/-}$/WT mixed bone marrow (BM) chimeric mice. In mixed bone marrow chimeric mice, approximately half the cells were reconstituted *Aicda*$^{-/-}$ BM, and the other half from WT (*Aicda*$^{+/+}$) BM, and can therefore conclude that any effect seen is cell intrinsic. An interesting observation was that the presence of the BCR^{low} DZ subset depends on AID, because it was almost absent in AID deficient (*Aicda*$^{-/-}$) GC B cell populations despite being present within the WT cells from the same mice (Figures 3.4a, b). Given these findings, we sought to understand whether the generation of BCR^{low} cells might be a part of a BCR exchange programme, associated with the SHM event. However, it was also possible that the BCR^{low} DZ populations might arise by SHM generating autoreactive antibodies, or structurally destroying the antibody gene. Therefore, we assessed these alternate hypotheses for the nature of the cells in the BCR^{low} DZ population.

We first explored the possibility that the BCR^{low} DZ population was attributable to the generation of autoreactive BCRs during SHM, because doing so might induce an anergy-like state. As mentioned in the introduction, anergy is a well characterised state that occurs in B cells that express BCRs specific for self, and it associated with reduced surface BCR levels[145,313–315]. To test the possible role of the induction of anergy in the creation of the BCR^{low} cells, we analysed the generation of their formation in GC B cells whose BCR is directed against a specific foreign antigen. We analysed the generation of these BCR^{low} cells in GCs derived from MD4 transgenic and SW_{HEL} "gene-targeted" B cells, in which all BCRs are specific for HEL[292,293]. The SW_{HEL} mouse has a HEL specific variable region knocked in to the endogenous *Ighv* locus, as well as a transgenic copy of the HEL specific Igκ light chain. Because the variable region is inserted into the endogenous heavy chain locus, the cells can undergo class switch recombination.[13] The MD4 mouse has a transgenic copy of the HEL specific IgM heavy chain and Igκ light chain.[4] The generation of these GCs from adoptively transferred cells was as described earlier. Either MD4 or SW_{HEL} HEL specific B cells were transferred into allotypically marked recipients along with OT-II $CD4^+$ T cells, and the following day mice were immunised with HEL-OVA in adjuvant. Mice were culled on day 7 and their spleens stained for FACs. We observed similar populations of BCR^{low} cells in the DZ even when BCR specificity is fixed (Figures 3.4c and d). Therefore, the generation of BCR^{low} DZ cells is unlikely entirely attributable to the generation of autoreactive B cells.

An alternative explanation for the presence of BCR^{low} cells only in AID-sufficient B cells was that their generation could be caused by SHM generating a structurally destroyed antibody genes

or proteins. For example, somatic mutations might disrupt gene coding, or proteins may be modified in such a way that prevents their proper coupling/trafficking to the cell membrane. In addition to implying that the BCR^{low} population is not related to the generation of auto-antibodies, the presence of BCR^{low} cells within MD4 GC B cell populations also suggested that the state is not caused only by SHM structurally damaging the immunoglobulin gene. This is because although cells from this mouse line can still undergo SHM, they carry multiple copies of the transgene, and therefore the induction of a premature stop codon or a disruption of a BCR framework region would be rescued by redundancy for alternate copies of the gene (Figure 3.4d)[293].

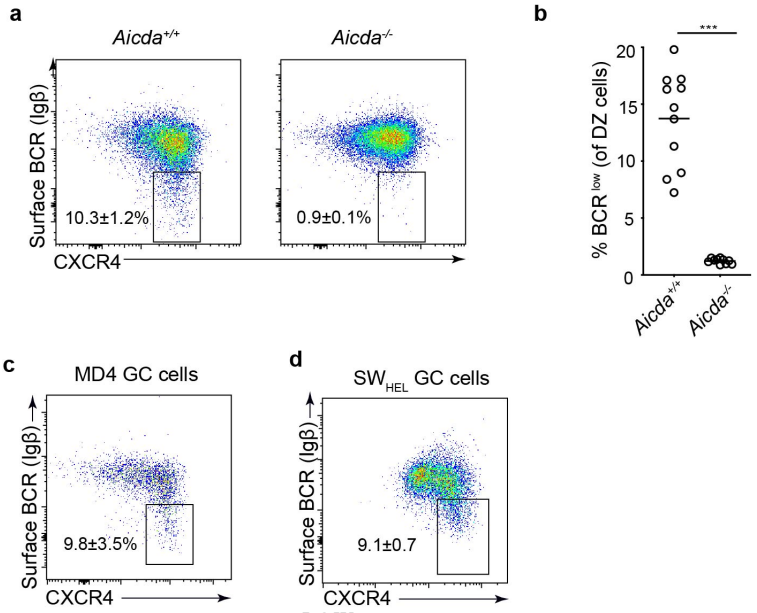


FIGURE 3.4| GENERATION OF BCR^{LOW} POPULATIONS IS RELATED TO SOMATIC HYPERMUTATION, BUT NOT DEPENDENT ON THE CREATION OF ABERRANT ANTIBODIES

(a) Surface BCR levels on *Aicda*$^{+/+}$ and *Aicda*$^{-/-}$ IgD^{low} $CD95^{+}$ $GL7^{+}$ GC cells from mixed bone marrow chimeric mice 8 days after SRBC immunisation (n=11). The results are summarised in (b). Analysis was performed using an unpaired two-tailed student's t-test, ***, P <0.001 (c, d) HEL-specific SW_{HEL} or MD4 B cells were adoptively co-transferred with OT-II $CD4^{+}$ T cells and mice were immunised with HEL-OVA and sigma adjuvant. Splenic IgD^{low} $CD95^{+}$ $GL7^{+}$ $CD45.1^{+}$ GC B cells were analysed on day 7. (c) DZ cells with low BCR levels in MD4 transgenic (n=7) (d) and SW_{HEL} gene targeted GC B cell populations (n=10). Numbers represent means ± SEM.

To further exclude this possibility, we next formally tested whether BCR^{low} cells were capable of producing intact antibodies, and were able to re-express the BCR on their surface. For the first of these questions, we cultured single cell sorted $CXCR4^{high}$ (DZ) GC B cells, expressing high or low BCR levels, using the recently described "Nojima" feeder cell method that allows single B cells to be expanded several hundred or thousand fold in 8 days[310]. FACS sorts were performed in "index" mode, so to save protein abundance and cell phenotype information for each individual cell. Supernatants were harvested after the culture period and screened by ELISA for the presence of intact antibodies (protocol outlined in Figure 3.5a). We again used the SW_{HEL} cell transfer/immunisation system for this assay, because all SW_{HEL} B cells utilise the Igκ light chain allowing ELISAs to be performed with a single detection reagent. This culture-based assay is more sensitive than *Ighv* sequencing approaches for detecting disruptive mutations.

Cells from both the $CXCR4^{high}$ BCR^{high} and BCR^{low} populations proliferated and differentiated to secrete antigen-specific antibodies during the culture periods (Figures 3.5b and c). The capacity of this method to relate level of surface BCR at the time of sorting to the capacity of the cell to produce detectable antibody when expanded *in vitro* provides valuable information. We observed that the cells that were capable of producing functional antibody were not only those cells on the edge of the BCR^{low} gate, but also included cells that clearly had undetectable surface BCR. Therefore, within the BCR^{low} DZ population, there are cells that are able to produce intact antibody. This is consistent with a model in which, the generation BCR^{low} population is not solely attributable to the induction of detrimental mutation in the *Ighv* genes. Interestingly, the frequency of BCR^{low} DZ cells successfully cloned was approximately half that of BCR^{high} DZ cells,

suggesting this population may contain cells at a mixture of states including some which may be destined to die.

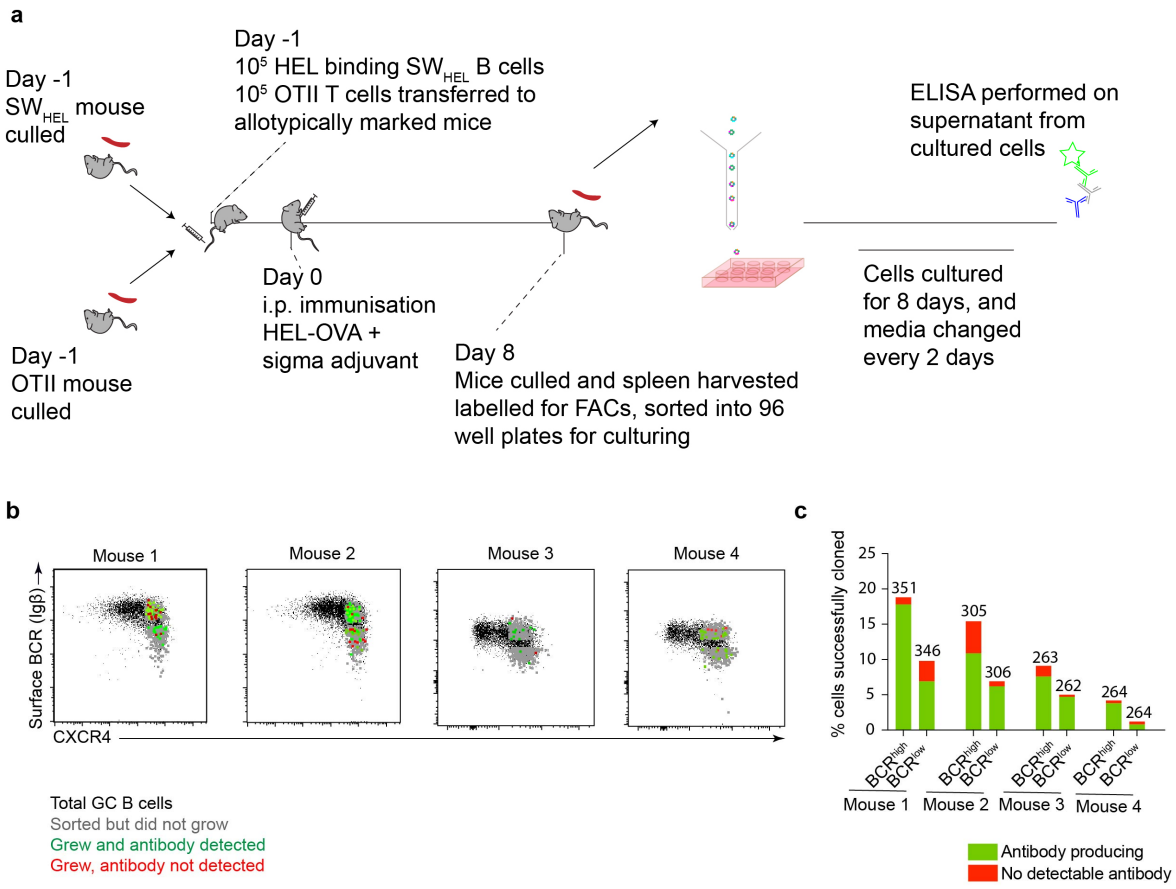


FIGURE 3.5| GENERATION OF THE BCR^{LOW} POPULATION IS NOT RELATED TO THE DAMAGE TO THE BCR

(a) Summary of protocol for (b, c) SW$_{HEL}$ GCs are generated as previously described. Single CXCR4^{high} BCR^{high} and CXCR4^{high} BCR^{low} DZ SW$_{HEL}$ GC B cells (gated IgD^{low}, CD38^{low}, GL7^{+}, CD45.1^{+} or CD45.2^{+}) were index sorted into pre-warmed culture with NB21 feeder cells and cultured for 8 days, with media changed every two days. Antibody production was analysed by ELISA. (b) The BCR and CXCR4 staining profiles (at the time of sort) of single GC B cells that grew and secreted detectable amounts of antibody after culture are shown. The plots for cells that did not grow, or that grew did not secrete detected immunoglobulin, are also shown and overlaid with the total GC B cell population for reference. (c) Summary of frequency of sorted cells that were successfully cloned, and of those that produced detectable antibody. Numbers of sorted cells in each group per experiment are indicated.

While the above results were clear in establishing that some or many BCR^{low} DZ cells retain the capacity to express soluble antibody, we wished also to exclude the unlikely possibility that these cells might have accumulated mutations in sites that prevent membrane expression of the BCR protein complex. To test this, we FACS sorted small populations of $CXCR4^{high}$ BCR^{high} or $CXCR4^{high}$ BCR^{low} GC B cells and cultured them *ex vivo* either for 8hrs alone, or for two days with "Nojima" feeder cells, and then analysed surface BCR expression by FACS. BCR^{low} GC B cells became BCR^{+} as early as 8 hours after sorting, confirming that the Ig genes in surviving cells were not damaged in a way that prevents receptor complex formation on the cell surface (Figures 3.6a and b).

If the presence of a BCR^{low} population in DZs depended on AID expression, but was not related only to the generation of autoreactive or non-functional BCRs, then we next considered whether their generation could be related to their increasing BCR affinity, with this in turn leading to increased protein turnover rates. The mechanism by which this would occur was unclear, but we addressed the possibility using *Aicda*$^{-/-}$ SW_{HEL} GCs, whose BCR at initiation of the GC is already very high affinity. In SW_{HEL} *Aicda*$^{-/-}$ GCs, the defect was still seen; therefore, the generation was unlikely related to the accumulation of higher affinity clones (Figure 3.6c and d).

In summary, GC DZs contain subpopulations of cells that have very low or undetectable BCR levels and that depend upon AID for their normal generation. The presence within the BCR^{low} DZ population of cells still capable of synthesising un-damaged immunoglobulin is consistent with,

although admittedly does not prove, a model in which BCR^{low} GC B cells are in the process of receptor replacement that is somehow triggered in response to SHM.

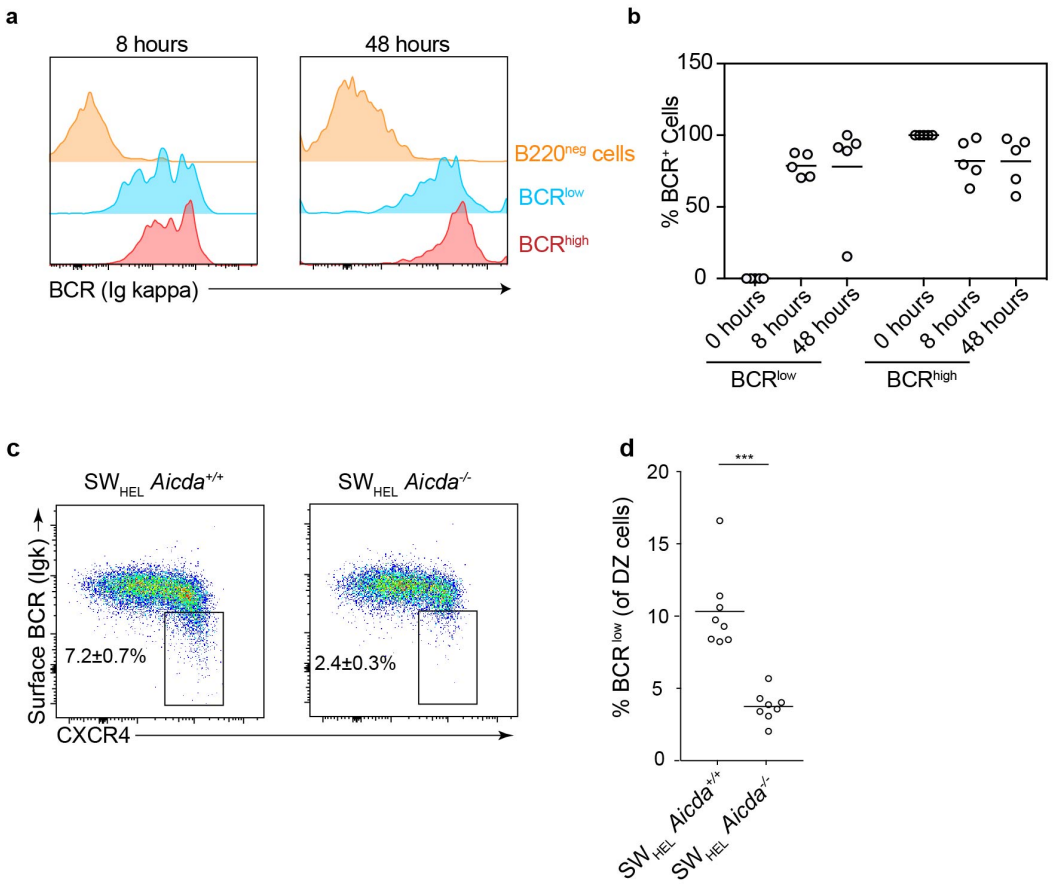


FIGURE 3.6| BCR^{LOW} CELLS CAN RE-EXPRESS SURFACE B CELL RECEPTOR *IN VITRO* AFTER SHORT-TERM CULTURE

(a, b) CXCR4^{high} BCR^{high} and CXCR4^{high} BCR^{low} GC B cells from SRBC immunised mice were sorted for 8hrs alone or for 24hrs under "Nojima" conditions. BCR levels at the end of culture were determined by FACs and compared to that of non-B cells. (a) Representative histogram, labels indicate phenotype at time of sort/plating, with data summarised in (b) Data in (a, b) are pooled from 2 experiments with each dot representing a single mouse. (c) Surface BCR levels in co-transferred *Aicda*$^{+/+}$ and *Aicda*$^{-/-}$ SW$_{HEL}$ GC B cells. SW$_{HEL}$ GC induced as in (Figure 3.5). Data summarised in (d). n=8, Numbers in (c) represent mean +/- S.E.M. of the shown GC B cells population. Analysis was performed using an unpaired two-tailed student's t-test, ***, P <0.001.

3.4 GERMINAL CENTRE B CELLS REPLACE THEIR ANTIGEN RECEPTORS FOLLOWING SOMATIC HYPERMUTATION BUT WHILE STILL IN THE DARK ZONE

As described previously, having diversified their BCRs in the DZ, GC B cells transition to the LZ where they encounter antigen on the surface of follicular dendritic cells. They internalise this antigen and present peptides from it to T_{FH} cells. Cells which have improved or maintained their ability to interact with antigen are said to be "selected" to re-enter DZs, and cells that have received a better quality or quantity of T cell help proliferate more upon re-entry into the DZ. Therefore, a presumed key requirement of selection in the LZ is that cells are selected on the BCR they encode. However, it is unknown whether DZ cells fully exchange their BCRs after SHM but before entering the LZ. Therefore, we investigated the critical issue of whether the turnover and replacement of pre-SHM BCRs with newly mutated versions may occur while cells are still in that zone.

The ideal assay for testing this important question would be to determine whether all the surface BCR proteins on a cell are post-SHM versions at the time of cells entering the LZ, and whether the protein sequence matches that encoded by the gene. However, SHM involves the induction of random mutations in the *Ighv* genes, therefore are no appropriate reagents exist for differentiating individual cell pre- and post-SHM BCR proteins, which may deviate by as little as a single amino acid residue. However, the one instance in which one may reasonably identify a cell which still expresses a surface BCR protein that it does not genetically encode is by identifying cells that have acquired structurally damaging mutations but that still have surface BCR protein. Therefore, we performed single cells sequencing of the BCR heavy chain variable region on indexed sorted cells,

such that we could correlate the surface expression of the BCR with the *Ighv* gene sequence; a cell expressing a functional BCR but genetically encoding a damaged BCR being a cell that has not yet exchanged its BCR following SHM. We reasoned that, by searching for cells that are expressing a BCR they no longer encode, it should be possible to determine to what extent BCR proteins are replaced within the DZ.

For this sequence analysis, we focused on the induction of premature stop codons in the variable region of the BCR heavy chain. Although premature stop codons are not the only mutation type that will be harmful to the immunoglobulin protein structure, they are invariably detrimental and so are an appropriate surrogate for structural disruption of the BCR. We predicted that cells with premature stop codons should present at measurable levels. Based upon published estimates of SHM rates (10^{-3} nucleotides/division), we calculated that in an unselected DZ population B cells should contain stop codons in the heavy chain variable region (*Ighv*) gene at a frequency of approximately 4.1% if DZ cells complete on average two cell cycles (as has been predicted)[112,120] and 6.4% when the average is three (details in methods).

For these experiments, we again used the SW_{HEL}/HEL-OVA immunisation system, with all being heterozygous for the SW_{HEL} IgH allele. The use of gene targeted B cells with a fixed *Ighv* gene allowed for the amplification and sequencing of gDNA sequences, thereby overcoming concerns regarding reverse transcriptase fidelity, non-sense mediated mRNA decay and non-productive allele transcription. VDJ recombination occurs in bone marrow at the pro-B cell stage. Ultimately, the VDJ region of a failed recombination event could look similar to the induction of a premature stop codon through SHM[316]. The use of the SW_{HEL} system also

eliminates this risk. Additionally, analysing genomic DNA rather than mRNA prevents underestimating the frequency of cells which have acquired a SHM, by avoiding the possibility that BCR mRNA from damaged genes is degraded more rapidly than that from undamaged genes, through nonsense mediated decay[317]. Single HEL-specific SW_{HEL} GC B cells (IgD^{low} $CD95^{high}$ $GL7^{high}$) were index sorted on day 8 post-immunisation and the presence or absence of premature stop codons in their *Ighv* genes was determined by PCR amplification and Sanger sequencing[292] (protocol summarised in Figure 3.7a). Premature stop codon encoding mutations were not detected in *Aicda*$^{-/-}$ SW_{HEL} GC B cells that cannot undergo SHM (n=25), confirming the fidelity of the assay (not shown).

We were able to detect cells encoding a premature stop codon, and these cells were more likely to have undetectable surface BCR. The BCR staining intensities of individual cells carrying the damaging mutations are shown in Figure 3.6b for each mouse where they are overlaid with the total GC (i.e. non sequenced cells, for reference) as well as with DZ B cells that did not carry premature stop codons. *Ighv* region stop codons were present in BCR^{high} DZ cells at a frequency of approximately 2.2% but were strikingly enriched in cells with undetectable BCR levels, being >7-fold more abundant (17.2%) (Figures 3.7b summarised in Figures 3.7c and d). Therefore, the majority of cells (66%) encoding a premature stop codon had removed most or all of their pre-SHM BCR in the period since that event. These results confirm that BCR^{low} DZ populations contain post-SHM unselected cells. Furthermore, these results suggest that GC B cells may turnover their BCR proteins following SHM while still in the DZ.

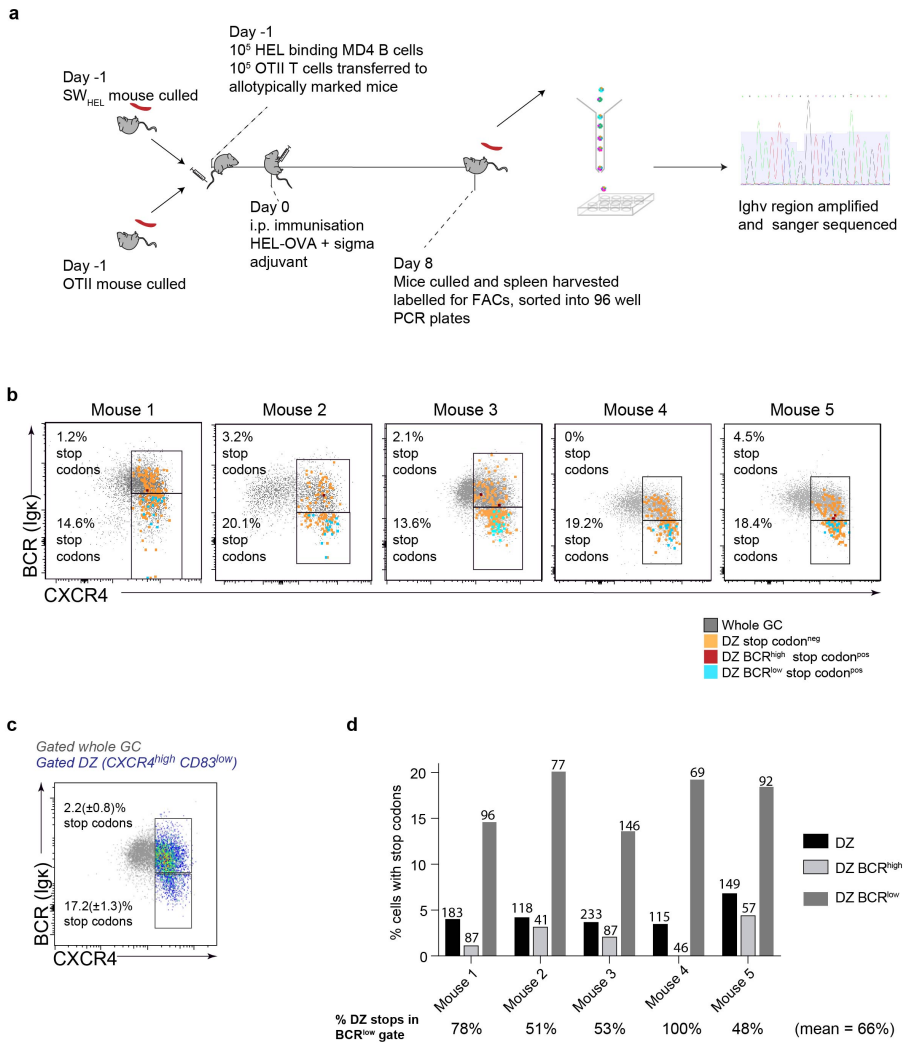


FIGURE 3.7| DARK ZONE CELLS ACQUIRING DETRIMENTAL MUTATIONS IN THEIR IMMUNOGLOBULIN V REGION GENES ACCUMULATE IN BCR^{LOW} GATES

(a) Summary of protocol. SW$_{HEL}$xR26p-Fucci2 B cells were co-transferred with OT-II CD4$^+$ T cells into WT hosts that were subsequently immunised with HEL-OVA/adjuvant. Single IgD^{low} CD95$^+$ GL7$^+$ SW$_{HEL}$ GC B cells were index sorted on day 8 and their *Ighv* region genes were PCR amplified, Sanger sequenced, and analysed for the presence of premature stop codons. (b) BCR and CXCR4 levels on individual indexed sequenced cells. Colours indicate presence or absence of *Ighv* region premature stop codons in cells of the indicated phenotype (pos, positive, neg, negative). Data are overlaid with cells that were not sequenced (grey), for reference. Less frequent BCR^{low} cells were enriched during FACs sorting. (c, d) Summary of results from multiple mice and experiments. (c) Frequency (mean +/- S.E.M., n=5) of premature stop codons in BCR^{high} and BCR^{low} DZ populations is shown. (d) Graphical representation of five experiments. Number of cells sequenced are indicated above bars and the proportion of total DZ stop codons that are in the BCR^{low} subset is marked below. Results are normalised for rare population enrichments performed during FACs sorting.

3.5 THE B CELL RECEPTOR IS REPLACED RAPIDLY IN THE DARK ZONE

GC B cells are in the DZ for an average of two divisions per cycle, each lasting 8 to 10 hours, meaning that each DZ stage lasts approximately 20 hours[45,120]. However, the half-life of the BCR complex in naïve cells was reported in early studies to be in the range of several days[269]. Therefore, full exchange of the BCR following LZ entry would require the half-life of the BCR to be significantly shorter in GC B cells than in naïve B cells, at least when they have undergone SHM.

Some estimates of BCR half-life in the DZ could be drawn from the above data. When observing the GC, one must recognise that it represents an asynchronous dataset of cells going through what is ultimately a linear process of SHM followed by BCR replacement. By asynchronous, I mean that some cells in the DZ would have very recently acquired a SHM while others may have acquired a SHM soon upon entry into the DZ and may be preparing to leave. One could consider 50% of cells with a premature stop codon to have acquired it within the last 10 hours before culling, and the other 50% to have acquired the mutation between 10 and 20 hours before culling (as mentioned above, DZ cells spend an average of 20 hours in that zone). Of the cells which encode a premature stop codon, those cells which are BCR^{high} represent those that have not yet had sufficient time to exchange their BCRs. Therefore, one could say that of the 33% of the cells which have acquired a premature stop codon have not yet "had time" to exchange their BCR, and are BCR^{high}. Similarly, one could say that 33% of cells had acquired their SHM in the 6.6 hours before culling; and 66% of cells acquired their SHM between 6.6 and 20 hours before culling. Therefore, it likely takes about an average of 6.6 hours for cells to down-regulate their BCR after SHM. It should be acknowledged however, this is an estimate based on assumptions

about DZ occupancy and assumes that all cells degrade their receptors at equal rates. In appendix two (section 7.2) I show some data which implies that the exchange of the BCR in the DZ unlikely controlled by modulating mRNA levels, either be degradation or by cessation of transcription, and is more likely to be the controlled by post-translational mechanism.

3.6 SUMMARY

We hypothesised that to ensure the efficiency of selection in the GC, cells would need to exchange their BCR while in the DZ. In this model, one would expect increased BCR turnover rates in GC B cells relative to naïve B cells, and also possibly increased turnover in DZ compared to LZ subsets. We observed that when naïve B cells differentiate into GC B cells they alter their handling of the BCR complex. The BCR is expressed at lower levels and internalised more rapidly in GC B cells relative to naïve B cells. In the DZ, the site of SHM, there is a population of cells with undetectable surface and intracellular BCR, suggesting that all antibody proteins have been degraded. These cells were capable of expressing BCR when cultured *in vitro,* indicating that their immunoglobulin genes remain intact. This raises the possibility that the BCR^{low} DZ populations may represent a pre-selection medley of cells, some of which are engaged in a process of receptor exchange. However, this model is still speculative. We have not been able to demonstrate *in vivo* that any of the cells in the DZ BCR^{low} population will subsequently re-express surface BCR and transition into the LZ. It is also plausible that receptor replacement is a relatively rapid but continuous process and that the seemingly "non-crippled" BCR^{low} cells observed here carry mutations that destabilize, rather than completely disrupt, the membrane BCR complex.

To address whether cells in the DZ are predominantly expressing the BCR they encode, we utilised the fact that there is one instance in which one may identify a cell which is expressing a BCR it no longer encodes. This is when cells express undamaged BCRs on their surface, yet carry genes encoding a SHM induced premature stop codon, which would prevent a functional protein synthesis. We correlated *Ighv* sequences with surface expression of BCRs to identify how commonly cells in the DZ express BCRs that they no longer encode. If cells frequently transitioned into the LZ having not exchanged their surface BCR, then one would expect the majority of cells which have acquired a premature stop codon to be BCR^{high}; alternately if cells exchange their BCR before entering the LZ then the majority of cells encoding a premature stop codon in the DZ would have already exchanged their BCR. 66% of cells which encode a premature stop codon are BCR^{low}, which is consistent with a model in which the BCR is exchanged while the cells are still in the DZ.

Early studies on BCR turnover in follicular B cells predicted protein half-lives to be in the range of 20–80 hrs[318]. However, we estimated that the half-life of the BCR in the DZ of the GC would be closer to 6 and a half hours. As already discussed, we would not claim these to represent accurate numbers but rather crude approximations, but there is good reason to believe they will be in the right range. It may be that increased BCR turnover rates in DZ GC B cells, relative to follicular B cells, allows for the BCR to be fully exchanged while cells are still in that phase. This might be sufficient for allowing the removal of pre-SHM BCR from the surface of the DZ cells in advance of LZ entry if there were a mechanism to restrict SHM activity to specific stages in the DZ cycle (elaborated in chapter four).

The existence of small numbers of BCR^{high} cells carrying detrimental *Ighv* mutations in the DZ indicates that SHM occurs at times of receptor expression, however the greater abundance of stop codons within BCR^{low} populations suggests that replacement may then proceed quickly. An interesting question, which we were not able to address, relates to the kinetics of the BCR exchange. It is possible that following SHM there is a period of replacement during which the pre-SHM and post-SHM BCR are co-expressed on the surface of the GC. The length of time the period of "dual specificity" lasts will be dictated by the half-life of the BCR in DZ GC B cells. Alternatively, cells may actively purge their old, pre-SHM BCR very quickly after SHM has occurred. In this second model, the period of time within which cells express BCRs they no longer encode is minimised. However, if cells maintaining pre-SHM BCRs on their surface are restricted to the DZ (as is investigated in the next chapter), then this should not pose any selection issues. The question of whether the BCR is "purged" following SHM, or whether instead there is a period of co-expression following SHM were-in pre- and post SHM BCR is maintained on the cell surface can be rephrased as two different questions: do cells have a mechanism to detect that SHM has occurred? And, is degradation of the BCR constant in the DZ?

If the purging model is correct, then BCR degradation must be an acute event that is "triggered" by, or co-ordinated with, SHM. For the former of these possibilities to be correct, cells must have a mechanism so detect SHM activity. Interestingly, we found that the presence of BCR^{neg} cells depended upon expression of AID, the key enzyme responsible for initiating SHM. Therefore, it is possible that the BCR^{low} cells in the GC, which encode functional, non-self-reactive BCRs, are cells which have recently purged their BCR following SHM. One

hypothesis might be that SHM activity is sensed through DNA damage response pathways that subsequently initiate BCR degradation however, this remains a purely speculative model. Secondly, if the BCR is purged from the surface of the B cell then the rate of degradation of the BCR in the DZ is not constant. We invested considerable effort and time in trying to develop new tools for determining whether BCR turnover occurs uniformly in all cells or if it is preferentially favoured at particular times, however our effort unfortunately did not prove fruitful. In appendix 1, I have described the various approaches tried. Addressing this important question remains an active area of interest for the group.

In this section I have described finding which are consistent with the GC being specifically adapted to support the rapid turnover of the BCR complexes in GC DZs following SHM. In the next chapter, we go on to investigate the key question of to what extent BCR replacement is completed in advance of cells testing their newly mutated antibody genes during selection.

CHAPTER FOUR: CELLS TRANSITION INTO THE LZ EXPRESSING THE B CELL RECEPTOR THEY ENCODE, BUT FAIL THE TRANSITION IF THEIR B CELL RECEPTOR IS DESTROYED

In the first results chapter, we observed that subsets of DZ cells show evidence of having degraded all their pre-SHM BCR complexes following SHM. In this chapter, we set out to address to what extent BCR replacement occurs after SHM but before LZ entry, by searching for cells in the LZ that express a BCR on their surface which they no longer genetically encode. We investigated the possibility that GC B cells facilitate the exchange of the BCR while cells are still in the DZ by restricting AID activity to a specific stage in the DZ cycle.

4.1 EVIDENCE OF SOMATIC HYPERMUTATION AT G1 IN THE DARK ZONE FOLLOWED BY CONTINUED INITIATION OF AND PROGRESSION THROUGH CELL CYCLE WITHOUT FUNCTIONAL B CELL RECEPTOR GENES

We hypothesised that, if cells exchanged their BCRs following SHM but before entering the LZ, then there could possibly be a restriction of AID activity to specific stages in the DZ cycle. Our single cell *Ighv* sequencing protocol represented an interesting opportunity to analyse the question of restriction of AID activity to specific stages in the DZ cycle. Cells expressing high levels of surface BCR, but whose *Ighv* region gene has been rendered non-functional through SHM, are cells which have not yet "had time" to degrade their BCR proteins. Therefore, these are cells most proximal to the SHM event than has been ever been identified *in vivo*. Exactly how proximal to the SHM event these cells are depends on the speed at which the BCR is

exchanged. The single cell sequencing experiments described in Chapter One were performed in R26-Fucci2 mice. This provided us with the opportunity to investigate whether any correlation existed between the timing of SHM and cell cycle staging.

We analysed the indexed *Ighv* region sequencing results to determine whether any correlation exists between the presence of disruptive stop codons and cell cycle stage. Interestingly, all DZ cells which express high levels of BCR yet genetically encode a disrupted BCR were restricted to G1 *in vivo* (Figures 4.1a, summarised in Figures 4.1b). In contrast, BCR^{low} DZ cells carrying premature stop codons were found at all cell cycle stages (Figures 4.1c). Despite catching our attention, these results were however at best suggestive, because BCR^{high} cells carrying premature stop codons were extremely rare and so the number of events interrogated was very small. Therefore, to further examine this possibility, we sought perform a similar analysis but on larger cell numbers by developing a next generation/Illumina sequencing approach.

To this end, bulk populations of BCR^{high} cells in early G1, post mitotic cells; late G1; and late S, G2, M, were sorted. The exact numbers of cells harvested varied for the individual subsets, but ranged from 180 to 500 cells. The *Ighv* genes from the bulk populations were amplified, indexed tagged, and sequenced on an Illumina MiSeq machine. We found that amongst the BCR^{high} cells in the DZ, reads encoding premature stop codons were restricted to G1, premature stop codons were detected in early G1 in two of seven mice, in late G1 in three of seven mice (Figures 4.1d and summarised in 3.1e). We observed slightly lower stop codon frequencies overall using the NGS sequencing method, which likely reflected the stringency of the BCR^{high} gates used in these particular experiments. While this method of measuring AID activity remains somewhat indirect, these results are highly suggestive that AID activity in the GC may be restricted to cells at the G1

stage of cell cycle. An association of AID activity and G1 was previously reported in *in vitro* B cell culture systems during class switch recombination[319,320].

Restriction of AID activity to G1 could possibly be a response to two presumed selective pressures in the GC: the requirement to preserve positively selected clones, and the pressure on cells to be selected on the basis of the BCR they encode. In regards to the first evolutionary pressure, the first division following selection is initiated in the LZ, and so cells mostly enter the DZ in S phase. Therefore, if SHM occurred only or preferentially in G1 DZ cells, this might help ensure that all newly "selected" cells complete at least one mitotic event before initiating SHM again. This could, in principle, ensure some degree of clonal expansion in advance of further diversification. As cells have approximately a one in two chance of introducing a mutation each division, it seems likely that the unmutated clone will be preserved through subsequent division.

Additionally, a possible restriction in AID activity to a specific cell cycle stage could ensure that cells have sufficient time to exchange their BCR following SHM. If SHM is restricted to G1, and cells take around six and a half hours to exchange their BCR, given that it takes about 10 hours to complete mitosis, cells would have had time to exchange their BCR by the completion of mitosis. This conceptualisation of exchanges raises interesting question about cells in G1, which has exhausted their DZ program and are awaiting transition into the LZ. It may be that cells which are preparing for LZ re-entry do not initiate SHM. Alternately, cells that do acquire a SHM in their final G1 phase may transition into the LZ having acquired a mutation but not exchanged their BCR, this may be the source of the rare BCR^{high} cells in the LZ which encode a premature stop codon.

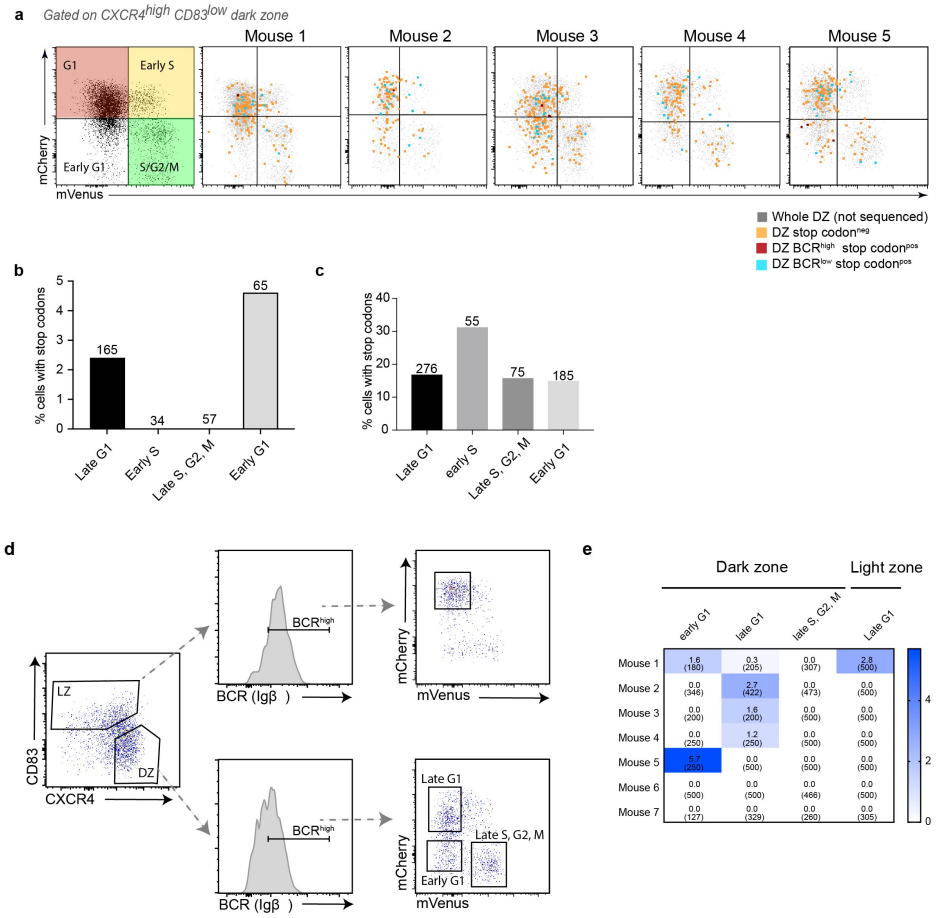


FIGURE 4.1| CORRELATING CELL CYCLE AND THE ACQUISITION OF STOP CODONS

As in Figure 3.6, SW_{HEL}xR26p-Fucci2 B cells were co-transferred with OT-II $CD4^+$ T cells into WT hosts that were subsequently immunised with HEL-OVA/adjuvant. Single IgD^{low} $CD95^+$ $GL7^+$ SW_{HEL} GC B cells were index sorted on day 8 and their *Ighv* region genes were PCR amplified, Sanger sequenced, and analysed for the presence of premature stop codons. (a) Gates indicate the cell cycle status of DZ SW_{HEL}xR26p-Fucci2 GC B cells (annotated in left plot). Cells with high and low BCR levels that have acquired premature *Ighv* region stop codons, as well as sequenced cells in which stop codons were not detected, are shown (right). Data are overlaid with cells that were not sequenced (grey), for reference. (b, c) Frequency of stop codons among DZ cells at the different cell cycle stages (b) DZ BCR^{high} and (c) DZ BCR^{low} (c) includes results from 3 additional mice from which rare BCR^{low} S and early G1 phase cells were enriched to increased cell numbers. (d, e) SW_{HEL}xR26p-Fucci2 GC were generated as above, and bulk populations were sorted for NGS sequencing. (d) Representative gates used to sort BCR^{high} cells at different cell cycle stages for bulk population based NGS analysis of stop codon presence. (e) The frequency of stop codons detected within each population is shown, with the lower numbers in brackets indicating population sizes sorted from each gate and mouse. 7 *Aicda*$^{-/-}$ control populations were included and showed the expected patterns of zero stop codons (not shown).

4.2 GERMINAL CENTRE B CELLS ACQUIRING DAMAGING IMMUNOGLOBULIN GENE MUTATIONS RARELY REACH THE LIGHT ZONE PHASE

To address the key question of whether few, most, or all GC B cells fully replace their BCR complexes after SHM but before entering LZs, we next performed a similar correlative analysis of BCR levels and the presence/absence of stop codons, but this time focussing on LZ cells. We hypothesised that if cells exchanged their BCR before LZ entry then the majority of cells encoding a premature stop codon would have removed the pre-SHM BCR from the surface of their cells, and will have undetectable levels of surface BCR. Alternately, if cells transition into the LZ before having completely exchanged their BCRs, one would expect cells genetically encoding a disrupted BCR to still express surface BCR complexes.

Current models for antibody affinity maturation involve GC B cells moving to the LZ following SHM in order to "test" the ability of the newly mutated BCR to interact with, internalise, and present antigen. If all cells transitioned into the LZ, and cells are in the DZ for an average of two divisions, then one would expect the frequency of cells in the LZ carrying a damaged *Ighv* gene would be about 5.7%, or even higher as cells accumulate, awaiting death, as their sister cells are selected back into the DZ. Surprisingly, contrary to these predictions, there was no accumulation of cells with premature stop codons in the LZ. Strikingly the opposite was true, rather than collecting in the LZ as a consequence of them failing the DZ re-entry bottleneck, cells in the LZ were three times less likely to carry a detrimental mutation than DZ cells (4.6% in the DZ versus 1.3% in the LZ) (Figures 4.2a-c).

As these data are from index sorted cells, their particular expression of LZ and DZ markers can be analysed, and compared between cells carrying premature stop codons and those which do not. The cells carrying premature stop codons were more commonly at the LZ/DZ border, however this is also generally where cells are more densely packed. Given the cells are so rare, it is difficult to say if LZ cells encoding premature stop are less fully "LZ like" than their sister cells confined to that zone. There may be benefit in the future to performing RNAseq analysis on these cells to more fully understand if how well they comport with the DZ/LZ binary. For this discussion, moreover, we will consider them fully LZ cells.

We can assess the surface BCR levels on the cells in the light zone encoding premature stop codons. Interestingly, in terms of the question of exchange of the BCR before LZ entry, LZ cells carrying a premature stop codon were more likely to still have high surface BCR levels; 74% of cells carrying premature stop codons were BCR^{high} (Figures 4.2c). If cells were to transition into the LZ before replacing their pre-SHM surface BCRs, then one would expect cells in the LZ which encode a premature stop codon in their *Ighv* region genes to express intact BCR on their surface. Conversely, if cells fully exchanged their BCR in the DZ before LZ entry, then one would expect the majority of cells in the LZ, that encode a damaged *Ighv* regions, to have undetectable surface BCR. However, the interpretation is less straightforward if the cells carrying premature stop codons are lost in the LZ.

If 74% of the LZ cells carry a detrimental mutation are expressing a functional BCR, that is to say they have not yet exchanged their BCR since undergoing SHM, then 0.96% of cells in the LZ are stop codon positive and BCR^{high} (i.e. 0.74 x 1.3 % = 0.96%). Given that the frequency of stop codons in the DZ is 4.6%, this would suggest that ~20% of cells have transitioned to the LZ stage without having exchanged their BCR; therefore, one could say that ~80% of cells exchange their

BCR before entering the LZ (0.96% is 21% of 4.6%). In fact, this estimate that 80% of cells exchange their BCR before LZ entry is likely an underestimate. The totality of cells in the DZ is in some ways not a representation population of those cells transitioning to the LZ, as this is an asynchronous data set. The cells present in the DZ include cells which have recently entered the GC, and have not yet initiated SHM, and those which not yet completed the number of divisions they are destined to complete, as well as those which have exhausted their proliferative potential in the DZ and are preparing to transition to the LZ. We estimated the frequency of premature stop codons in the DZ, if cells are in the DZ for an average of two divisions, would be 4.1% (calculations in the methods section) in reality the frequency was remarkably similar to this at 4.6%. Using the same formula, we used to estimate the frequency of stop codons, but working backwards from the final frequency of cells with premature stop codons in the DZ, we would find that the likelihood of acquiring a premature stop codon per division is 3.26%. Therefore, of those cells that are about to transition into the LZ, having completed their average of two divisions, 6.4% would have acquired a premature stop codon. If only 0.96% of cells in the LZ carry a recently acquired damaging mutation while still expressing a BCR on their surface, and 6.4% of cells having finished their DZ program will encode a premature stop codon, then those cells which have not exchanged their BCR only represent 15% of the cells with premature stop codons, therefore cells exchange their BCR before LZ enter ~85% of the time.

The presence of cells in the LZ which express high levels of surface BCR but encode an *Ighv* gene with a premature stop codon most likely represent a leakiness in the system on two fronts; either they are cells which have transitioned to the LZ before having sufficient time to exchange their BCR, or these represent instances of SHM that have occurred in the LZ. The estimates

above, do not account for the cells acquire SHM has been initiated in the LZ, but this is likely to be a more rare event.

The clear deficit of cells in the LZ that have acquired premature stop codons, and have corresponding lost surface BCR was striking. Only 24% of the cells carrying a premature stop codon in the LZ have lost surface BCR; therefore only 0.3% of the LZ are cells encode a premature stop codon and which have lost surface BCR. There were two possible explanations for this result. The first hypothesis first was that the cells that have lost surface BCR were rapidly deleted in LZ, and as such they do not accumulate in the LZ awaiting death by neglect. The second hypothesis is that these cells fail to transition to the LZ.

The second model predicts presence of a previously unappreciated selection bottleneck at the DZ to LZ stage; we have tended in the past to only consider bottlenecks and selection to occur at the LZ to DZ transition stage. In this model, cells losing expression of a functional antigen receptor may die while still in the DZ. Using the more conservative estimate, if 80% of cells exchange their BCR before LZ entry, then having finished their DZ cycle 5.1% of cells (80% of 6.4% of cells) will encode a premature stop codon and have lost surface expression of the BCR. If only 0.3% of cells in the LZ encode a premature stop codon and have lost their surface BCR, then only 5.9% of cells which this phenotype transition into the LZ, or conversely 94.1% of cells will not make the transition.

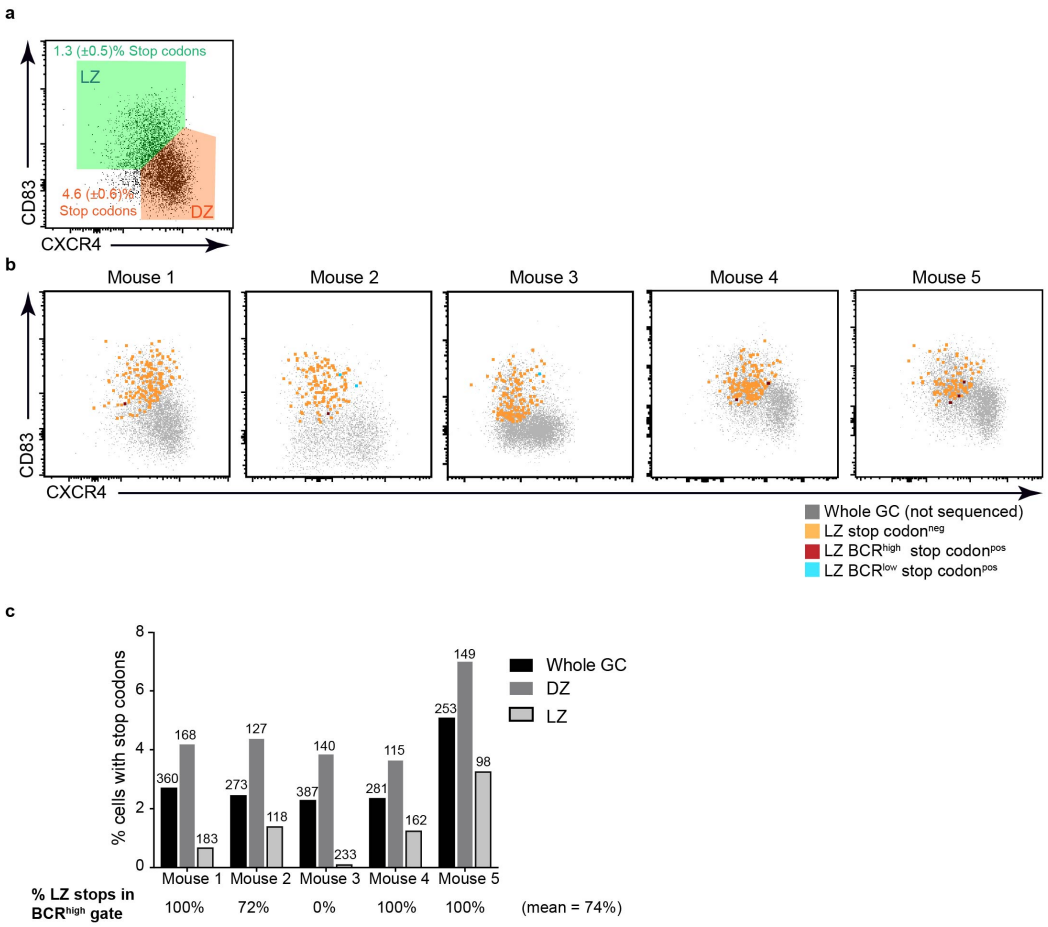


FIGURE 4.2| CELLS CARRYING PREMATURE STOP CODONS FAIL TO ACCUMULATE IN THE LIGHT ZONE.

As in Figure 3.6, SW_{HEL}xR26p-Fucci2 B cells were co-transferred with OT-II $CD4^+$ T cells into WT hosts that were subsequently immunised with HEL-OVA/adjuvant. Single IgD^{low} $CD95^+$ $GL7^+$ SW_{HEL} GC B cells were index sorted on day 8 and their *Ighv* region genes were PCR amplified, Sanger sequenced, and analysed for the presence of premature stop codons. (a) The frequency of cells carrying *Ighv* region premature stop codons in SW_{HEL}xR26p-Fucci2 LZ and DZ populations was determined. Plot and gate are from a representative mouse, numbers indicating means (+/- S.E.M.) from five independent experiments. (b) The phenotype of LZ cells with premature stop codons is shown for all three mice. Data are overlaid with total GC B cells that were not sequenced (grey), for reference. (c) Summary from five independent experiments. Numbers above bars indicate number of each cell type sequenced. Numbers below indicate frequency of detected LZ stop codons that were BCR^{high} and have been normalised for where rare populations were enrichments performed during FACs sorting.

It is worthwhile to note that there are low numbers of cells with very low levels of surface BCR in the LZ, (~5% of the LZ). We hypothesised that the cells in this population were not predominantly cells that have lost surface BCR, but rather were cells that have differentiated into plasma cell, which down-regulate surface BCR. We addressed this question using we examined Blimp-1 expression by GC B cells using a GFP transcriptional reporter mouse (*Prdm1*$^{wt/gfp}$ heterozygous mice)[321]. We immunised these mice were SRBC and analysed on day 8, GC B cells were gated as previously described. We found the CXCR4^{low} BCR^{low} cells were enriched for BLIMP expression, and therefore, likely represented pre-plasma cells (Figure 4.3).

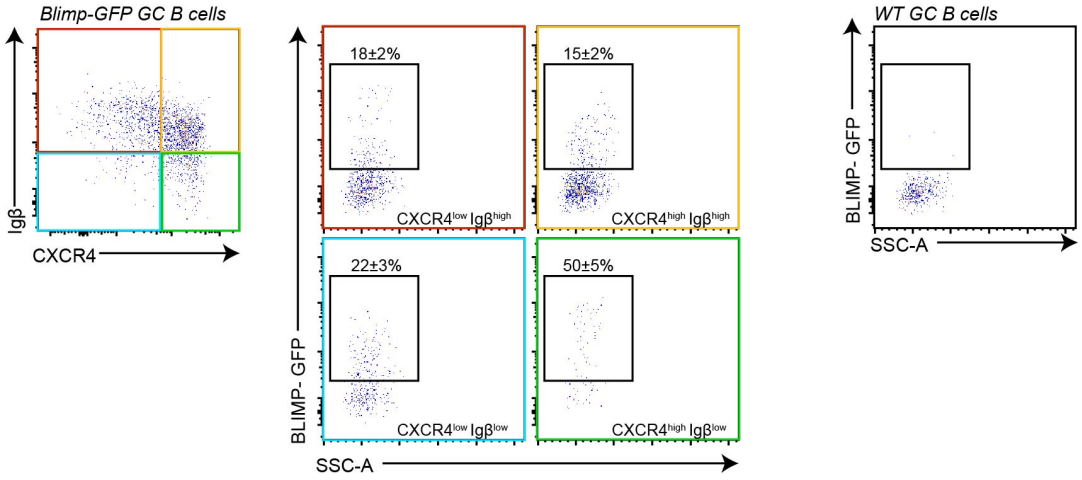


FIGURE 4.3| BCR^{LOW} LIGHT ZONE CELLS ARE ENRICHED FOR PRE-PLASMA CELLS

Prdm1$^{+/GFP}$ mice were immunised with SRBC and culled on day 7, spleens were harvested and stained for FACS analysis. GC were defined as IgD^{low} CD95^{+} GI7^{+} B cells. Cells are divided in quadrants based on CXCR4 (DZ marker) and Igβ (BCR component), and GFP fluorescence for each quadrant is displayed in plots with corresponding coloured borders. Below, in black, the background fluorescence in WT GCs. (n=7, numbers represent mean ± SEM).

4.3 GC B CELLS MAY UNDERGO CELL DEATH AS AN ALTERNATIVE FATE TO INITIATING ANOTHER CELL DIVISION OR TRANSITIONING TO THE LIGHT ZONE STAGE.

In order to address the question of where cells that have lost functional BCR die in the GC, we investigated the site and timing of apoptosis in GC. According to current models of selection, all cells are equally competent to transition to the LZ, and upon entry into the LZ cells that have lost their surface BCR should die as a consequence of neglect. These models of selection would predict the most cell death in the GC occurs in the LZ, as cells which have lost the ability to interact with antigen fail selection. However, if the alternate hypothesis were correct, and cells which have lost surface BCR fail a LZ entry checkpoint and do not transition into the LZ, then there would be significant death in the DZ, especially of BCR^{low} cells.

We assessed these hypotheses by flow cytometry, by intracellular staining of fixed and permeabilised cells with a monoclonal antibody specific for the active form of caspase3 (act-Casp3)[322]. GCs were induced by SRBC immunisation and GC B cells were gated as previously described (IgD^{low} $CD95^{+}$ $GL7^{+}$). Consistent with our second hypothesis, Act-$Casp3^{+}$ GC B cells were detected in both LZ and DZ gated populations (gated as shown, on the basis of CD83 and CXCR4). Interestingly the frequency of Act-$Casp3^{+}$ cells was slightly higher in the latter subset in this particular immunisation setting (3% in DZ, compared to 1% in the LZ), which is also numerically larger (Figures 4.4a and b). Therefore, most GC cell death occurs in the DZ. Furthermore, cells dying in the DZ were more likely to be BCR^{low}- BCR^{low} cells were about 4 times more likely to be apoptotic that BCR^{high} cells (Figures 4.4c). This pattern of cell death is consistent with a model in which cells, having introduced a detrimental mutation into their

immunoglobulin genes, will remove their pre-SHM BCR from the surface of the cell, and having failed to transition to the LZ, will die in the DZ.

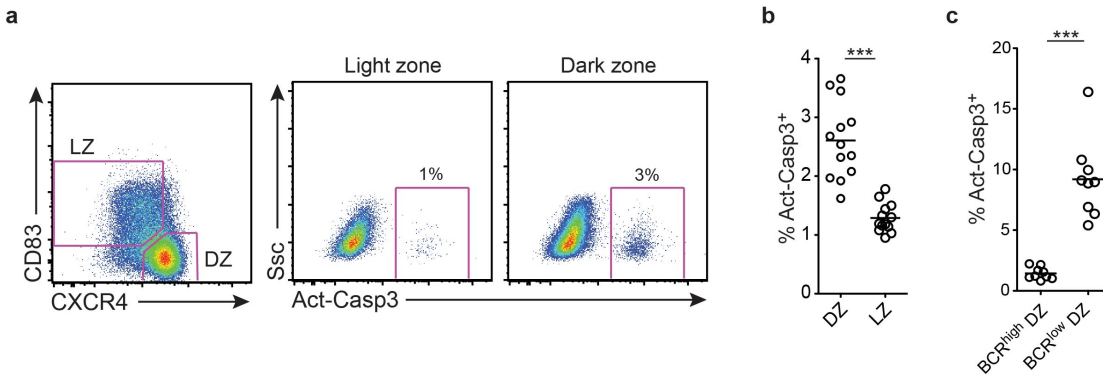


FIGURE 4.4| BCR^{LOW} GERMINAL CENTRE B CELLS UNDERGO APOPTOSIS IN THE DARK ZONE

Mice were immunised with SRBCs and splenic IgD^{low} CD95^{+} GL7^{+} GC B cells were analysed on day 10. (a) GC B cells were stained with LZ/DZ markers, fixed and permeabilised and stained with anti- act-Casp3. Data summarised in (b). (c) BCR (Igβ) levels on act-Casp3^{+} GC B cells were measured, frequencies of BCR^{high} and BCR^{low} DZ cells that were act-Casp3^{+} are displayed. N=13 mice from 4 experiments; plots and means. Analysis was performed using an unpaired two-tailed student's t-test, ***, P <0.001

To investigate if cells that have lost functional BCR simply die before they have had time to enter the LZ, or whether instead they die upon entry into the LZ, we analysed if delaying or preventing cell death might be sufficient for GC B cells with detrimental mutations to transition to the LZ state. If cells that have lost function surface BCR are more prone to death than cells with functional BCR, but are generally just as capable of transitioning to the LZ state, then delaying apoptosis would allow cells encoding non-functional BCR to transition into the LZ. To address this question, we analysed GCs from Bcl2-tg mice. Bcl2-tg mice, as previously described, have

multiple copies of the anti-apoptotic a *Bcl-2* transgene. In order to characterise the GCs generated in the presence of this transgene, we immunised Bcl2-tg mice with SRBCs and analysed splenocytes by flow cytometry on day 10, with GCs being gated as previously described. GCs from mice carrying a B cell specific Bcl2 anti-apoptotic transgene contained larger DZs and an increased frequency of $CXCR4^{high}$ BCR^{low} cells (Figures 4.5a and b). This phenotype was consistent with delayed apoptosis in the DZ being associated with an accumulation of cells in the DZ which have lost surface BCR.

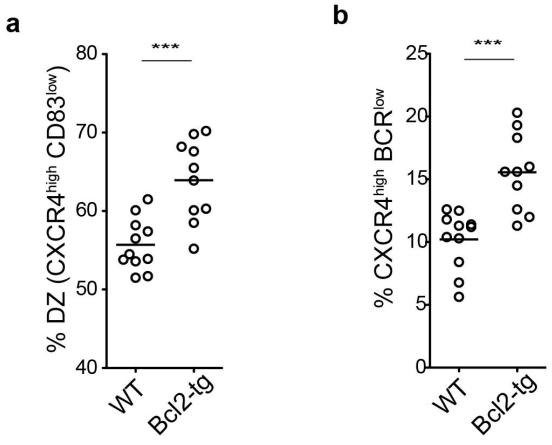


FIGURE 4.5| BCL2 GERMINAL CENTRE PHENOTYPE

(a-b) WT or BCL2-tg mice were immunised with SRBCs splenic IgD^{low} $CD95^{+}$ $GL7^{+}$ GC B cells were analysed on day 10 (n=10, from three experiments). (a) Frequencies of $CXCR4^{high}$ $CD83^{low}$ DZ cells and (b) $CXCR4^{high}$ $BCR(Ig\beta)^{low}$ cells in GCs ($CD38^{low}$ IgD^{low} $GL7^{+}$). Analysis was performed using an unpaired two-tailed student's t-test, ***, $P < 0.001$.

We investigated whether delaying apoptosis will allow cells with non-functional BCRs to enter the LZ, by performing similar single cell *Ighv* sequencing experiments to those described earlier, but this time using SW_{HEL} cells that carried the Bcl2-tg. These GCs were induced in the same manner as in previous experiments. Premature *Ighv* stop codons were present in Bcl2-tg^{+} SW_{HEL} GC B cells populations at approximately 2.5-fold that in WT mice (11.6% vs 4.6% for WT DZ cells), and were 45% higher within the BCR^{low} subset than in the WT equivalent (25.0% vs 17.2%), supporting the notion that the transgene allows cells to persist for longer after a detrimental SHM event has occurred (Figures 4.6a and b). Surprisingly, the frequency of detrimental somatic mutations was also increased within the DZ BCR^{high} gates, perhaps indicating either higher SHM rates in these cells, possibly related to general increased resistance to genotoxic stress[319].

Despite the striking increases in the DZ subsets, the promotion of cell survival through expression of the Bcl2 transgene was not sufficient to increase the frequency of cells with premature stop codons present within LZ gates (0.8% of $CXCR4^{low}$ $CD83^{high}$ cells in Bcl2-transgenic vs 1.3% in WT cells) (Figures 4.6c-e). While it is possible that SHM and LZ re-entry are temporally separated to a degree that cannot be rescued by the transgene, these results raise the possibility that BCR-dependent cues may be necessary not only for survival in the DZ but also for the transitioning to the LZ state.

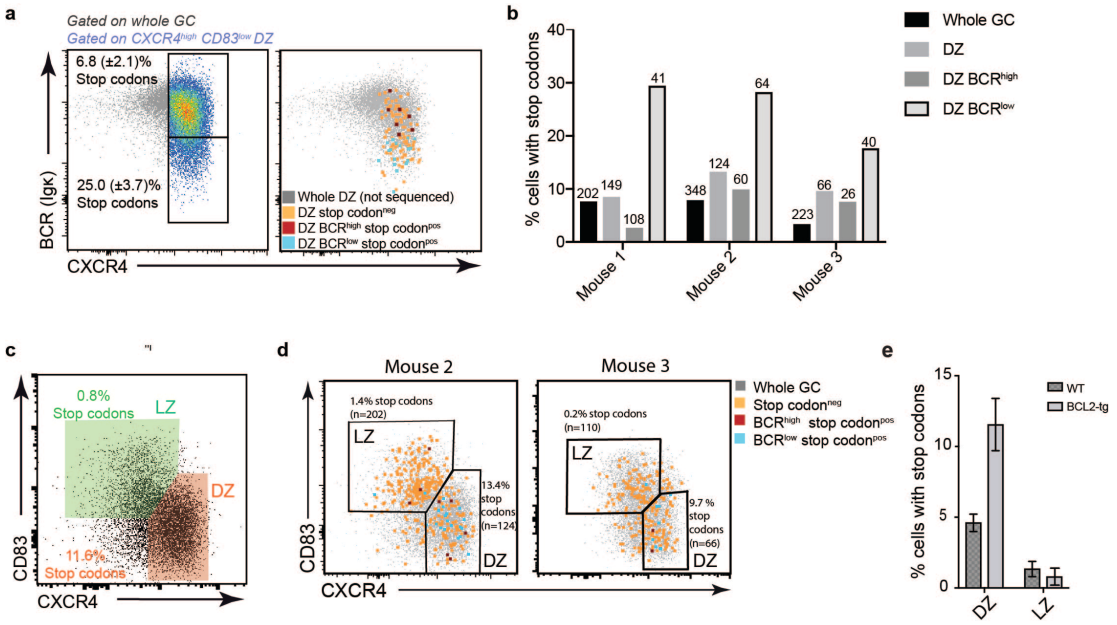


FIGURE 4.6| DELAYING APOPTOSIS IS NOT SUFFICIENT TO DRIVE CELLS INTO THE LZ

Similar to Figure 3.7 B cells were co-transferred with OT-II $CD4^+$ T cells into WT hosts that were subsequently immunised with HEL-OVA/adjuvant. Single IgD^{low} $CD95^+$ $GL7^+$ SW_{HEL} GC B cells were index sorted on day 8 and their *Ighv* region genes were PCR amplified, Sanger sequenced, and analysed for the presence of premature stop codons. (a) Frequencies of Bcl2-tg SW_{HEL} DZ cells with high and low BCR levels that carry *Ighv* region premature stop codons are shown (left). The phenotype of sequenced cells with stop codons is shown to right. Numbers represent mean ± SEM. (b) Summary of results from multiple mice and experiments. Numbers of cells sequenced are indicated. Mouse 1 DZ is gated using only CXCR4. (c) Frequencies of DZ and LZ cells with premature stop codons. Numbers are means from two independent experiments which are shown separately in (d). Cells with high and low BCR levels that have acquired premature *Ighv* region stop codons, as well as sequenced cells in which stop codons were not detected, are shown (right). Data are overlaid with cells that were not sequenced (grey), for reference. (e) Comparison of frequency of cells with premature stop codons in DZ and LZ in WT and Bcl2-tg mice.

Given that delaying apoptosis does not allow cells with non-functional BCRs to transition into the LZ, it is possible that these cells might be negatively selected in the DZ, and are actively restricted from the LZ. We hypothesised that if cells which have lost functional BCR are negatively selected from entering the LZ, then cell death of BCR^{low} cells could be restricted to a specific stage of the DZ program. It has been shown that GC B cells move to the LZ a number of hours after exiting cell cycle in the DZ[323].

The R26-Fucci2 model allows us to analyse the relative amount of time a cell has spent in G1. Analysis of GC B cells from the R26-Fucci2 mice revealed that the timing of CXCR4 down-regulation is tightly co-ordinated with a specific period in late G1. BrdU pulse-chasing experiments revealed that this period corresponds to 8-10 hours after the completion of the last S phase because it took labelled cells this period of time to reach the relevant mCherry levels (Figures 4.7a(i)). By comparison, S phase re-entry in the DZ occurs 5-8hrs after the previous period of DNA synthesis (Figures 4.7a(ii)). As such, the period in which CXCR4 down-regulation occurs follows only slightly that of S phase re-entry by DZ cells initiating more cellular divisions. This late G1 period can therefore be considered a fate junction wherein cells must commit to S phase re-entry or to CXCR4 down-regulation (period corresponding to shaded yellow area on Figures 4.7b). With this in mind, it was notable to us in earlier experiments that GC B cells with damaged *Ighv* genes and very low BCR levels were reasonably abundant at this late G1 phase and in the populations of cell re-entering S phase from it despite their being almost absent from the LZ which is only separated by a short period of time (Figures 4.1a and c). We therefore sought to ask whether apoptosis might preferentially occur at the period when cells might otherwise be trying to make the DZ to LZ transition.

DZ GC B cells staining for act-Casp3 were present at all stages of cell cycle indicating that apoptosis can occur at any time (Figures 4.7c). However, we observed a striking enrichment within the apoptotic gate of cells at late G1 (mCherry^{high} mVenus^{neg}) indicating that a disproportionate amount of cell death occurs at this stage, and 60% of apoptotic BCR^{low} DZ cells were at this cell cycle stage (Figures 4.7c, summarised in d). Therefore, GC B cells may undergo cell death at any time while in the DZ but they are most likely to do so in late G1 when other cells are re-entering S phase or transitioning to the LZ. Consequently, mCherry levels in apoptotic DZ cells were higher than in non-apoptotic CXCR4^{high} CD83^{low} cells but lower than in the LZ (Figures 4.7e). These data imply that death of cells that have lost surface BCR may be favoured to occur at specific stages in the DZ cycle. It provides evidence for a model of GC selection in which cells carrying damaged *Ighv* region genes negatively selected as an alternative fate to entering LZs. These results are consistent with earlier findings that the DZ to LZ transition is determined by an intrinsic timer but also suggest that cells may not all be equally competent for the change[44]. Rapid cell death may be the default pathway when cells exhaust their DZ proliferative program but also do not receive, or respond to, LZ promoting cues.

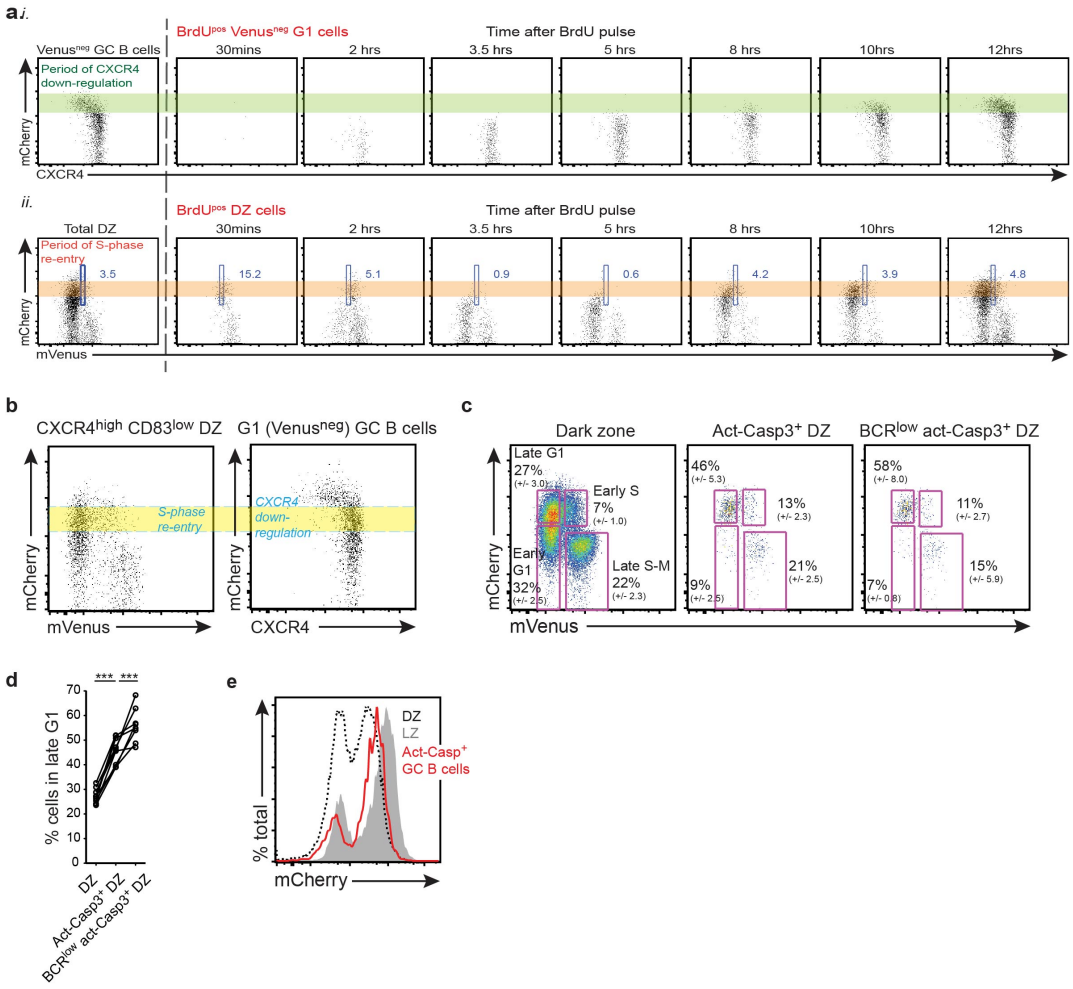


FIGURE 4.7| THE TEMPORAL RELATIONSHIP BETWEEN G1 SUB-STAGE AND S PHASE RE-ENTRY OR CXCR4 DOWN-REGULATION BY DARK ZONE CELLS

(a) R26p-Fucci2 mice were immunised with SRBCs and received single BrdU injections at the indicated time points before analysis. Plots to left of dash show whole populations irrespective of whether they are $BrdU^{+}$ or $BrdU^{neg}$, plots to right of dash show just $BrdU^{+}$ cells. (i) mVenus (G1) GC B cells were gated to determine the timing of CXCR4 down-regulation after the last cellular division. (ii) $CXCR4^{high}$ $CD83^{low}$ DZ cells were gated. Green (i) and orange (ii) shaded areas indicate ranges of mCherry at which CXCR4 down-regulation or S phase re-entry occurs. Very early S phase DZ cells are gated (blue rectangles) in (ii). (b) The timing of cell cycle re-entry from the DZ or CXCR4 down-regulation by G1 GC B cells was determined by examining mCherry levels. Yellow area indicates a likely "fate junction" where cells must be committed one of these processes. (c) DZ, act-$Casp3^{+}$ DZ, and BCR^{low} act-$Casp3^{+}$ DZ cells were gated and levels of the cell cycle regulated Fucci2 florescent reporters were examined, mean frequencies ± SD are shown, with summaries in (d). (e) mCherry levels in LZ, DZ, and Act-$Casp3^{+}$ cells are shown. Numbers on plots are means (+/-SD). Data in (a) are representative of 4-5 mice per time point from, 2 independent experiments (b) are representative of 13 mice from 4 experiments (c-e) are compiled from, or representative of 9 mice from 3 experiments Analysis was performed using an unpaired two-tailed Student's t test. ***$p < 0.001$

4.4 SUMMARY

The random nature of SHM means that any given mutation is much more likely to be deleterious than improve antigen binding ability. While, as discussed earlier, the GC functions both to amplify high affinity clones and increase the breadth of the immune response, there must still be limits on the breadth of BCR maintained in the GC. GCs are only useful if they can maintain breadth. It may be that uncontrolled narrowing of the repertoire until only the highest affinity clone prevails would be worse for protective immunity then no affinity maturation at all, because it would sacrifice breadth and the humoural response and allow the pathogen to easily mutate into an escape variant. In order for the GC to provide the plasticity needed to develop antibodies against potentially infinite number of antigens, it must be able to maintain breadth. However, while a BCR that has lost or decreased antigen specificity may be auxiliary in subsequent infections, or along a pathway to generate BCRs that are specific for difficult to access epitopes, a BCR that has lost structural integrity will unlikely be redeemable. Therefore, there is value to quickly negatively selecting cells that have lost intact BCR expression. If cells are only being selected for increases in affinity, then the presence of an old pre-BCR on the surface of the cell would not impede the selection on the basis of a new, higher affinity post-BCR. However, in order to remove cells that have lost functional surface BCR, all post-SHM BCRs would need to be removed and replaced. Therefore, it could be argued that some form or rapid negative selection is necessary for GC permissiveness, and that this requires full exchange of the BCR in the periods between SHM and LZ entry.

We addressed the extent to which cells are found in the LZ expressing BCRs that they no longer encode, and we found that BCR replacement usually occurs within a single DZ cycle. We estimated that between 80-85% the BCR is fully exchanged before LZ entry. Additionally, our calculations may in fact be underestimates, because they do not take into account the cells which carried premature stop codons, but which underwent apoptosis before they could be detected. The apparent rapid turnover of the BCR in the DZ provides the opportunity to screen out detrimental mutation before the cells enter the LZ.

We had postulated in Chapter Three that the predicted half-life of the BCR in the DZ would be sufficient for BCR exchange to be completed while cells were still in the LZ if there were a restricted time period in which SHM occurred. We found that SHM may be restricted to G1, and that cells transition into the LZ at a tightly controlled time point in late G1. Therefore, if cells have acquired a SHM in the previous cycles G1 phase, they would have time to exchange their BCR.

The extensive apoptosis that occurs in the GC has been well documented[102,189,324,325], and its necessity is intuitive given that GCs are such extensive sites of division, yet during the peak of the response their size remains somewhat stable. Bursts of immune cell divisions are intense, and they are associated with some degree of waste. Furthermore, while DZ cells are more prepared to deal with DNA damage, it is still likely burdensome of GC B cell. AIDKO GCs have less death than WT GCs, and this was attributed to the DNA damage burden of AID activity[326]. However, this published phenotype is constituent with a model of the DZ to LZ transition wherein cells with non-functional BCR are removed in the DZ. In the absence of AID, cells with non-functional BCR would not be generated, and as such one would expect a decrease in death in the DZ.

We found evidence of extensive apoptosis in the DZ, and our finding suggested that the majority of cell death occurs there, rather than in cells failing selection in the LZ. This was consistent with a study by Mayer et al which was published while this work was in preparation[327]. We found that cells in the DZ, and particularly those cells which have lost function B cell receptor, were more likely to die during late G1. We described this period as a fate junction, because it appeared to represent a someone specific phase during which the cells appeared destined to either undergo another cell division or enter the LZ, or seemingly to die. Cells may die at this juncture if they have exhausted their proliferative potential in the DZ, but have failed to receive LZ promoting cues. Given that delaying apoptosis does not allow cells which have lost functional BCR to enter the LZ, it is likely that the cues which trigger transition to the LZ phenotype to be more active in manner. The possible candidates for these LZ promoting cues will be discussed in Chapter Four.

Based upon observations that positioning GC B cells in the LZ is not enough to make them look or behave like cells of that zone, it was proposed that the DZ to LZ transition is controlled by a ‘‘timer’’ that is part of an intrinsic cellular program[44]. This model was supported by later reports that T cell derived signals set the DZ dwell time and that the transcriptional regulator FOXO1 contributes to driving that state[120,128,135,138]. These results are not inconsistent with this model, but do imply that when the internal ‘timer’ dictates that the cells should transition from a DZ to LZ phenotype if cells fail to receive LZ promoting cues, they may die when their sister cells down-regulate CXCR4 and transition into the LZ.

CHAPTER FIVE: INVESTIGATION INTO A POSSIBLE LIGHT ZONE PROMOTING CUE

Given that cells expressing a disrupted *Ighv* gene region do not accumulate in the LZ, we next sought to address whether DZ cells need to receive LZ-promoting cues to enter that zone, and we wished to understand whether successfully receiving them was related to the cells capacity to express a functional BCR on their surface.

5.1 RELIEF OF COMPETITION BETWEEN DARK ZONE CELLS DOES NOT ALLOW CELLS WITH DISRUPTED B CELL RECEPTORS TO ENTER THE LIGHT ZONE

Following the publication of the work described in chapters three and four, Casola et al. postulated that the rapid removal of GC B cells carrying premature stop codons in the DZ may be due to their sister cells outcompeting them before they can enter LZs[328]. They made an analogy between DZ cells acquiring a detrimental mutation and lymphoma cells losing functional BCRs in competitive culture settings[329].

The apparent paradox that the GC is both a site of extensive growth and maintains relatively stable size during the peak of the reaction can be accounted for by the well-documented phenomenon of extensive death in the DZ. It is possible that, just as T cell help is limiting in the LZ[101,330], there are resources required for division that are limiting in the DZ. Indeed, hypoxia has been described in the GC LZ (and has been somewhat tenuously linked to control of class switch recombination)[331]. Controlled restriction of access to oxygen with microenvironments, inducing

hypoxia, has been described for other niches to control cell growth[332,333]. Therefore, it seems plausible that in the DZ environmental pressures create a selective environment where only the fittest can survive, and such a model would be commensurate with a requirement for a tonic BCR signal. While naïve B cells appear to require a BCR for survival, the half-life for cells which have lost their BCR is several days[269]. GC B cells may require a tonic BCR signal that is no different in nature to that needed by follicular B cells, but GC B cells may die more rapidly in its absence as a consequence of the DZ niche being inherently more competitive. In this scenario, the DZ is similar to a cancer microenvironment, in which the cells are predisposed to divide extensively, but due to the ultimately limited resources within the niche, clones which are more fit will prevail, and those which are less fit will fail.

In order to address the hypothesis that cells which have lost surface BCR fail to enter the LZ because they fail to compete with their sister cells for resources, we analysed the capacity of cells that have structurally destroyed the BCR to transition into the LZ in experimental settings where competition is greatly reduced. We reduced competition for potential DZ niche factors by inducibly deleting a portion of the B cells in the GC. For this experiment, we utilised AID-Cre^{+} Rosa26-floxDTR mice. These mice express a Cre recombinase under the control of the AID locus that "switches on" diphtheria toxin receptor expression only in antigen-experiences (principally GC) B cells. We generated mixed bone marrow chimeric mice containing mixtures of WT and AID-Cre Rosa26 Flox DTR cells and, following reconstitution, the chimeric animals were immunised with SRBCs. This generated GCs in which ~75% of the cells expressed the diphtheria toxin receptor. 12 hours before culling, the mice were treated with diphtheria toxin

(DT) to specifically ablate the DTR^+ cells (protocol summarised in Figure 5.1a). DT treatment efficiently induced apoptosis within 5-8hrs post-treatment (data not shown).

If the phenotype of cells with no surface BCR being restricted from the LZ is related to competition between sister cells in the DZ, then the release of this competitive pressure through a sudden and significant depletion of cells in the DZ should result in an increase in cells in the LZ with no surface BCR. However, we did not see a relationship between the depletion of cells in the DZ and the presence of BCR-negative cells in the LZ (Figures 5.1b, c). Therefore, these findings do not support the concept that DZ cells compete in a BCR dependent manner for short-term survival or LZ entry. Therefore, even when competitive pressures within the DZ are relieved, cells that do not meet the entry requirements fail LZ entry.

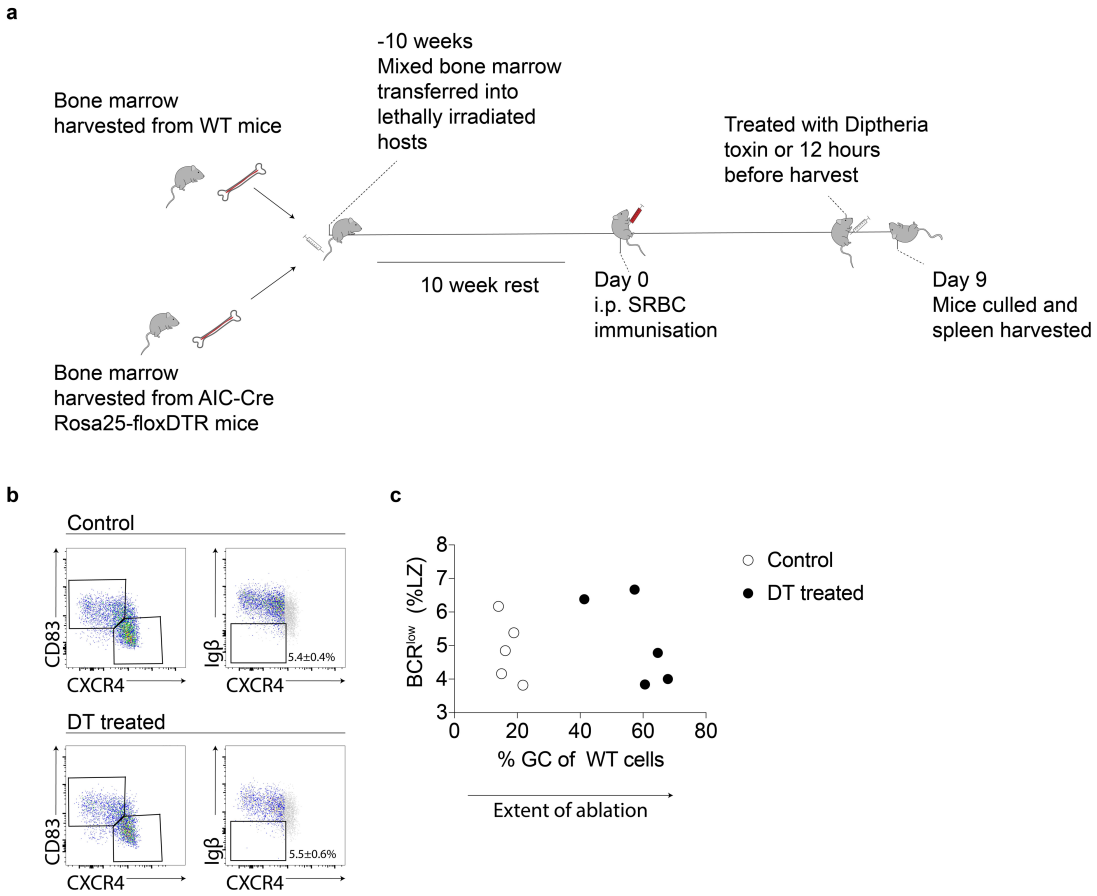


FIGURE 5.1| RELIEVING COMPETITION BETWEEN B CELLS IN THE GERMINAL CENTRE DARK ZONE

(a) Outline of protocol for experiment. Bone marrow from WT and AID-Cre Rosa Flox-DTR mice were co-transferred i.v. into lethally irradiated mice; mice were rested for at least 10 weeks, then immunised with SRBC i.p. 12 hours before culling mice were treated with diphtheria toxin; mice were culled on day nine, spleens were harvested and stained by flow cytometry. (b) Comparison of BCR^{low} cells among WT LZ cells in GCs of mice treated and not treated with (GCs gated as IgD^{high}, $CD95^{+}$ $Gl7^{+}$ B cells; WT cells differentiated at $Ly5.2^{+}$ cells; LZ gated as $CD83^{high}$ $CXCR4^{low}$). Numbers represent mean ± SEM. (c) Frequency of BCR^{low} LZ cells against frequency of WT cells in the GC as a marker for extent of B cell ablation (n=5)

5.2 BLOCKING ANTIGEN ENGAGEMENT DOES NOT PREVENT CELLS FROM ENTERING THE LIGHT ZONE

If DZ cells require a BCR-specific signal to enter the LZ, it can either be tonic in nature or antigen-driven. We therefore sought to address the possible role of an antigen-mediated BCR signal in providing LZ-promoting cues. In order to investigate the potential necessity of an antigen-mediated signal, we sought to directly block the antigen interaction. We focused on SW_{HEL} Fucci2 GCs, because the binding relationship between the BCR and antigen has been well defined[334]. Following adoptive transfer of SW_{HEL} Fucci2 B cells, mice were immunised with SRBC, conjugated to a variant of HEL to which the B cells have lower affinity (HEL3X). HEL3X carries mutations at three residues with which the SW_{HEL} BCR makes direct contact[335]. Cells in the GC can affinity mature towards higher affinity for the antigen, and treatment with the higher affinity Hy10* antibody blocks SW_{HEL} BCR-antigen engagement. We sought to address whether blocking BCR-antigen engagement with this antibody caused defects in the DZ to LZ transition. Mice were treated with the Hy10* antibody or PBS eight hours before culling (Protocol outlined in 3.2a).

We hypothesised that, if blocking the antigen-mediated BCR signal were to disrupt the DZ to LZ transition, treatment with the Hy10* antibody should cause increased apoptosis in DZs. Furthermore, if this caused a blockage at our hypothesised "fate junction," we might expect an accumulation of G1 apoptotic DZ cells and a decrease in the frequency of recent LZ entrants. Recent LZ entrants are identifiable as being CXCR4-intermediate and $mCherry^{low}$ (relative to other LZ cells). However, none of these signs appear, with treatment causing no detectable changes in the frequencies of dying cells in LZs or DZs (Figure 5.2b, summarised in 3.2c). There was also no measurable accumulation of cells in late G1 in the DZ in response to treatment, nor was there a

measurable defect in the cells which had most recently transitioned into the LZ (Figures 5.2d, e, f). Furthermore, no changes in the LZ/DZ ratio were observed (Figures 5.2d, and g). While it is possible this blocking does not cause complete ablation of BCR-antigen interactions, one might still expect the blockade to have some effect, if it indeed were the case that cells did require an antigen-mediated signal to enter the LZ. Therefore, when the specific interaction between the BCR and antigen is blocked, GC B cells still appear to transition into the LZ.

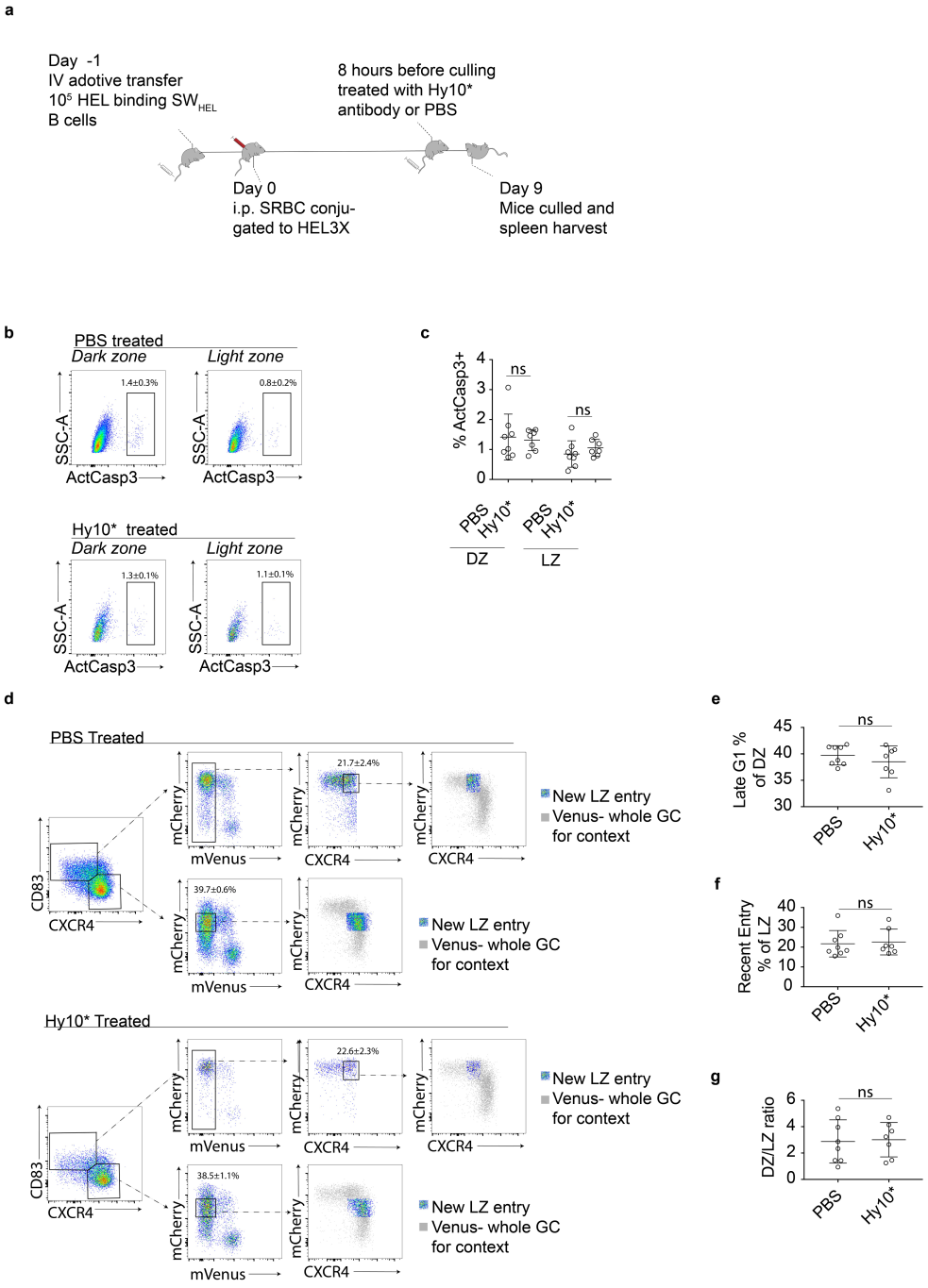


FIGURE 5.2| BLOCKING ANTIGEN ACCESS IN THE GERMINAL CENTRE

(a) Outline of protocol for experiment. SW_{HEL} B cells and OTII T cells were co-transferred into allotypically marked recipient mice, mice were immunised with SRBC conjugated with HEL3X. 12 hours before culling mice were treated with Hy10* antibody or PBS, mice were culled on day nine and spleens were harvested and stained for FACs analysis. (b) Having stained for surface markers, cells were fixed, permeabilised and stained for active caspase 3 ($ActCasp3^{+}$). Death in the DZ and LZ were compared for between treated and untreated mice, these results are summarised in (c). (d) Experiments were performed in Fucci2 mice, and so the DZ to LZ transition

could be analysed. The frequency of cells which had recently re-entered the LZ (CXCR4intermediate mCherry high) and cells were blocked at the fate junction in the DZ (cells in late G1) were compared between Hy10* and PBS treated mice. Results summarised in (e) and (f). (g)DZ/LZ ratio in PBS and Hy10* treated mice. PBS treated n=8 over two experiments, Hy10* treated n=7; Numbers represent mean ± SEM; in graphs crossbars represent, error bars represent SEM. Analysis was performed using an unpaired two-tailed student's t-test

5.3 THERE ARE CELLS IN THE LIGHT ZONE WHICH HAVE LOST THEIR ABILITY TO INTERACT WITH ANTIGEN

If it is correct that DZ cells do not require an antigen-mediated signal to transition into the LZ, then it would follow that we should find measurable populations of LZ cells that have lost their antigen binding ability. To test this, we used flow cytometry to search for cells that had transitioned to the LZ after having apparently lost the ability to interact with cognate antigen. To this end, SW_{HEL} B cells and OTII T cells were transferred into WT hosts which were subsequently immunised mice with HEL-OVA in sigma adjuvant. Mice were culled and spleens were harvested on day seven and splenocytes were analysed for their capacity to bind fluorescently-labelled HEL (using Igκ light chain to normalize for BCR levels). We saw that a population of cells had lost the ability to bind HEL, but still had high levels of BCR on their surface, evidenced by their expression of high levels of Igκ. As controls for this population, we analysed SW_{HEL} GC B cells on an $Aicda^{-/-}$ background because these cannot undergo SHM, as well as MD4 GC B cells that contain multiple BCR transgene copies (making them less likely lose BCRantigen affinity through SHM) (Figure 5.3a, summarised in b). The finding that we could identify population LZ cells that have apparently lost the ability to interact with antigen is inconsistent with a model that requires an antigen-mediated BCR signal for cells to transition into the LZ.

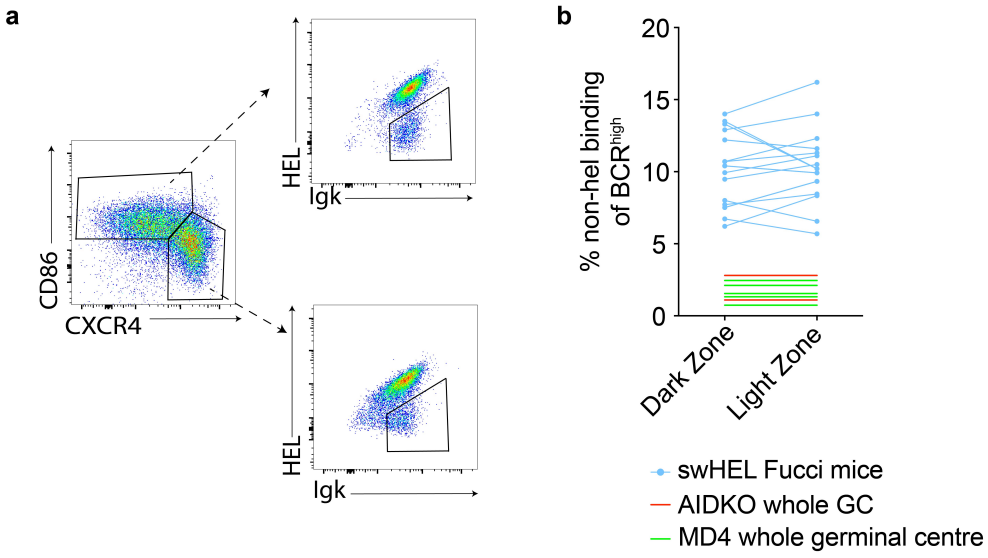


FIGURE 5.3| PRESENCE OF NON-HEL BINDING CELLS

(a) SW_{HEL} germinal centres were induced after SW_{HEL} B cells and OTII T cells were co-transferred, mice were immunised with HEL-OVA and GCs were analysed on day 7. SW_{HEL}GC B cells were gated as IgDlow $GL7^+$ $CD95^+$ $Ly5.1/2^+$ (depending on reciprocal allotypic markers of donor and recipient cells). DZ and LZ cells were gated on the basis of the expression of CD86/CXCR4. In each zone cells which have apparently lost HEL binding were gated on Igκ levels and capacity to bind HEL conjugated to AF647. Results summarised in (b) Long red and green lines represent AIDKO and MD4 GC B cells for context. (n=15, over four experiments)

Given these observations, we sought further evidence that non-HEL-binding LZ cells had definitively lost their antigen-binding ability, first using a HEL-Ea-GFP/antigen presentation approach. This approach had the advantage of labelling cells in vivo, thereby ensuring that antigen is encountered in its "natural" setting. HEL-EaGFP is a conjugate of cognate antigen (HEL), GFP, and a well characterised peptide (Ea52–68), which can be detected by Y-Ae monoclonal antibody

staining when presented in complex with I-Ab [299,336]. While theoretically cells that have internalised HEL-EaGFP could be detectable by flow cytometry as being GFP^+, this measurement may be influenced by cells that interact with antigen during tissue preparation. The HEL-EaGFP system allows us to differentiate between cells that are capable of engagement with antigen and those which are not, by assessing whether they present the Y-ea peptide on their surface in the context of MHC-II.

GCs containing SW_{HEL} B cells were generated by immunising mice with HEL-OVA in sigma adjuvant by subcutaneous (s.c.) injection. Six hours before culling, mice received a further injection of either HEL-eaGFP or HEL-BSA, as a control (Protocol demonstrated in Figure 5.4a). Draining inguinal lymph nodes were subsequently harvested and antigen-binding ability determined using similar HEL-AF647 labelling approaches as outlined above. Interestingly, GC B cells that had lost detectable HEL binding also had much reduced Ea peptide-MHCII staining intensities. While there was still some antigen presentation within the non-binding cells, it was significantly less (Figures 5.4b, summarised in c). Low levels of GFP were detected for "non-binding" cells, however given that it was poorly presented this may be caused by serum antibodies being adsorbed via Fc receptors. Therefore, it appeared that a significant proportion of these cells did not interact with antigen *in vivo*.

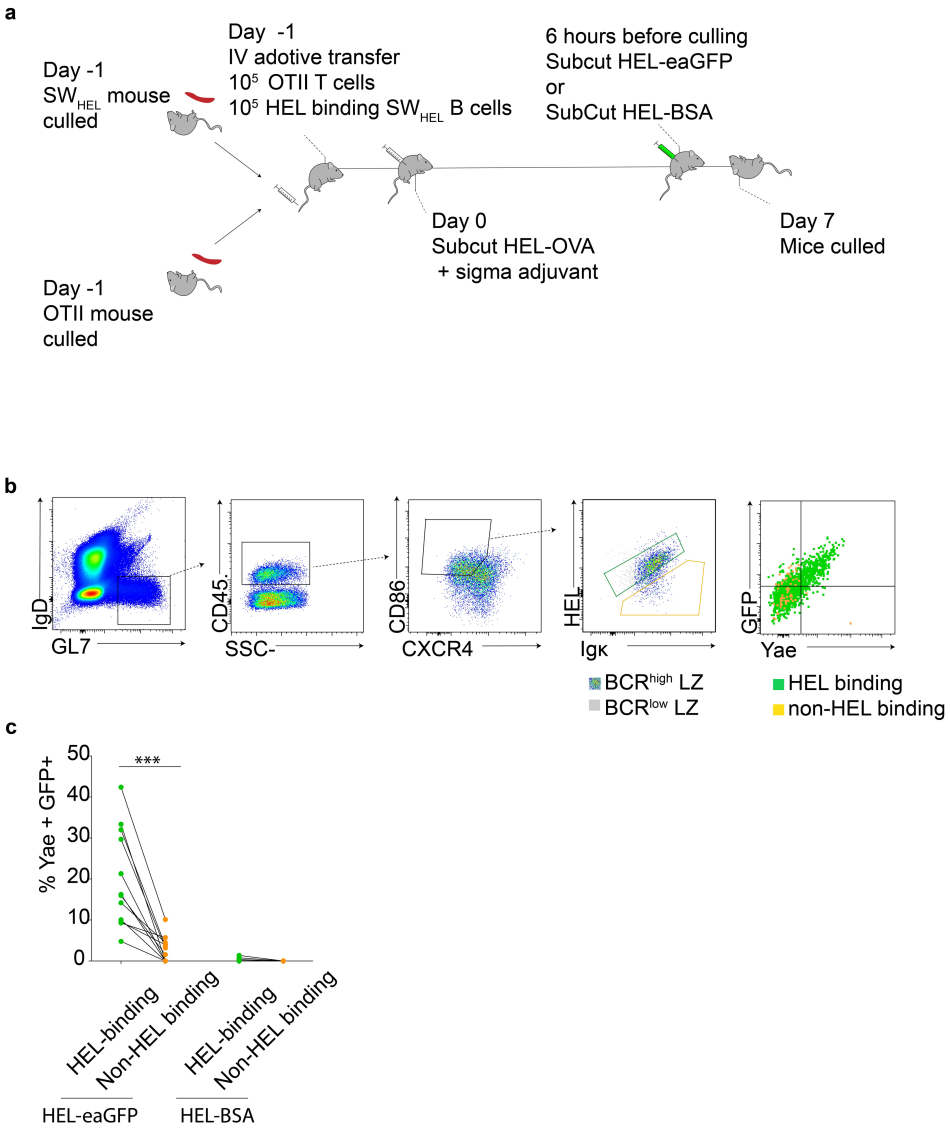


FIGURE 5.4| CELLS WHICH HAVE APPARENTLY LOST CAPACITY TO BIND HEL FAIL TO PRESENT ANTIGEN

(a) Outline of protocol for experiment SW_{HEL} B cells and OTII T cells are co-transferred into allotypically marked recipient mice. Mice were immunised subcutaneously in the flank, and 6 hours before culling they were subcutaneously injected with HEL-ea-GFP and HEL-BSA. On day seven mice are culled and LN harvested. (b) GC B cells were gated at IgD^{low} $Gl7^{+}$ $Ly5.1/2^{+}$ (depending on reciprocal allotypic markers of donor and recipient cells). LZ cells were gated at $CD86^{+}$ $CXCR4^{low}$. Non-binding cells are gated on the basis of Igκ levels and capacity to bind HEL conjugated to AF647. Plot shows overlay binding (shown in green) and non-binding (shown in yellow) GC cells, showing GFP fluorescents as an indicated of antigen internalisation, and Yae presentation, as a marker of antigen processing. Comparison of frequency of cells which are GFP^{+} and Yae^{+} is shown in (c) (n=10, over three experiments). Analysis was performed using an unpaired two-tailed student's t-test, ***, P <0.001.

To further validate the above findings, we sought to analyse the variable region gene sequences of the cells that had seemingly lost or reduced their antigen-binding ability. As mentioned previously, the interaction between the SW_{HEL} antibody and the HEL antigen has been well characterised. Therefore, we performed single cell sequencing on the *Ighv* region genes, to compare the variable region sequences of those cells that have lost antigen binding capacity and those which have maintained it. We focused only on the *Ighv* region because the light chain is encoded by a transgene and so not usually subject to SHM. We analysed the sequences for any amino acid divergences from the parent sequence.

Firstly, the majority of mutations observed in the HEL-binding and non-HEL-binding gates were unique to those gates, meaning that they were not seen in the alternate gate. Surprisingly, the non-antigen-binding populations were found to contain cells without coding somatic mutations. These cells, with an apparently unmutated heavy chain variable region, might have acquired mutations in their light chain or in heavy chain regions outside of the V region analysed. In addition, some mutations were shared in between cells from the binding and non-binding subsets (Figure 5.5a). These mutations were less abundant among HEL-binding cells. While the "non-binding" populations did contain "unmutated" and "similarly mutated" cells, these populations also contained at much higher frequencies cells with somatic mutations that are expected to be deleterious to antigen binding (Figure 5.5b). The somatic mutations found in these cells were more likely to change polarity or charge of the residue. The sequence analysis is demonstrated visually in Figure 5.6.

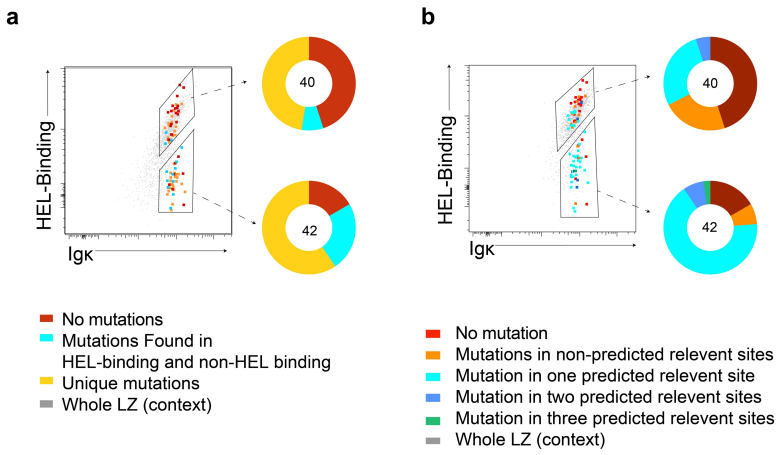


FIGURE 5.5| SEQUENCE ANALYSIS OF CELLS IN NON-BINDING GATE

SW_{HEL} GCs were generated as previously described. Spleens were harvested and stained for FACs. GC were gated as IgD^{low} $Gl7^{+}$ $Ly5.1/2^{+}$ (depending on reciprocal allotypic markers of donor and recipient cells), LZ cells were gated as CD86+ $CXCR4^{low}$. HEL binding and non-binding cells were gated on Igκ levels and capacity to bind HEL conjugated to AF647. Individual LZ cells in HEL binding and non-HEL binding were single cell sorted into 96 well PCR plates, sorted and their *Ighv* region genes were amplified and analysed by sanger sequencing. Frequencies and staining intensities of cells with (a) unique mutations or mutations found in both populations (b) mutations at predicted relevant sites. Number in centre indicates number of cells sequenced.

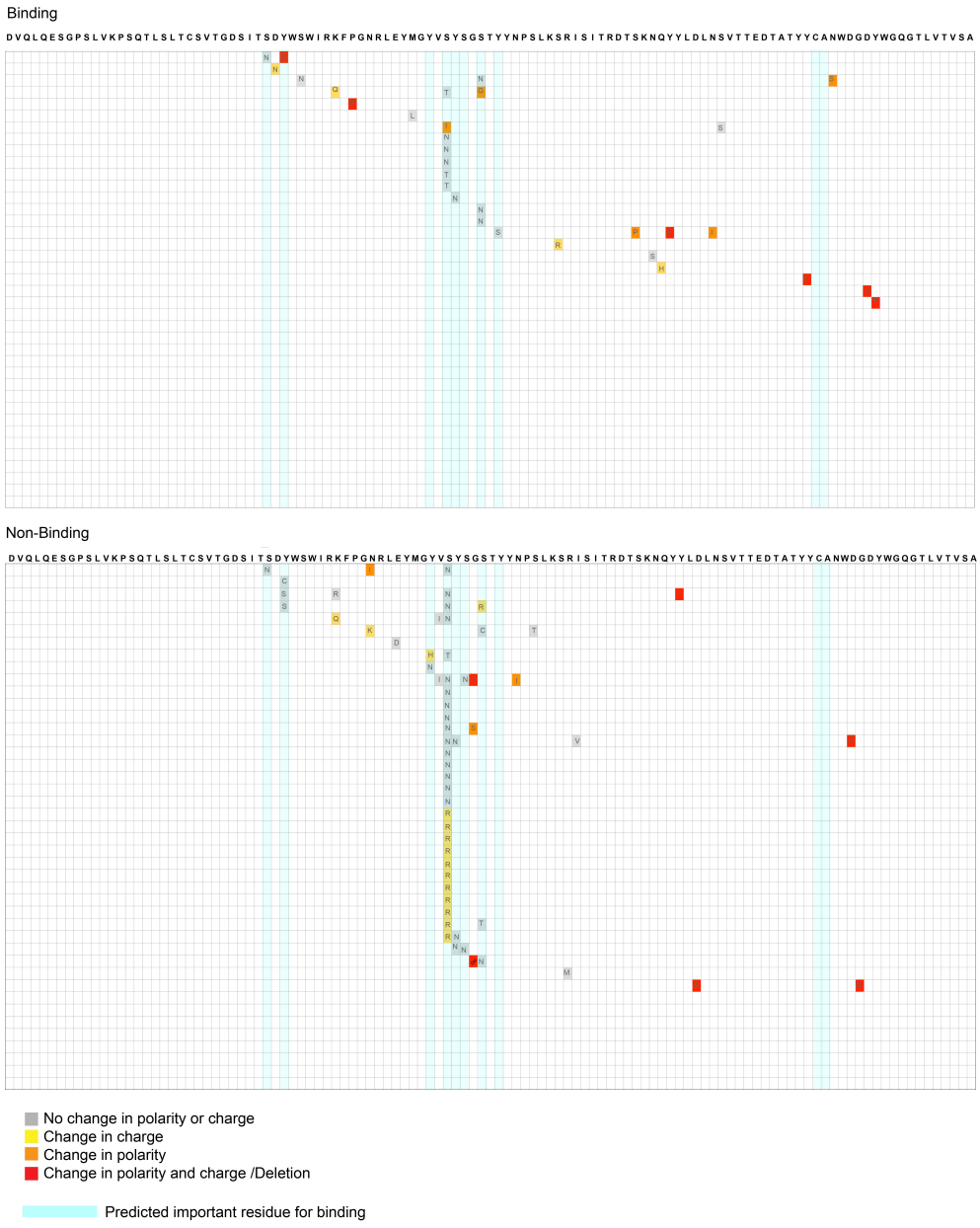


FIGURE 5.6| SUMMARY OF MUTATION PROFILE IN HEL BINDING AND NON-BINDING CELLS

Visual representation of HEL binding and non-HEL binding LZ sequences from SW_{HEL} GC, from Figure 5.5. The two tables show sequences from HEL binding and non-binding cells. Each horizonal line represents an individual sequence. The unmutated variable region sequence is displayed above each table. Mutations in each sequence are shown, as a change in the colour of a square, relating to a change in the residue indicated at the top of the table. The nature of the mutation is shown by the colour of the square, the residue to which the residue changed is indicated by the single letter code inside the box. Predicted important binding sites are highlighted in pale blue.

In order to assess the antigen-binding function of antibodies that had apparently lost the capacity to bind antigen *ex vivo*, seven binding and non-binding variable region genes were subcloned into human IgG1 backbones. We expressed these genes together with, the SW_{HEL} Igκ light chain in 293T cells to generate recombinant antibodies which could then be tested by ELISA. While some of the non-binding sequences displayed a significantly impaired capacity to interact with the antigen, others did successfully interact with antigen (Figure 5.7). Furthermore, of the sequences with significantly impaired antigen binding ability, the binding was still above background, although only slightly. Therefore, these results strongly argue that strong antigen mediated BCR signals are probably not required for LZ entry, but like with the Hy10* blocking experiments, we are not able to completely exclude a role for antigen binding in facilitating LZ entry.

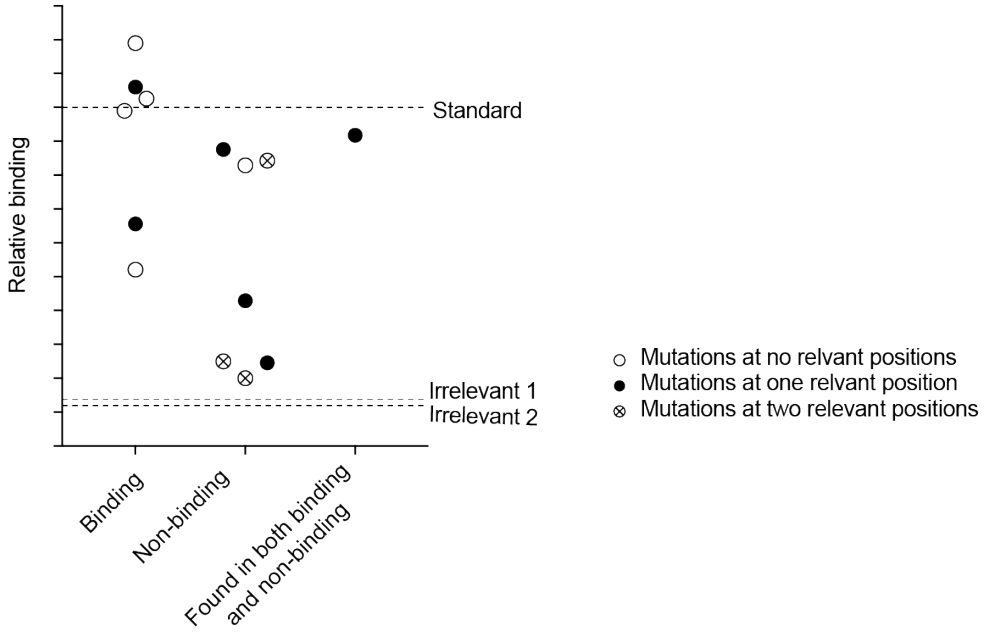


FIGURE 5.7| HEL BINDING ASSESSED *IN VITRO*

Heavy chain variable regions from HEL binding and non-HEL binding LZ cells, as analysed in figure 5.5, were cloned into human IgG1 backbone and co-expressed with humanised Hy10 light chain in 293T cells, HEL binding was measured by ELISA and binding was normalised to unmutated sequence. Sequences were selected to be representative of the mutations found in the respective populations. HEL binding of two irrelevant chains is presented for context. (sequencing results from one mouse).

5.4 INDUCIBLY ABLATING B CELL RECEPTORS FROM THE SURFACE OF GERMINAL CENTRE CELLS LEADS TO AN INCREASE IN CELL DEATH

We saw previously that cells which have acquired detrimental mutations in their BCR genes do not appear to transition from the DZ to the LZ. Our working hypothesis is that a tonic, non-antigen-mediated BCR signal may be required for cells to transition from the DZ to the LZ. To begin directly testing the requirement of a BCR signal for LZ entry in a system where BCR loss can be temporally separated from SHM, we made use of B1-8f gene targeted mice, in which the variable region of the NP specific heavy chain is flanked by two loxP sites. To allow temporal deletion after tamoxifen treatment, these were crossed to R26-CreERT2 Fucci2+ mice. B1-8f CreERT2+ Fucci2+ B cells and OT-II T cells were adoptively transferred into allotypically marked mice that were subsequently immunised with NP conjugated to OVA in alum adjuvant. On day seven, mice were treated with tamoxifen or a vehicle control, with FACS analysis 48hrs later (protocol summarised in Figure 5.8a).

According to our hypothesis, we expected that ablating surface BCR should lead to a measurable defect in the DZ to LZ transition. Gene excision in this model is relatively inefficient, as reported previously, but increases in frequencies of BCR-negative cells after tamoxifen treatment were observed. 48 hrs after treatment, the frequency of cells with undetectable surface BCR in the DZ increased from 17.0% to 32.8%, a change of 15.8% (Figure 5.8b, summarised in d). Despite BCR loss being independent of SHM activity in this system, it was striking that induced BCR-negative cells were over-represented in DZs as compared to LZs, where we observed only a 3.3% increase in BCR^{low} cells. These findings are consistent with two non-mutually exclusive principles of BCR handling which we have postulated in the

GC. Firstly, that LZ cells may exhibit BCR turnover at a slower rate than DZ cells and, second, cells may indeed require a BCR signal for LZ entry. 50% of DZ cells transition to the LZ every six hours. If it were the case that the DZ to LZ transition does not require a BCR signal, and 15.8% of the DZ have lost their BCR due to tamoxifen induced ablation, we should expect a significant proportion of the LZ cells to have lost their BCR 48 hours after tamoxifen treatment.

While we saw some impacts on the DZ to LZ transition in this system, given that the loss of the BCR was only induced in a small fraction of the cells, these impacts were somewhat muted (Figure 5.8b, summarised in c). An increase BCR^{low} cell in response to tamoxifen treatment was restricted to the DZ. If the an intact BCR were required for the DZ/LZ transition, then increasing the frequency of cells in the DZ which have undetectable surface BCR might have led to an accumulation of cells in that zone, and therefore an increase in the DZ to LZ ratio. However, a measurable impact would likely require a more robust deletion of surface BCR. We analysed cell death by flow cytometry, again using an antibody against active caspase 3 as described in Chapter Four. We saw no significant increase in cell death in both the DZ and LZ (Figures 5.8e, and f). However, those cells dying in the DZ were more likely to be BCR^{low} (Figure 5.8g). There was a statistically significant difference in the frequency of ActCasp3+ BCR^{low} cells in the DZ and LZ, as a frequency of each zone respectively (Figure 5.8h). While there was an increase in dying cells with undetectable surface BCR in both the DZ and LZ, this difference was much larger in the DZ. Therefore, we concluded that induction of loss of surface BCR is associated with an increase in the death of cells that have lost surface BCR, especially in the DZ.

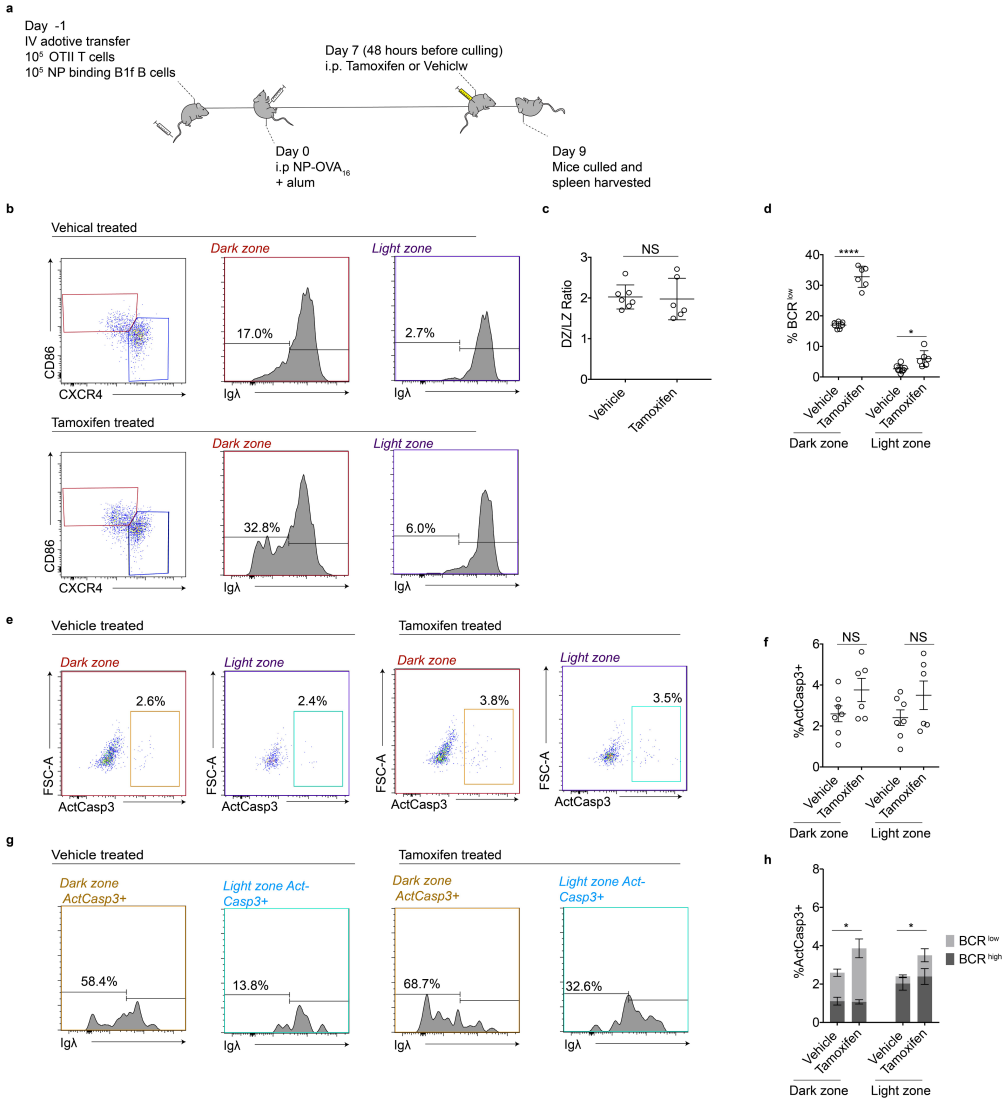


FIGURE 5.8| INDUCED ABLATION OF SURFACE B CELL RECEPTOR

(a) Outline of protocol for experiment. 10^5 NP binding B1-8f B cells and 10^5 OTII T cells were co-transferred into allotypically marked mice; mice were immunised the next day with NP conjugated to Ovalbumin (NP-OVA_{16}) with alum. On day seven mice were treated with tamoxifen or vehicle control. On day 9, 48 hours after treatment mice were culled, and spleens were harvested and stained for FACS analysis. (b) Extent of surface B cell ablation in B1-8f in DZ and LZ. GCs gated as IgD^- $CD95^+$ $GL7^+$; DZ and LZ gated on the basis of CD86 and CXCR4 expression. Surface BCR expression measured by Igλ light chain expression. (c) Summarised DZ/LZ ratio for B1-8f GC B cells in vehicle versus tamoxifen treated mice. (d) Summarised increases in BCR^{low} cells in the DZ and LZ. (e) Staining for intracellular active caspase 3 in LZ cells and DZ cells. Numbers represent means. (f) Summary of increase in frequency of cells with undetectable surface BCR in the DZ and LZ after tamoxifen treatment. (g) Surface BCR expression on active caspase 3 cells in the DZ and LZ; gates represent

positivity and negativity for surface BCR, numbers represent means. (h) Bar graph showing Caspase+ cells in DZ and LZ, demonstrating relative contributions of BCR high and BCR^{low} cells; statistical significance indicated relates to differences in BCR^{low} cells. n=7 for vehicle treated, n=6 tamoxifen treated. Analysis was performed using an unpaired two-tailed student's t-test *, $P<0.1$

5.5 SUMMARY

Based upon our surprising finding that GC B cells with non-functional BCRs do not accumulate in LZs at the expected frequencies, and that LZ entry was not enhanced when apoptosis was inhibited via ectopic Bcl2-expression, we hypothesised that LZ entry may require some form of active BCR-dependent signal, which may be either "tonic" and antigen-driven. However, it was also postulated by others that, rather than failing to receive LZ promoting cues, GC B cells which had lost surface BCR suffered a fitness defect in the DZ that prevented their competing efficiently with neighbouring cells for survival cues, therefore causing them to die before LZ entry[328]. In this chapter, we began exploring these possibilities. Our initial experiments argued against the latter of the proposed options, because reducing competition had no detectable impact of the likelihood of remaining cells entering LZs without surface BCRs. This was consistent with a model in which cells may need a specific LZ promoting cue to transition into the LZ, which we explored further.

We next sought to address whether cells required a BCR mediated signal to enter LZs, and if so whether this was antigen-mediated or "tonic" in its nature. We investigated this first by asking whether LZ entry occurred normally when BCR-antigen interactions were disrupted through the delivery of a masking antibody. While we cannot be certain as to the completeness of this block, the same antibody/dose was previously shown to be functionally active in GCs in that it prevented

plasma cell differentiation[190], and we have observed the same batch of antibody at the same concentration prevent HEL specific B cells from establishing in an adoptive transfer model after immunisation. Our experiments indicated that blocking (or reducing) BCR-antigen interactions had no detectable impact on capacity of DZ cells to transition into the LZ. In separate experiments, we also noted that acquiring SHMs that strongly reduced antigen-binding ability also did not prevent cells entering LZs. Taken together, these results imply that cognate BCR-antigen interaction may not be necessary for the LZ transition, although we cannot yet exclude the possibility that weak engagement with antigen might be necessary and sufficient.

The decision to analyse the BCR-antigen binding using the SW_{HEL} system was based on the fact that this system is well-characterised, and the antibody-antigen relationship well annotated[334], and that reagents exist to efficiently block the relationship. While these systems have been of great use for studying increases in antigen binding affinity, there may be restrictions to their utility for analysing loss of antigen binding. Cells may have lost their ability to bind HEL, but while doing so also gained specificity for other unknown antigens that are present in the GC. While this possibility seems unlikely, the concept that cells can mature towards new targets has been documented[337].

To begin directly assessing whether BCR cues in general (i.e. regardless of specificity) are required for LZ entry, we made use of the B1-8f system, in which antibody heavy chain variable region genes can be inducibly deleted. This approach was suboptimal in that we only managed to achieve a 15.8% increase in the frequency of BCR-negative DZ cells after tamoxifen treatment, presumably indicating incomplete gene excision. However, the treatment did

preferentially impact DZ cells, supporting a model in which cells that have lost their surface BCR fail to receive BCR-dependent LZ-promoting cues. We hope in future experiments to refine this approach such that we can confidently also analyse when and where cells losing surface BCRs die in GCs. While the results of deleting surface BCRs from GC B cells are inconclusive at this stage, there would likely be benefit to attempting to address the role of BCR signalling in the DZ to LZ transition by pharmacologically inhibiting downstream components of the BCR signalling cascade, for example by using Btk inhibitors or Syk inhibitors.

CHAPTER SIX: DISCUSSION

GC B cells encounter unique challenges during antibody affinity maturation. A key example is that, following SHM, GC B cells express surface BCR protein complexes that do not strictly reflect the cell's genetic identity. It does so in an environment in which it will soon be selected on the basis of the surface BCR that it presents, and the efficiency of selection relies on the correlation of the genetic identity of the cell and the protein expression. In this work, we addressed the question of whether the pre-SHM BCRs are fully replaced by DZ cells before they transition to LZs for selection. We were able to conclude that GC B cells exchange their BCRs before LZ entry at least ~80% of the time, and that cells failing to re-express an intact surface BCR are quickly deleted from the GC. The first of these findings is presumably essential for the second to occur; that is, in order to test the structural integrity of the new post SHM-BCR, pre-SHM BCRs will need to be removed from the cell surface.

The negative selection of cells encoding non-functional BCRs is critical for a permissive model of selection because it enables cells to quickly distinguish between when a mutation has destroyed the structure of the BCR, and when a mutation has merely diversified the antibody pool. I should note that the term "negative selection" can also be used to describe the selective pressures existing in the GC to guide cells away from autoreactivity, but here I will use it specifically to describe the removal of cells with non-functional BCRs. Below, I will discuss models for the kinetics of the exchange of the BCR within the DZ; the conceptual evolutionary benefit of allowing mutations that are more likely to structurally destroy the antibody; the possibility that BCR-mediated signals are required for LZ entry; and different ways of conceptualising permissive selection in GCs (involving the necessity for actively removing cells encoding non-functional BCRs). A revised model for selection is shown in Figure 6.1.

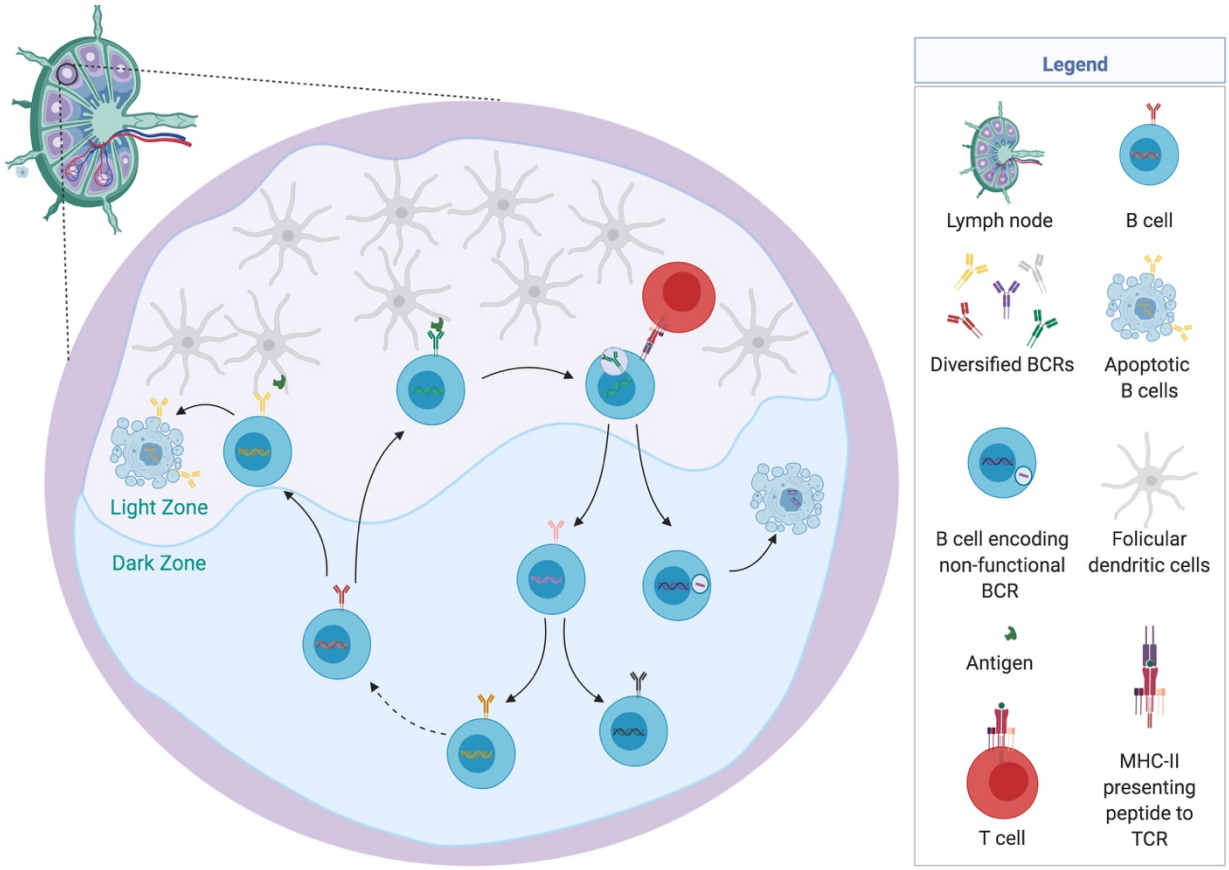


FIGURE 6.1| REVISITED MODEL OF SELECTION IN THE GERMINAL CENTRE

Germinal centre B cells divide and undergo somatic hypermutation in the DZ, where there exchange their pre-SHM BCR for post-SHM BCR. Cells which have acquired a non-functional BCR will die before LZ entry. Cells with functional BCR will transition into the LZ, and cells which have maintained the ability to interact with antigen will re-enter the DZ.

6.1 B CELL RECEPTORS ARE MAINLY EXCHANGED WHILE CELLS ARE IN THE DARK ZONE

While we have made strides regarding the extent to which the BCR is exchanged in the DZ before LZ entry, important questions remain about the nature of exchange of the BCR in the DZ. More specifically, we do not yet know whether the kinetics of BCR turnover differ in DZs to compared to LZs. It is possible that a component of the DZ program involves a temporal switch in how cells "handle" their BCR complexes in order to promote exchange following SHM. However, it is also possible that GC B cells have generally increased surface BCR turnover rates, and that this facilitates the exchange of BCRs within the DZ before LZ entry.

If BCR protein trafficking and turnover are differentially regulated in DZ compared to LZ cells, it would be of interest to investigate whether exchange is directly triggered by the SHM event, as is implied by the dependence of the BCR^{low} DZ population on AID expression. Additionally, if there is a difference in rates of BCR exchange in DZs compared to LZs, a second question of interest for our group would be the kinetics of exchange of the BCR. We propose two models for the exchange of the BCR following SHM. The first model is that the SHM event causes a BCR surface protein "purging" event, in which pre-SHM BCRs are removed from the cell surface and targeted for degradation. The second model is this that, following SHM, pre- and post-SHM BCRs are co-expressed on the cell surface. The length of time these "co-expressing" B cells persist would depend on the half-life of the BCR. The presence of a population of DZ cells that lack detectable surface BCR complexes, but that can produce functional surface and secreted antibodies when cultured *ex vivo*, is consistent with a model of BCR purging. However, we have not so far been able to directly prove or disprove whether these cells actually do come to re-express BCRs *in vivo*.

We sought to address these questions about the nature of BCR exchange in GCs by developing approaches for allowing pre-existing BCR complexes to be labelled, for example by using photo-conversion methods to turn chimeric BCR-photoconvertible fluorescent proteins from green to red. We hoped that this would permit us to perform *in vivo* pulse-chase experiments, thereby addressing the questions of relative rates of BCR degradation in GC B cell and naïve B cells, and more importantly, in DZ compared to LZ cells. We also aimed to use these methods for assessing the impact of AID activity on the half-life of the BCR, by comparing turnover rates in AID-deficient and WT GC B cells. Furthermore, the patterns of expression of labelled constructs, whether there was measurable "old" and "new" co-expressed on the surface of cells could have been investigated. However, despite the significant time and energy investments made in these approaches, none proved fruitful because we encountered technical roadblocks for each. In appendix one, I briefly highlight some of the main approaches that we tried.

Beyond our BCR labelling efforts, we also hoped to develop an approach for triggering AID activity by delivery of tamoxifen, using an AID-ERT2 gene[338–340]. We hoped to induce SHM in *Aicda*$^{-/-}$ AID-deficient GC B cells *in vivo*, and ask whether BCR^{low} cells appear more quickly that would be predicted based upon predicted average half-lives for BCRs in GC, calculated from one of the techniques described in appendix one. In this way, inducing AID activity might tell us whether BCRs are degraded steadily in the DZ, or in rapid bursts after SHM in GC B cells. To this end, we expressed the AID-ERT2 using a retroviral vector and we confirmed the construct to be active *in vitro* as evidenced by induction of class switching in *Aicda*$^{-/-}$ B cells. However, class switching was not observed after using the same construct *in vivo* after reconstituting irradiated mice with transduced bone marrow, and the induction of BCR-negative cells in the GC was also not achieved. Therefore, further studies are needed to test the appropriateness of this approach.

6.2. CELLS WITH NON-FUNCTIONAL B CELL RECEPTORS ARE DELETED IN THE DARK ZONE

In this work, we demonstrated that cells carrying premature stop codons in their *Ighv* region genes do not transition to the LZ. Our findings indicate that significant apoptosis in GCs occurs in DZs, and that the cells dying in the DZ are more likely to have undetectable surface BCR levels. As discussed in greater detail in later sections, it is possible that BCR-mediated signals are required for LZ entry. These conclusions are consistent with results of an independent study that was performed at approximately the same time as ours by the Nussenzweig group (Mayer et al. 2017)[327].

Mayer et al. developed a FRET-based Caspase3 reporter mouse line which they used to investigate how BCR specificity correlates with apoptosis in GCs. This FRET reporter approach is conceptually similar to that applied by others for studying apoptosis[341,342], however this was the first study of this type in GCs and the authors made numerous important discoveries. While they did not, in their work, correlate single cell BCR sequences with surface BCR expression as we did to observe the extent of BCR replacement, they had the advantage of directly demonstrating preferential apoptosis by DZ cells acquiring damaging mutations. As such, this study and ours provide complementary advances.

While earlier models for the GC have mostly involved selection occurring almost exclusively in LZs, or during the LZ-DZ transition, we add to this by proposing that a form of selection also occurs as DZ cells attempt to enter LZs. Consistent with this model, Mayer et al. found that apoptotic cells encoding non-functional BCRs were highly enriched in the DZ as opposed to the LZ (43%-58% of DZ apoptotic cells carried premature stop codons, compared to 9-14% in

LZs). Mayer et al. saw that about 35% of the cells which had acquired premature stop codons had done so through out of frame insertions or deletions (indels)[327]. This was similar to the proportion of stop codons attributable to frameshift mutations that we saw (data not shown), interestingly we also very rarely saw in-frame indels. Therefore, indels are very frequently damaging, and cells encoding indels are usually rapidly deleted.

Further to their analysis of BCR sequences, Mayer et al. also cloned and expressed antibodies from apoptotic GC B cells, finding that ~50% of FRET+ DZ cells no longer carried functional antibody genes despite their not encoding premature stop codons[327]. Therefore, in addition to those cells which have acquired premature stop codons by frameshift or substitution mutations, dying cells in DZs are more likely to carry mutations that results in the BCR being structurally disrupted. A significant proportion of DZ apoptotic cells had acquired mutations that perverted antibody structure so profoundly that they were either unable to associate with the light chain, or had some other severe structural defect that prevented the formation of functional antibodies. Therefore, we estimate that for every two cells carrying a premature stop codon in their BCR genes, there is one cell that has acquired a different type of (undetected) mutation that structurally destroys the BCR. If this holds true in the BCR^{low} DZ population, then an additional ~9% (50% of 17.2%) would be structurally damaged, possibly by FWR mutations; bringing the total frequency of cells carrying structurally damaged BCR to 25.8%. It is likely that these mutations were enriched in antibody framework regions. As discussed in the introduction, SHM preferentially targets “hotspots” in CDRs and avoids FWRs. While this an impressive intricacy of AID activity, the recalcitrance of FWRs to DNA damage appears to have been mythologised to some extent, as mutations in these regions still do occur somewhat frequently.

Given the data discussed above, we can conclude that a significant proportion of mutations that disrupt BCR expression in GCs are insertions and deletions that lead to frameshift mutations or FWR gene disruption. The biology of the GC is a map of the evolutionary pressures that have been applied to the adaptive immune system over millions of years. Mayer et al. estimated that almost half of all GC B cells die every 5 hours[327], which corresponds to ~3% of the GC dying every ~21 minutes. Like previous groups[343], they noted that a large proportion of the death was related to SHM, because AID deficiency was clearly associated with reduced apoptosis, particularly in the DZ[327]. If ~60% of GC apoptosis occurs in DZs, which is a somewhat conservative estimate, and ~75% of this is the result of cells generating non-functional BCRs, then ~23% of the cells in the GC die every five hours due to mutations that yield non-functional BCRs. Evolution abhors waste, yet this process is incredibly wasteful. It is worthwhile to ask why the SHM process involves the induction of indels, or allows for SHM in FWRs. It appears that, while these mutations are very often harmful, they can be critical for the development of broadly neutralising antibodies, like those which are directed against influenza and HIV.

A small proportion of people infected with HIV develop broadly neutralising antibodies (bNAbs)[344,345], but these only appear 2-3 years after infection[346–348].These antibodies can protect macaques from infection[349–351], and control infections in humanised mice[352]. bNAbs differ from more commonly occurring antibodies; they not only carry more mutations (a normal antibody may carry 15-20 variable region mutations in the heavy chain[353], whereas broadly neutralising HIV antibodies carry 40-100[10,354–356]), but also exhibit unusual structural features. bNAbs are also more likely to have insertions and deletions, and they more commonly have FWR mutations[357,358] This phenotype is not specific to HIV, as mutations in FWRs are also

important for broadly neutralising antibodies against influenza[12,359]. The opposing evolutionary pressures on the activity of the AID molecule, and the "targetability" of the Ig variable sequences are, in one direction, the pressure to increase diversity, and, in the opposite direction, the pressure the yield functional antibodies. The adaptation we have described, wherein the pre-SHM BCR is fully exchanged, such that the structural integrity of the newly mutation BCR can be tested, relieves the evolutionary pressure potentially restricting AID activity and the sequence diversity of the variable regions. This creates a GC in which "high risk" mutations occur often, and freely, such that very occasionally they can generate an unusual, broadly neutralising antibody.

FWR mutations, while spatially separated from the points of antigen contact, can impact antigen binding. In "normal" antibodies (i.e. non-bNAbs), mutations within FWR are more likely to be incidental to antigen binding, and rarely contribute directly to association with antigen. However, in bNAbs, experimental reversal of mutations in FWRs often reduces the breadth and potency of the antibodies[30]. FWR mutations can also allow the antibody to interact with the antigen in an unconventional manner, for example by forming a salt bridge with residues in conserved regions of target antigen[360,361]. In some bNAb clones, like 3BNC60, which has low germline affinity for cognate antigen, FWR mutations occur early in affinity maturation, to reorient the CDRs in order to appropriately bind the epitope on the antigen. Once these FWR mutations have accumulated, the CDRs are appropriately orientated to interact with the difficult to access epitope, and mutations that improve affinity will be able to be selected[362,363]. While FWR mutations are not always necessary for broad neutralisation[364], the flexibility or altered orientation rendered by FWR mutations may expand the targetable epitopes for GC B cells[362]. Therefore, although they are more often than not disastrous for the structural integrity of the BCR, the evolutionary pressure to develop mechanisms that entirely prevent AID from targeting FWRs is weak.

Another unusual feature of HIV bNAbs is the presence of multi-base in-frame indels, induced by SHM[365]. The indels found in normal post-GC B cells are generally short, and their frequency decreases with the length of the insertion or deletion[366]. The proportion of indels among post-GC populations is very low, and has been estimated to range from ~0.005% to as high as ~4.4% in memory B cells[366,367]. The rate at which SHM actually leads to indels can be analysed by looking at the unselected introns between the J_H and constant region; Smith et al. found that 1-2% of the unselected SHM events were single base insertions or deletions, which, were they to occur in exons, would lead to frame shifts[53]. However, indels are much more prevalent in HIV bNAbs, with a prevalence of ~40%[365]. Indels are also found in bNAbs against influenza A viruses, although at a much lower frequency[368]. Similar to the FWR mutations, the indels found in HIV bNAbs are often found to be critical for binding[365,369]. It is likely that, in some cases, as with FWR mutations, indels provide plasticity to the antibody by allowing it to reorient the CDRs[367].

Furthermore, a large insertion of a collagen-binding domain of *LAIR-1* gene has been found in broadly neutralising malaria antibodies in multiple people. The 100 amino acid collagen-binding domain of *LAIR-1* is encoded on chromosome 19, but has in these individuals been copied and pasted to the *Ighv* locus on chromosome 12. This allows the antibody to interact with the antigen in an unconventional manner. However, it is not clear if this particular trans-chromosomal insertion occurs in the GC or during RAG recombination. Insertions occur both in the third CDR region, between V, D, and J regions, which would be more likely to occur during recombination; and in the switch region immediately following the *Ighv* region, which is more likely to be the result of AID targeting. Given that it has been found in so many people,

mutations of this kind most likely occur often[14,370]. These trans-chromosomal duplications are another example of the incredibly "high risk" mutations observed in the GC.

Similar to the mutations in FWRs, indels are introduced less frequently than simple substitutions in CDRs, but are still allowed to occur at somewhat alarming frequencies considering how often they are detrimental to the structural integrity of the BCR. That positive selection is "permissive", while still frequently introducing "high risk" mutations events, necessitates a selective force restricting the accumulation of cells encoding non-functional antibodies. In the absence of antibody "quality control" as a component of selection, there would be selective pressure to prevent AID from introducing more "high risk" mutations, like indels and FWR mutations. This would risk narrowing of the potential repertoire of the humoral response and would leave gaps in immunological protection which could be exploited by pathogens.

6.3. CELLS MAY REQUIRE ACTIVE LIGHT ZONE PROMOTING CUES TO TRANSITION FROM DARK ZONES TO LIGHT ZONES

In addition to finding that cells which have lost their surface BCRs failed to transition into the LZ, we also observed that delaying apoptosis through the enforced expression of the *Bcl-2* transgene was not sufficient to rescue this effect. This led us to hypothesise that cells that have acquired deleterious immunoglobulin mutations and have lost surface BCR fail to receive LZ-promoting cues. This was consistent with a model in which the DZ to LZ transition may require a LZ-promoting transition signal, rather than the LZ transition occurring simply when DZ cells survive sufficiently long enough for the process to occur. However, the evidence for this conclusion remains somewhat indirect at this stage, therefore it is important to consider our findings in the broader context of current knowledge.

6.3.1 MATURE B CELLS MAY REQUIRE "TONIC" B CELL RECEPTOR SIGNAL, OR MAY "SENSITISED" TO DEATH UPON LOSS OF SURFACE BCR

Our finding are far from the first to uncover an apparent need for BCR expression for B cell survival – it is more than 20 years since Rajewsky and colleagues demonstrated that naïve B cells die when their BCR is experimentally deleted[269]. It is possible, therefore, that GC B cells that have acquired deleterious mutations in their immunoglobulin genes may simply fail LZ entry as a consequence of them not living long enough to make it there. However, while our findings do not formerly exclude this possibility, there are several reasons why it seems unlikely.

The first argument to be made relates to kinetics- naïve B cells survive with a half-life of approximately 3-6 days after their antibody heavy chain genes are experimentally deleted[269]. Such a time frame would represent several DZ/LZ transitions in the GC (as has been noted 50% of cells transition into the LZ every six hours[330]), and would therefore expect to lead BCR^{neg} cells being found at far higher frequencies of cells carrying disrupted BCRs in LZs than we observed. Given that GC B cells are in a very different state to naïve B cells that includes them dividing (and diluting proteins) quickly, it is plausible that the underlying biology is similar to that reported for naïve B cells but that the amounts of time involved are much reduced. The second argument is that, for the reasons discussed in the introduction, the BCR "maintenance signal" is unlikely to exist. Given that when mature Igα is ablated naïve B cells will last for as long as 20 days[271], and that mature cells tolerate extended periods of time in the absence of Syk[237,282], it does not appear that cells require a BCR signal to survive. The inconsistency in survival between cells that have lost their heavy chain, and cells that have lost their signalling

chain, necessitates a re-examination of the "maintenance" signal apparently required by mature resting cells.

As Levit-Zerdoun et al. rightly pointed out, loss of the variable chains is more likely to occur than loss of the signalling chains. It may be that the observed increased sensitivity to of UPR-mediated apoptosis in mature B cells may have evolved to eliminate cells which have introduced disruptive mutations in the immunoglobulin variable chains, and GC B cells could be especially sensitive to it, such that when unpaired alternate chain builds up they are deleted especially quickly[271]. It is possible that cells are sensitised to apoptosis upon loss of the variable chains, and this sensitisation can be overridden by BCR signals. In this model, enforced activity of PI3K does not recapitulate a lost BCR signal, but rather counteracts the UPR, or some other signal that the cell has received communicating loss of functional heavy chain. The question of the existence of a LZ promoting cue could perhaps be rephased as a question of what the default pathway is for a cell once it has exhausted its proliferative program in the DZ. If the default program is for the cell to die, in order to transition to the LZ it will need to receive an active positive signal, possibly a BCR mediated signal. Alternatively, if the default program is to live and to transition into the LZ, the cell may die if it receives a negative signal, possibly related to the build-up of unmatched variable chain. However, our experiments in which we extended GC B cell survival through expression of Bcl2-transgene seemingly make this possibility unlikely. Instead we favour the hypothesis that more "active" LZ promoting cues may be necessary.

6.3.2 SIGNALS DOWNSTREAM OF THE BCR PROMOTE LIGHT ZONE PHENOTYPE

While the previously described BCR mediated "maintenance" signal is unlikely our hypothesised LZ promoting cue, the LZ-promoting cue may be provided through an acute BCR

signal. The induced deletion of Syk caused a reduction in the frequencies of CXCR4^{low} CD83^{+} LZ phenotype cells[130]. It has been postulated that the LZ cells are predisposed to die if they do not receive a BCR mediated signal[327]. However, the putative role of a BCR signal in the generation, as opposed to maintenance of, the LZ phenotype has not been investigated.

We attempted to uncouple loss of BCR heavy chain from the SHM event using the B1-8f mouse line, in which the heavy can be inducibly ablated. While the induction of loss of the BCR was not complete, it did indicate that cells which have lost their surface BCR are more likely to die in the DZ. As mentioned, there would be merit in repeating these, experiment in the absence of AID, such that the DZ BCR^{low} cells could be analysed as a more homogenous group of cells which have lost endogenous heavy chain, rather than a mixed population of cells which have lost surface BCR, and cells which are exchanging their BCR. Additionally, there would be value in attempted to induce loss of the BCR by genetically ablating Igα with the Igα/BAP31-eGFPinv mouse line[271]. This would allow us to differentiate between defects related to losing surface BCR, and defects related to a build-up in unpaired chain (while, the build-up of paired chain unable to traffic to the surface should in theory cause similar problems, it appears from the comparison of the defects in cells having lost Igα and IgH that they do not[269,271].)Furthermore, although it would likely cause more global changes to BCR biology, there would also be value in blocking BCR signalling using a Btk or Syk inhibitor.

Conversely, we would wish to recapitulate BCR signalling by inducing the expression of constitutively active downstream components. BCR engagement can activate PI3K signalling, leading to the phosphorylation and subsequent activation of Akt, which itself will then phosphorylate and inactivate FOXO1. Inactivation of FOXO1 results in the release of the DZ program, and PI3k-Akt activation facilitates the LZ phenotype. Furthermore, BCR signalling

through PI3K/Akt/FOXO1 is a good candidate for the LZ promoting cue not only because it links BCR signal with LZ phenotype, but also because Akt signalling promotes the survival of GC cells[43].

Given that phosphorylation of Akt as a consequence of BCR engagement can lead to the inactivation of FOXO1 and as such drive cells into a LZ phenotype, BCR mediated activation of Akt is an interesting candidate for a LZ-promoting cue. This is especially interesting given that Akt had been shown to be necessary for controlling the size of GCs. The sphingosine 1-phosphate receptor S1P2, and its downstream mediators Gα12, Gα13 and p115RhoGEF, antagonize Akt. S1P2 is expressed by GC B cells and is necessary to control the size of chronically stimulated GCs[43]. Human GC B cell like diffuse large B cell lymphoma is associated with mutations in S1P2, which disrupt the repression of Akt phosphorylation. This de-repression of Akt contributes to lymphomagenesis by increasing survival[371]. The demonstrated pro-survival role of Akt is consistent with this signal being part of a LZ-promoting cue. However, it is noteworthy that Akt signalling has been reported to be "rewired" in GC B cells, as described in the introduction. In addition to its association with negatively regulating FOXO1 activity, it also has a negative feedback role in BCR signalling. The putative dual role of Akt in the GC does not preclude it from being the conduit for a BCR signal mediate LZ promoting cue, however it presents issues in how to recapitulate this this signal in the without disrupting the system too profoundly, in order to try to attempt to understand the LZ promoting cues.

Given the potential relationship between downstream BCR components and induction of the LZ phenotype, it will be helpful to ask whether activating these specific pathways is sufficient to drive cells with a non-functional BCR into the LZ. If recapitulating BCR signal with a constitutively active PI3K (P110*), or a constitutively active Akt (myr-Akt), or by inducibly

ablating the PI3K inhibitor PTEN, drives cells with a non-functional BCR into the LZ, then it is possible that the LZ promoting cue is a BCR signal. However, if cells with a non-functional BCR do not transition to a LZ phenotype, and instead die, even after downstream BCR signals are received, then it is likely that disruption of the BCR heavy chain sequence disrupts the DZ to LZ transition in some other way.

There are two major limitations in this approach. The first problem relates to the conceptual difficulty in disrupting the DZ/LZ binary. Enforced activity of PI3K, or ablation of FOXO1, leads to loss of the DZ. It is possible that when cells are forced from the DZ to the LZ, a potential DZ to LZ checkpoint is maintained. However, it is equally likely that a fundamental disruption of polarity overrides any DZ to LZ selection bottlenecks. Cells with a non-functional BCR may therefore transition into the "LZ" of these disrupted GC, because the DZ phenotype has been ablated, rather than because the previously disrupted LZ promoting cues have been restored. The second issue is that when the DZ is ablated, the "LZ" cells are somewhat aberrant, in that they proceed to induce SHM[135]. Therefore, if cells have lost surface BCR through induction of a deleterious mutation in the *Ighv* regions, it would not necessarily be clear if this were a cell that had acquired the deleterious mutation when it was in the DZ state, and been allowed to enter the LZ having recapitulated the LZ promoting cue; or if it were already in the LZ when it acquired a SHM which disrupted the BCR gene. We could possibly utilise the Fucci2 mice to ensure that the cells we are observing are more conventional LZ cells by selecting non-cycling cells based on mCherry levels. However, there is value in being conservative about what can be deduced about the in intricacies of the GC polarity once the polarity has been perverted.

Given that recapitulation of downstream BCR signals disrupts GC polarity, we attempted to recapitulate the BCR signal through expression EBV protein LMP2a. LMP2a gives a false BCR

signal without fundamentally disrupting DZ/LZ polarisation[74]. It recreates the signal much further upstream than constitutively active Akt or Pi3K[372,373]. LMP2a can both deliver a BCR signal and block BCR signalling. The LMP2a N-terminal cytoplasmic domain caries ITAM motifs similar to those present in the Igα and Igβ (the signalling domains of the B cell receptor complex)[372]. LMP2a signals through critical signalling cascade molecules Syk, Lyn, Stk, BLNK, Akt and Pi3K, thereby delivering signals similar to those induced by BCR stimulation[373]. However, it is also noteworthy that LMP2a has been reported to also block endogenous BCR signalling[372,374], by acting as a "decoy receptor" that sequesters Lyn and Syk and triggers their degradation[373], and by physically preventing BCR complexes from entering lipid rafts[375].

There is strong evidence that expression of LMP2a allows GC B cells that have lost endogenous BCR to persist. GCs develop Payer's Patches in BCR deficient mice expressing LMP2a[376]. Furthermore, EBV infection allows for the survival of GC derived lymphoma cells which have lost endogenous BCR[377,378]. Analysis of the Ig genes in Hodgkin lymphoma cells showed that almost all cells with "crippling mutations", as opposed to those with functional BCRs, were EBV positive[379]. *In vitro*, pro-apoptotic BCR negative cells will be rescued by the expression of wild type EBV[378], but not EBV lacking the viral LMP2a gene[380]. Therefore, it is likely that after a cell acquires a premature stop codon in its immunoglobulin genes in the DZ, it could be allowed to transition to the LZ, and this may be the least disruptive way of recapitulating that signal.

To test the capacity of the LMP2a derived BCR-like signal to drive cells which have lost endogenous BCR into the LZ, we constructed a viral vector that could be used for the inducible expression of the LMP2a protein. While the LMP2a had been expressed in GC B cell *in vivo* before, in this setting induction of LMP2a appeared kill the highly transduced cells. It is possible that, under these conditions, the protein was cytotoxic. Given in a different setting that expression

of LMP2a leads to smaller GCs[126], it is also possible, but less likely, that in a mixed setting (these GCs contain both transduced and untransduced cells) the cells do not compete efficiently. Not all of the LMP2a transduced cells died upon tamoxifen treatment, but those that remained had lower levels of transduction. However, as this model avoids those issues discussed above for constitutively active BCR downstream component, this is a model the lab wishes to pursue possibly with a transgenic mouse.

6.3.4 A B CELL RECEPTOR MEDIATED LIGHT ZONE PROMOTING CUE MAY BE ANTIGEN DEPENDENT OR INDEPENDENT

If the LZ promoting cue is related to an BCR mediated signal, an interesting question is whether it is mediated by cognate antigen in the DZ; or by low affinity interactions with non-cognate antigen, possibly self-antigen; or a "tonic signal", like that which occurs in pre-B cells through the pre-BCR. Following SHM, it makes conceptual sense that a cell would have the same requirements for the BCR that it does after recombination of the heavy chain. This requirement not being that the BCR interacts with any antigen specifically, but that a functional BCR signal would be generated. However, the extent to which these are analogous are probably limited. Firstly, during the pre-B cell development phase, PI3K has the opposite role to that in the GC. In the GC PI3K signalling promotes a LZ phenotype overall, which is correlated with a decrease in cycling. However, in B cell development PI3K controls proliferative expansion of the cells[381,382.] Furthermore, as mentioned in the introduction, the pre-BCR mediated signal is related to autoaggregation caused by the surrogate light chain's unique capacity to bind to other pre-BCRs[288]. The chronic signalling seen in chronic lymphocytic leukaemia has also been

attributed, at least in some cases, to homotypic interactions between BCRs[206,278]. The BCRs in the cells in the DZ are normal and are very unlikely engage in homotypic interaction such as this. However, GC DZ B cells may signal in a way that is totally antigen independent, and there may be a restricted, acute reprieve in the activity of the negative regulators of the BCR signal which allow a basal signal to be amplified if the BCR is present on the surface of the cell.

We began to address this question by asking if cells require a cognate antigen-mediated BCR signal to enter the LZ. Using the SW_{HEL} system we identified a population of cells in the LZ which have severely defective capacity to bind to cognate antigen. Similar to our results regarding cells which had lost the HEL binding, Kuraoka et al. found a significant proportion of cells on both day 8 and day 16 did not show measurable binding to the native immunogen[310]. However, they were not differentiating between DZ and LZ cells, and so could not make inferences about this transition. Just as we have been cautious about the somewhat blurry line between BCR that can no longer interact with antigen, and BCR which has very low affinity for antigen, they noted that some unspecific GC B cells could simply be very low affinity B cells, as has been observed in other contexts[223,383]. They found that those cells which have lost antigen binding and those which had maintained antigen binding had similar levels of V_H mutations. However, in our analysis of cells that had lost the ability to bind HEL, we observed higher levels of mutation.

Kuraoka et al. postulated that non-antigen binding antibodies they observed may be specific to non-native confirmations of the antigen, and that these may be cryptic epitopes exposed by degradation, or complement fixation. Haptens, like NP, are generally resistant to these sorts of modifications; this is consistent with Mayer et al.'s finding that fewer than 1% of cells encoded

antigen which had lost antigen binding capacity in the NP response (this represented a single cell, which was found in the DZ)[327]. On one hand, the only relevant question is whether the cell that has apparently lost antigen binding is able to productively interact with the antigen *in vivo*. We sought to address this question using the HEL-Ea-GFP model, and saw severely reduced antigen presentation among the non-binding cells. However, the fact that there is measurable *in vitro* antigen binding, even if it is very low, means that it may be technically possible that the cell is able to interact with antigen *in vivo*.

We attempted to address whether delivery of BCR signal through a BCR variable region for which is directed against non-cognate antigen was sufficient to drive cells which have lost their endogenous BCR into the LZ. We hypothesised that inducing the expression of a non-cognate BCR would be sufficient to allow cells to enter the LZ, and that if we induced expression of a non-cognate BCR we would be able to identify cells that had acquired premature stop codons in the variable region of their endogenous BCR in the LZ. If introduction of neither the cognate nor the non-cognate heavy chain allowed cells which had lost endogenous BCR to enter the LZ, then it is likely that the presence of truncated protein disrupts the DZ to LZ transition. If the antigen binding heavy chain, but not the non-cognate heavy chain, allows cells which have lost endogenous BCR to transition into the LZ, then it would follow that cells do in fact require a BCR mediated signal to enter the LZ. However, if both cognate and non-cognate heavy chain expression allows cells which have acquired premature stop codons in their endogenous BCR genes to enter the LZ, then it would follow that cells only require a BCR signal, which does not need to recognise antigen, to enter the LZ. We cloned a NP specific IgG1a heavy chain and a HEL specific IgG1a heavy chain into the loxp MSCV vector (the same as that which we used for inducing the expression of LMP2a). We then made chimeras with SW_{HEL} Rosa-CreERT2

mice, which activate Cre recombinase upon tamoxifen treatment. However, while the transferred cells which bound HEL normally were transduced at high levels, these transferred cells failed to establish GCs, and those SW_{HEL} cells which did establish GCs were largely untransduced (were Thy1.1-). This was an unexpected technical failure. However, this is remains an area of interest for the lab, and we plan to make a transgenic mouse to address this issue.

6.3.5 NF-κB SIGNALLING IS UNLIKELY THE LIGHT ZONE PROMOTING CUE

It is unlikely that the LZ promoting cues required by DZ cells are related to the canonical or non-canonical NF-κB pathways. While components of both ways have been identified as related to GC B cell survival, this is more likely linked to more generalised cell survival, in the form of transmitting BAFF signals; and selection, in the form of transmitting signals related to CD40/CD40L interaction. The transcriptional programmes activated through the canonical and non-canonical NF-κB programmes are distinct, indicating that these are complementary but independent biological programmes that are required for maintenance of the GC programme[384].

In terms of canonical NF-κB, signalling is necessary for mature B cells under conditions of competition, but it is not sufficient to rescue cells which have lost surface BCR[270,385]. This is consistent with evidence from Bc110-deficient mice, wherein canonical NF-κB activity is decoupled from BCR signalling[386]; while B cell development and proliferation upon activation were effected, these mice still had sizable B cell compartments[387]. The canonical subunit of the NF-κB pathway, c-Rel is required for GC maintenance, but RelA is disposable[388]. This was considered to be related to ablation of c-Rel disrupting positive selection in the LZ, as CD40 stimulation is known to induce the nuclear translocation of NF-κB subsets[389], and disruption of the

CD40-CD40L interaction leads to involution of the GC[390]. This group hypothesised that upon CD40 ligation, c-Rel was activated and induced a metabolic programme that licenced B cells for cyclic re-entry. Therefore, we do not have any explicit plans to target these pathways to trigger LZ entry in DZ cells with newly acquired damaged BCR genes.

6.4. SUMMARY

That cells with non-functional BCRs are removed from the GC in the DZ, in a process distinct from classical selection occurring in the LZ, is critical for current conceptualisations of the paradoxical nature of selection in the GC. If the GC is to appropriately amplify high affinity clones and maintain diversity in the humoural response, then there must be a separate culling of cells encoding non-functional BCR. The negative selection which is required for the deletion of these cells necessitates full exchange of the BCR. Therefore, the discoveries in this work are critical for our understanding of the paradoxical selection requirements of the GC, that they maintain breadth while amplifying high affinity clones.

CHAPTER SEVEN: APPENDIX

7.1 APPENDIX ONE: EFFORTS TO ANALYSE EXCHANGE OF THE B CELL RECEPTOR IN THE GC

We developed and tested several models for analysing the kinetics of the BCR turnover in the GC *in vivo*, with the goal of being able to compare the BCR complex half-lives in GC versus naïve B cells, as well as between LZ and DZ cells. Furthermore, we hoped to use these tools for investigating the pattern of BCR exchange in the DZ, asking whether there is a period of co-expression of pre- and post-SHM BCRs following SHM, or if instead SHM is associated with a BCR purging event.

7.1.1 IGM-KIKGR

In order to differentiate between "old" and "new" BCRs, we attempted to label the IgM heavy chain with a photoconvertible monomeric protein, mKikGR. mKikGR undergoes a rapid and permanent change from green (mKikGR-green) to red (mKikGR-red) states upon exposure to violet light[391]. In this model, a chimeric gene was constructed that was composed of the monomeric photoconvertible protein mKikGR fused to the intracellular tail of the membrane bound IgM constant region using a short flexible linker. The mKikGR gene was genetically linked to the c-terminus of the *Ighm* membrane bound constant region, and this was knocked-in to the endogenous *Ighm* locus using a CRISPR/Cas9-mediated approach. This mouse was generated by Cyagen (Figure 7.1a). The monomeric form of the protein is less prone to aggregation and therefore would not be expected to induce BCR aggregation. A similar flexible linker was previously used to link the IgM heavy chain to non-photoconvertible fluorescent proteins[391]. A similar protein

tagging/photoconversion approach was also successfully used for monitoring MHCII turnover rates *in vivo*[300].

Our plan was to surgically expose and photoconvert the skin draining inguinal lymph nodes in immunised mice, converting the IgM-mKikGR protein from green to red. Analysis of the rate of loss of red fluorescence in different populations should have allowed for a comparison of the half-life of the BCR in different cell populations, and between individual cells within given subsets. We would be interested in comparing the half-life of the BCR in in GC B cells to that of follicular B cells, and also between DZ cells and LZ cells. Additionally, analysis of the patterns of loss of photoconverted IgM signal may give clues about the model of degradation of the BCR in the DZ. If cells in the DZ are degraded at a uniform rate, then one may observe loss of photoconverted IgM signal ass a single peak of photoconverted (red) BCR decreasing over time. However, if rates of loss of surface BCR were not uniform, instead involved purging events following SHM, then one would not observe cells with intermediate levels of photoconverted IgM accumulating, but rather we may expect to see bimodal peaks of photoconverted IgM (schematic of possible patters of loss of signal demonstrated in Figure 7.1b).

Disappointingly, the mKikGR labelling approach appeared to disrupt BCR signalling *in vivo*. Circulating B cell numbers were dramatically reduced in IgHM-KikGR homozygotes (34.4% in WT vs 3.5% in homozygotes), and IgM levels on the few splenic B cells recovered were lower than on control animals. In heterozygote mice, there were near normal B cell frequencies and these B cells did express IgM, however these cells appeared to be preferentially using the alternative allele because there was very little KikGR-green expression (Figure 7.1c, d). That the mKikGR label disrupted the number of resting B cells implied that it interfered with BCR

signalling, and so blocked B cell development at the pre-B stage, wherein the heavy chain of the BCR is tested[289,392].

When homozygote mice were immunised, there was a disproportionate expansion of class switched B cells, and BCR^{low} B cells (Figure 7.1e). The mKikGR-green-positive and negative cells were analysed separately. The mKikGR-green-positive GC cells were enriched for BCR^{low} cells, which implied that the IgM was not being trafficked to the surface of the cell (Figure 7.1c). The mKikGR-green-negative GC B cells showed a normal amount of BCR^{low} cells; however, while these cells expressed higher levels of Igβ they were all IgM-negative, and as such had likely class switched and were using an alternative constant chain. In the follicular B cell population, the mKikGR-green-negative cells showed higher levels of surface IgM, therefore the fluorescent protein might have been cleaved from the protein and degraded (Figure 7.1f). The mKikGR-green-positive follicular B cell populations still contained some $Ig\beta^{+}$ cells, however most of the surface Igβ was probably coupled with IgD because these cells were negative for surface IgM (Figure 7.1e, f, g, h). That the cells exit the BM at all, and that they are able to form a GC and class switch imply that signalling isn't totally disrupted. However, within the GC, the cells expressing the fluorescent protein were not expressing surface IgM, therefore the model is not of use for addressing our questions.

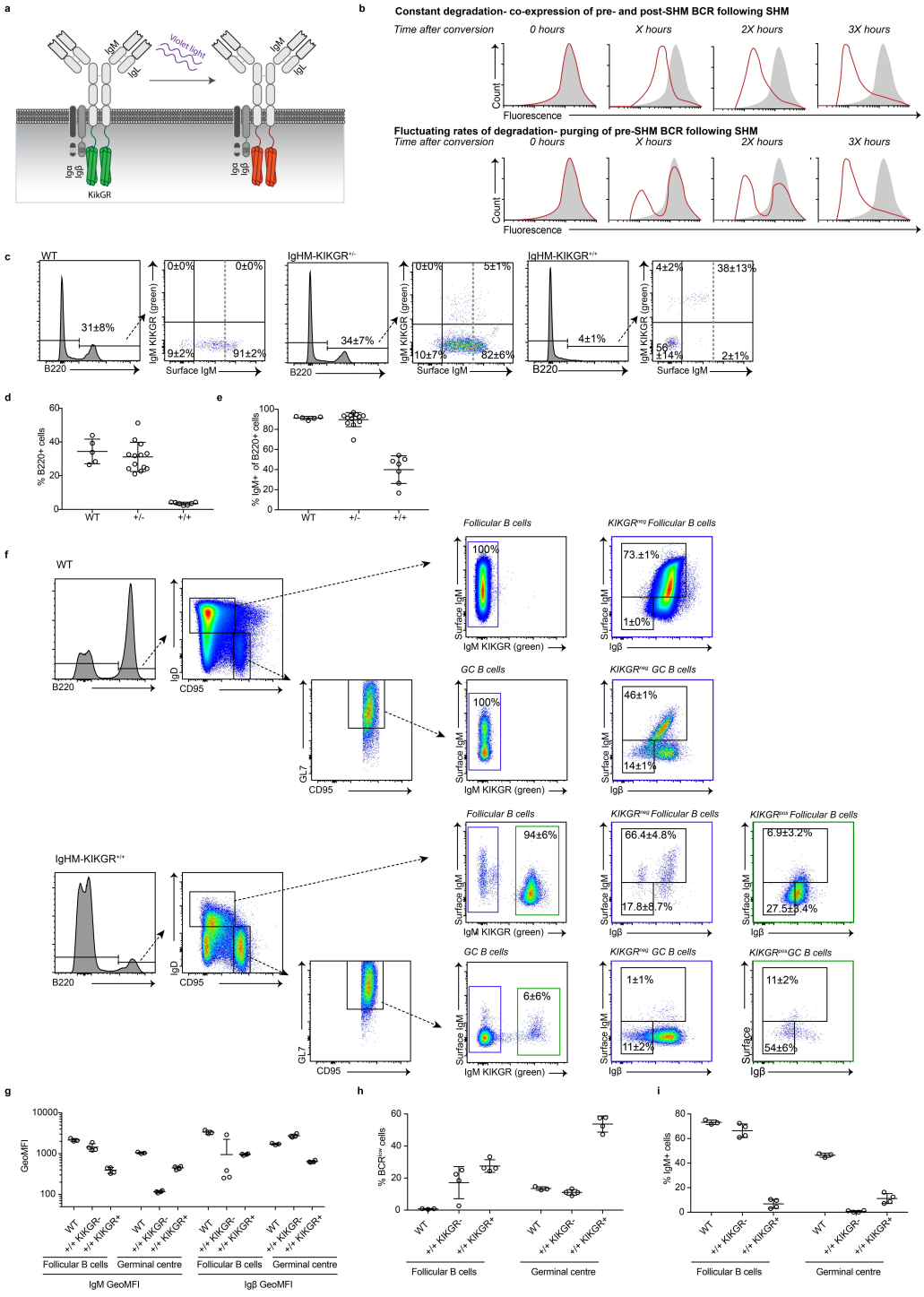


FIGURE 7.1| LABELLING B CELL RECEPTOR COMPONENT IGM WITH PHOTOCONVERTABLE PROTEIN KIKGR

Mice with a mKikGR protein conjugated to the IgM heavy chain were produced by Cyagen. (a) Schematic of mKikGR protein associated with IgM heavy chains switching from green to red when exposed to violet light. (b) Schematic of patterns of loss of photoconverted mKikGR

in the BCR purging model and co-expression model (c) Blood for from unimmunised WT, heterozygotes, and homozygotes mice were analysed for frequency of B cells (B220+ cells) and of those B cells the IgM- mKikGR green fluorescence was analysed against surface IgM. Data summarised in (d, e) (d) Frequency of B cells WT, heterozygotes, and homozygotes. (e) and frequency of IgM+ cells of B cells in WT, heterozygotes, and homozygotes. (WT, n=5, $KikGR^{+/-}$ n=13, $KikGR^{+/+}$ n=7) (f) Homozygotes and WT mice were immunised with SRBC and analysed on day 7, plots show histogram demonstrating frequency of B220+ cells, GC cells are gated as IgD^{low}, CD95+, GL7+. Cells positive and negative for IgM- mKikGR green fluorescence were analysed in GC B cells and follicular B cells for expression of total surface BCR (Igβ) and surface IgM. (g, h, i) Summary of data (f) comparing in WT, mKikGR green fluorescence negative (+/+mKikGR -), and mKikGR green fluorescence positive (+/+mKikGR +) in follicular B cells and GC B cells. (g) GeoMFI of IgM and Igβ (h) Frequency of BCR^{low} cells (i) Frequency of IgM+ cells, bar in graphs represent SEM). (WT, n=3, $KikGR^{+/+}$ n=4) (numbers on plots indicate mean ± SEM

7.1.2 IGB FLUORESCENT TIMER

Given that attaching a protein to the heavy chain disrupted signalling, we next attempted to instead label the Igβ signalling chain (schematic shown in Figure 7.2a). A benefit of this system is that we would not need to make accommodations for class switching. In this instance, we labelled the chain with a fluorescent timer proteins (FT)[391]. Fluorescent timer proteins are fluorescent proteins whose fluorescent properties change over time, essentially they change from one colour to another with time. We used two different forms of a monomeric fluorescent timer protein which transitions from red to blue, a "fast" converting protein and a "medium" converting protein. At 37^{o}C, the maxima of the blue fluorescence intensities were observed at 0.25 and 1.2 hours for the "fast"-FT, and "medium"-FT respectively[393]. The half-maxima of the red fluorescence intensities were reached at 3.9 and 7.1, respectively, which corresponds to the half-lives of the maturation for the red FT forms. As such, one can compare the relative "age" of tagged proteins between different cells populations and make assessments about the half-life of the proteins in that cell, by comparing

the ratio of “old” and “new” fluorescence. Therefore, one could compare the relative age of proteins in different populations: for example, follicular B cells and GC cells, and the DZ and the LZ. For example, if the BCR has a shorter half-life in the DZ than in the LZ, one might expect an enrichment of “old” BCR in the LZ and more of a spread of “old” and “new” BCR in the DZ.

Furthermore, one might be able to draw conclusions about the rate of degradation of the BCR in the DZ by analysing the ratios of “old” and “new” signal in the DZ. If BCR degradation is uniform in the DZ then one might expect the spread of ratios for different cells to be somewhat continuous. Moreover, if the rate of degradation is not constant, and associated with concentrated purging events, then there would be a bimodal distribution of cells with mainly “old” BCR, which express the same molecules they had on their surface in the LZ, and “new” BCR, having only recently replaced it. Additionally, the spread of “old” and “new” proteins among the BCR^{low} population would be somewhat telling. If the low levels of BCR bimodally segregated into mainly “old” or mainly “new”, while the cells removed or replaced BCR respectively, this would be consistent with a purging event. These possible situations are schematised in Figure 7.2b

We constructed a retroviral viral vectors with the Igβ protein fused to “fast” and “medium” switching fluorescent timer proteins[393]. Expression of the protein using viral vectors is faster and more affordable than the generation of new mice. However, it also has its limitations. Firstly, different cells will have different levels of viral integration, and so there will be some variability in the level of total fluorescence. However, the ratio of the two fluorescences in any given cell should give an indication of the age of the BCR on the surface of the cell. Secondly, the conjugated Igβ protein is not under the control of the Igβ promoter, and so any control of

expression that is occurring on the genetic level will be lost. However, the single cell analysis of mRNA levels of BCR components (described in appendix two) do not indicate that the transcriptional regulation of the BCR is the main driver of BCR exchange, so these caveats appeared to be appropriate concessions for the increased affordability and expediency.

The retroviral vector use (MSCV) contains an internal ribosomal entry site (IRES) driven Thy1.1 reporter gene for the identification of transduced cells. Having synthesised MSCV- Igβ-FT constructs (one in which the signalling chain is fused to the "fast" protein, and one in which it is fused to the "medium" protein), these constructs were expressed functionally in transduced B cells, as evidenced by blue "new" Igβ fluorescence for both the "fast" and "medium" forms of the protein (Figure 7.2c). The fluorescence correlated with transduction and was absent in the non-transduced cells and in the empty vector controls. All transduced cells were fluorescent, which indicated the FT proteins were not being degraded immediately. However, there was no red "old" Igβ fluorescence, possibly indicating that the protein is rapidly degraded before it had time to convert, or that the "red" emission was too weak for easy detection.

To formally test the possibility that these constructs may be usable for studying BCR turnover in GCs, we transduced activated bone marrow with this viral construct, and transferred the bone marrow into lethally irradiated mice. Figure 7.2d shows total lymphocyte viral transduction efficiency, by the expression of biscistronic IRES Thy1.1. The transduction efficiency was higher for B220+ cells for the "medium" FT, but not for the "fast" FT.

When the Igβ- FT medium was assessed, there was a small proportion of cells within the transduced (Thy1.1+, outlined in red) cells that were fluorescent (Figure 7.2d). However, these fluorescent cells represented less than 1% of transduced B cells. Furthermore, there was clearly

some background fluorescence in the non-transduced (Thy1.1-, outlined in yellow) cells. In the Igβ-FT-fast cells there was no fluorescence in the transduced (Thy1.1+ cells, outlined in red) cells. We concluded that the fluorescent timer conjugated Igβ was unlikely toxic to the cells, because there were reasonable levels of transduction, as evidenced by the Thy1.1 expression. However, neither the "old" or "new" FT signal correlated with surface Igβ in the majority of cells, therefore this signal was more likely background fluorescence. We concluded that it was more likely that the FT protein (either along with the Igβ protein, or having been cleaved from it) was being degraded.

This construct was being expressed in mice which expressed endogenous Igβ. Therefore, if there were fluorescence in transduced bone marrow chimeras, it would be necessary to ensure that the fluorescently tagged constructs were being trafficked to the surface with the rest of the BCR complex. This could be achieved by performing a pulldown and Western blot analysis to ensure that the Igβ-FT proteins are associating *in vivo* with the other components of the BCR complex.

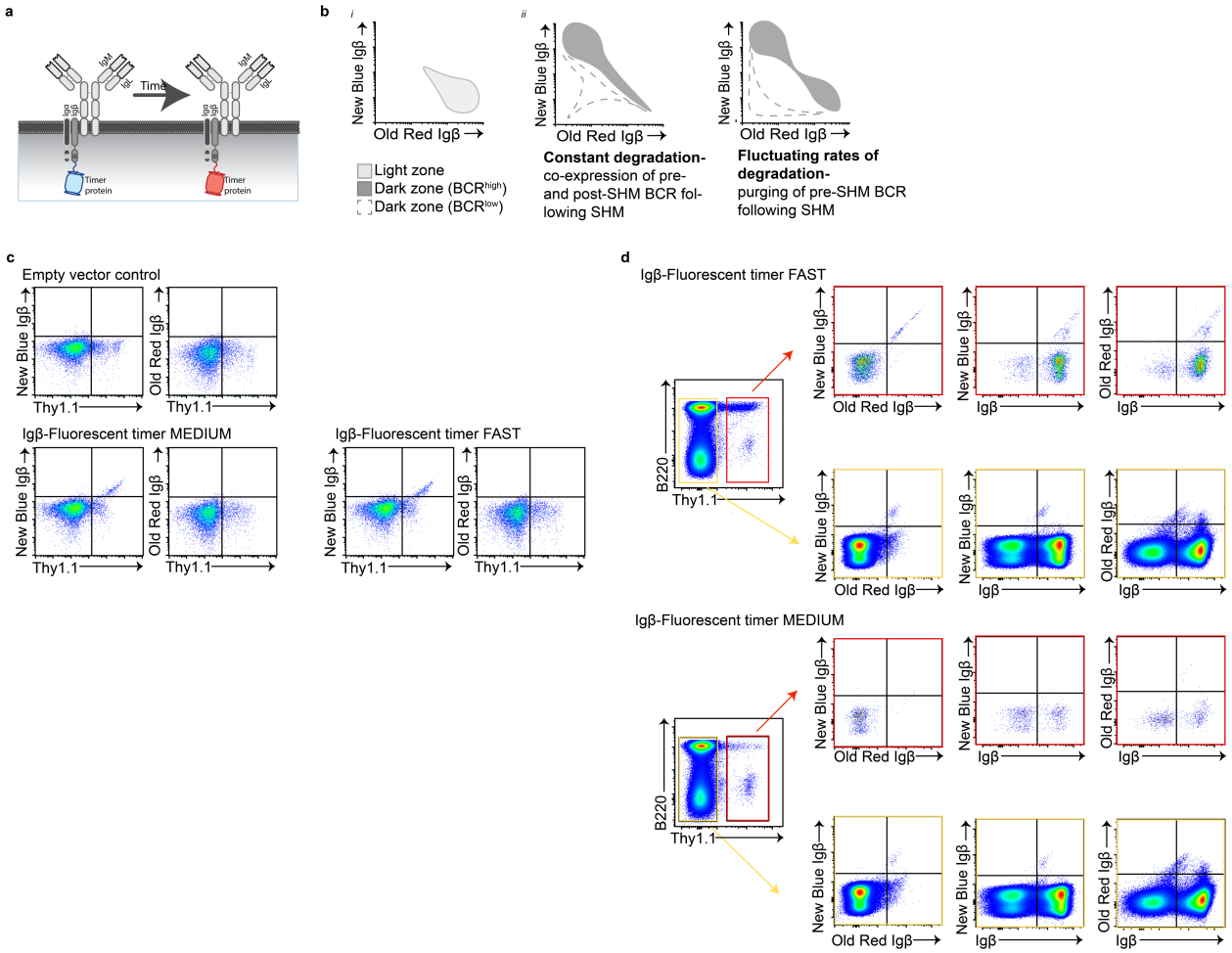


FIGURE 7.2| LABELLING B CELL RECEPTOR COMPONENT IGβ WITH FLUORESCENT TIMER PROTEINS

Igβ was genetically conjugated to either a fast or medium converting blue to red fluorescent timer (FT) proteins, these were cloned into the pQY vector. (a) Schematic of Igβ-FT converting to blue to red over time. (b) Schematic of patterns Igβ-FT "old" and "new" BCR fluorescence (i) LZ (ii) DZ in the BCR purging model and co-expression model (c)Igβ-FT (Fast or medium) pQY were transduced into activated B cells, fluorescence from the FT proteins was analysed by FACS as fluorescence in the red and blue channels versus expression of rat Thy1.1, as a marker of successful transduction. (d) Igβ-FT (Fast or medium) pQY were transduced into bone marrow, which was transferred into irradiated hosts. After reconstitution fluorescence from the FT proteins was analysed by FACS. Plots show red and blue FT fluorescence and surface BCR expression in Thy1.1+ cells (successfully transduced cells) and Thy1.1- cells (un-transduced cells, for background fluorescence context) (n=3).

7.1.3 IGκ-SNAP TAG

We hypothesised that these constructs may be disruptive because the labelling protein was too large, although the signalling chains and variable chains had previously been labelled with proteins of similar size with the same linker[239]. We therefore attempted to label the light chain with a "SNAP" construct. SNAP proteins are about 19.4kD in size, compared to the 30.0kD of mKikGR and 28.8kD of the two FT proteins. SNAP proteins can be covalently labelled *in vivo* by intravenous injection of O^6-benzylguanine (BG) derivatives carrying fluorescent molecular probes, followed by treatment with unlabelled BG molecules, to prevent continuous labelling by saturating unlabelled SNAPs[394]. We chose in these experiments to label the light chain because, unlike for the heavy chain, the C-terminus is extracellular and not associated with signalling subunits. This approach should in principle then allow for an in vivo pulse-chase type analysis, where we would follow the loss of labelled light chain over time.

We associated the HEL-specific light chain from HyHEL10 with the SNAP protein using three different linker sequences (Figure 7.3a), and cloned these constructs into the bicistronic MSCV retroviral vector. The constructs were expressed in murine B cells expressing the HEL-specific heavy chain that should pair with the light chain to confer HEL-binding. These experiments were initially performed in vitro using activated B cells. We assessed heavy chain-light chain pairing by assessing the capacity of the cells to bind fluorescently tagged HEL (Figure 7.3b). All three vectors caused B cells to successfully bind HEL as efficiently as the unlabelled HEL specific light chain. Empty vector virus did not allow cells to bind HEL.

Despite the above promising results, when we attempted to express this construct *in vivo* by making bone marrow chimeras with transduced Hy10 heavy chain-positive bone marrow, the labelled light chain constructs did not produce HEL binding B cells, whereas a control vector containing an unlabelled light chain construct did (Figure 7.3c). The different constructs with alternative linker lengths showed differential transduction efficiencies, with two of them giving transduction levels similar to that of the unlabelled light chains (linkers 1 and 2), while the third had lower efficiency. That the bone marrow chimeric mice made with the third construct had low levels of transduction implied that this construct may be toxic to the cells in some way, although this was not explored further. However, the first two constructs did not seemingly kill cells, suggesting that they may instead not pair and traffic to the cell membrane efficiently with the immunoglobulin heavy chain and as such did not confer HEL-binding ability.

The expression unlabelled Igκ chain in bone marrow chimeras results in peripheral B cell that did successfully bind HEL, and so this system did not appear to be affected by endogenous light chain (Figure 7.3c). The labelled light chains were able to bind HEL *in vitro*, and so it's unlikely that the edition of the SNAP protein prevented proper folding. The reasons for these discrepancies are not clear, however it is possible that the edition of the SNAP tag caused the light chain to be degraded *in vitro*.

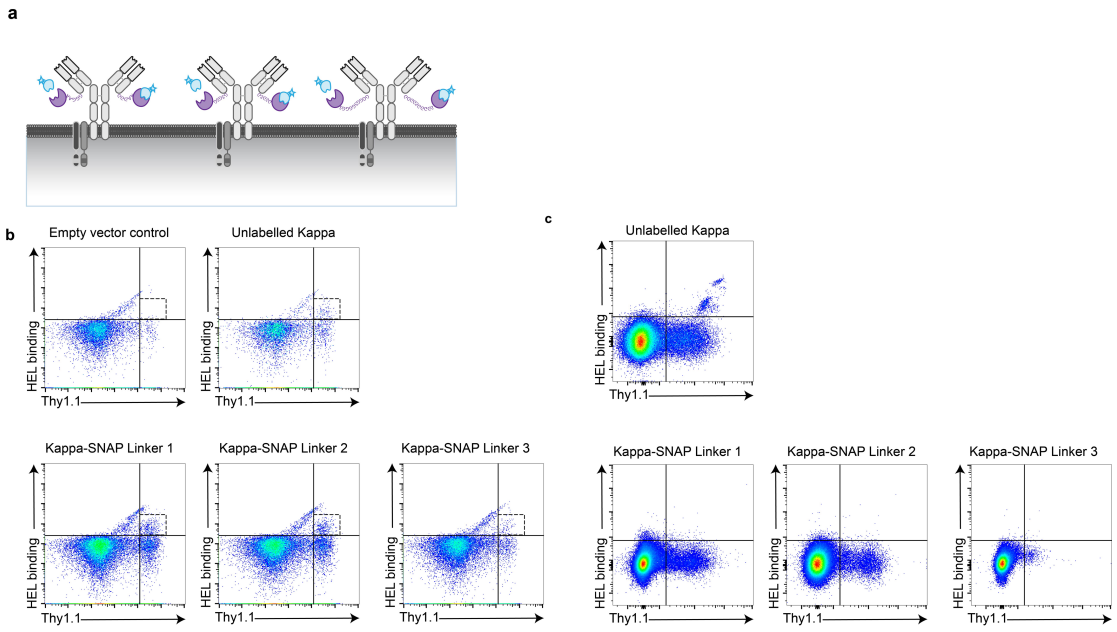


FIGURE 7.3| LABELLING B CELL RECEPTOR COMPONENT IGκ WITH SNAP TAG

Igκ was genetically conjugated to SNAP tag protein, using one of three different linker proteins, these were cloned into the MSCV Thy1.1 vector. (a) Representation of the three difference constructs of the Hy10 light chain genetically conjugated to SNAP-tag with different linkers. (b) The constructs represented in (a) were cloned into MSCV. Activated splenocytes from mice positive for the Hy10 heavy chain were transduced with the Igκ-SNAP-tag MSCV constructs, and also unlabelled constructs. Successful expression of the HEL specific chain was verified by staining of cells with fluorescently conjugated HEL. Quadrants demarcate positive and negative for Thy1.1 expression as a marker for successful B cell transduction, and HEL binding. Dashed line box represents HEL binding of transduced cells. (c) Constructs were transduced into Hy10 heavy chain positive bone marrow, which was subsequently transferred into irradiated hosts. After reconstitution expression of the constructs was analysed by FACS by staining for Thy1.1 and staining with a fluorescently conjugated HEL. (n=3)

7.2 APPENDIX TWO: EXCHANGE OF PRE AND POST SHM BCR MRNA

In this thesis, we describe that, following SHM, "old" BCR complexes are mainly exchanged while cells are still in DZs. We also observed that, at least at the bulk population level, GC B cells have seemingly slight lower immunoglobulin mRNA levels. Following SHM, a cell may not only carry on its surface copies of a pre-SHM BCR protein it no longer encodes, but within it pre-SHM mRNAs may persist for some time. We therefore sought to understand the extent to which exchange of the BCR receptor in the DZ following SHM is facilitated by control of BCR mRNA levels. This work was in part prompted by important findings from the Neuberger group a number of years ago that antibody mRNAs have much increased half-lives in plasma cells to facilitate antibody production[395], therefore we hypothesised that the opposite may be true in GCs.

There were two models of exchange of pre-SHM BCR mRNA, which mirror the models of exchange of the BCR protein. The first model GC B cells may reduce *Igh* and *Igl* transcription, or induce rapid mRNA turnover, during and following SHM in DZs. The latter of these possibilities is perhaps analogous to the BCR protein "purging" model described in other sections of this thesis. The second model involves the SHM event being associated with a purging of pre-SHM BCR mRNA from the cell, similar to the possible purging of pre-SHM BCR protein from the surface of cells. In this model it was conceivable that the SHM event is associated with a cessation of BCR transcription, which allows for the pre-SHM BCR mRNA to be removed following SHM. It was possible that the purging of pre-SHM BCR mRNA was co-ordinated with the possible purging of BCR protein from the surface of DZ cells, and so this single cell qPCR was performed on indexed sorted cells to correlate changes in surface protein levels with mRNA levels.

To begin to address the possibility that following the SHM event there was a clearance of pre-SHM BCR mRNA before it was replaced with new, post-SHM BCR mRNA, we performed single cell qRT-PCR on index sorted GC B cells, using the Fluidigm Biomark platform. If we were to discover an observable frequency of cells in the GC that have undetectable levels of BCR mRNA, this might support the concepts outlined above being correct. In addition to their "productive" *Igh* transcript that is derived from the productively rearranged BCR allele, there is also often detectable "sterile transcript", which is mRNA transcribed from the unproductively recombined alternate allele[396]. To avoid the problem of detecting sterile transcript, we used SW_{HEL} GCs that were heterozygous for the HEL specific heavy chain. Furthermore, we designed two sets of primer probes for each gene to overcome the additional concerns that isotype class switching may impact detection, or that SHM might damage primer/probe binding sites. Two sets of probes were designed, one spanning from the variable region into the IgG2b constant region, and one restricted to the variable region (Figure 7.4a). The concern with performing qPCR on the BCR heavy chain is that a significant proportion of GC cells are have undergone class switch recombination. BCR^{high} cells can be gated on the basis of their isotype during the FACS sort, but the BCR^{low} cells cannot as they have undetectable surface BCR and so it is unclear which isotype they are using. Alternatively, restricting the qPCR probes to the variable region runs the dual risk of a false positive after amplifying un-degraded genomic DNA, and of a false negative if the SHM yields the template too far deviating from the probes. With these limitations in mind, we proceeded with two sets of probes, one spanning from the variable region into the IgG1 constant region, and one restricted to the variable region (Figure 7.4a). We found that all cells gave a signal above background for at least one of the probes (Figure 7.4b). Furthermore BCR-encoding transcripts were not lower in the $CXCR4^{high}$ BCR^{low}

as compared to other GC B cell populations (Figure 7.4c-g). While the range of levels of BCR transcription was expansive, and may speak to dynamic regulation of BCR mRNA in the GC, it was not consistent with a model of GC B cells directing the exchange of the BCR protein in the DZ by purging cells of pre-SHM BCR mRNA following SHM.

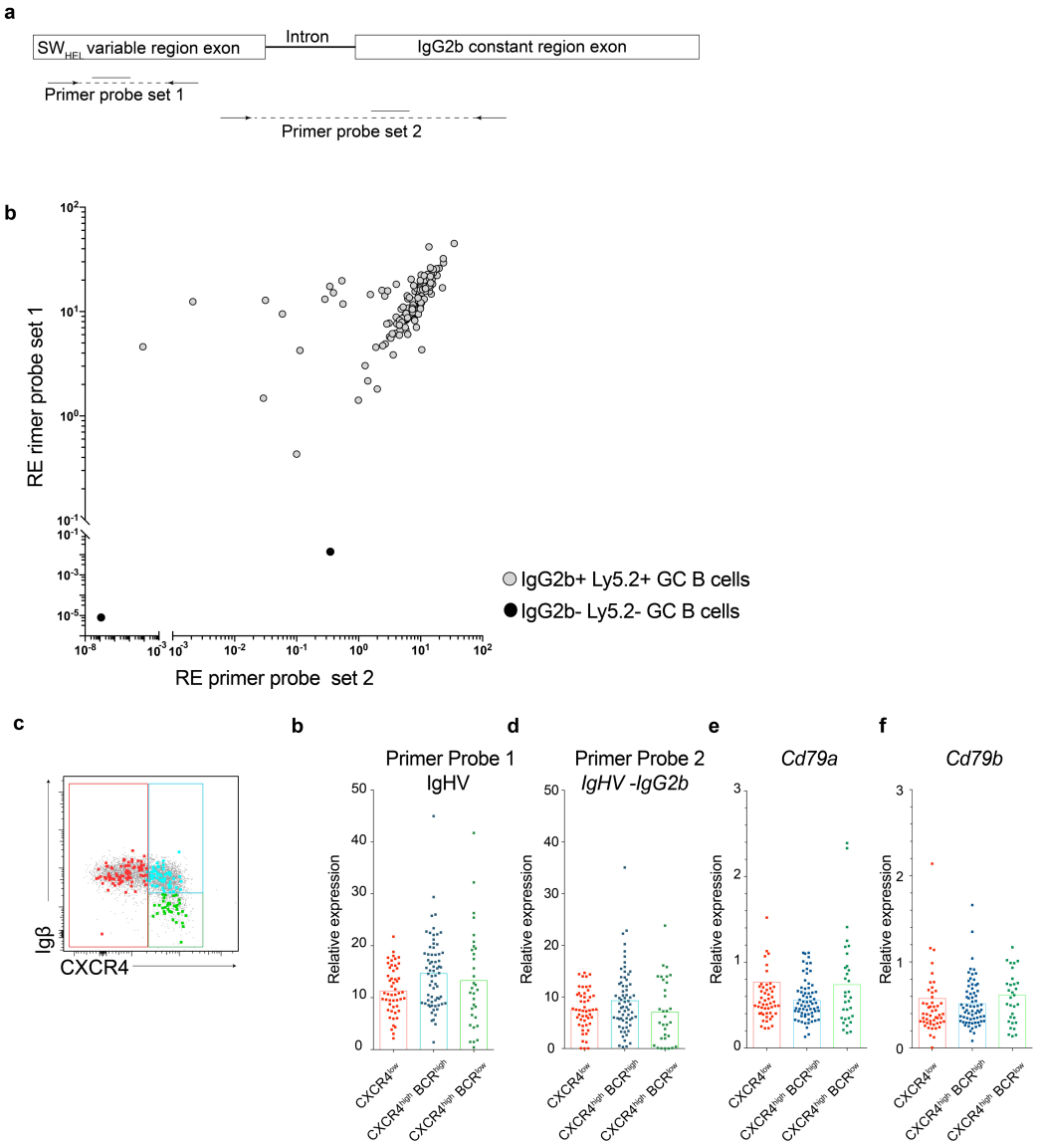


FIGURE 7.4| CELLS DO NOT APPEAR TO BE PURGING PRE-SHM BCR MRNA

(a) Schematic of primer probes used to quantify expression of the SW_{HEL} BCR mRNA (b, c) SW_{HEL} GCs were established as previously described earlier, and analysed on day seven. Individual IgD^{low} CD95+ GL7+ Ly5.2+ cells, which were gated either as BCR^{low} or BCR^{high} IgG2b+ were sorted, and mRNA expression of BCR components was analysed. (b) Plot of SW_{HEL} Ly5.2+ cells and WT Ly5.1- cells expression of the two SW_{HEL} heavy chain primer probes against one another. (c) Expression of different BCR components in $CXCR4^{low}$, $CXCR4^{high}$ BCR^{high}, $CXCR4^{high}$ BCR^{low} populations.

Therefore, we sought to identify cells that have recently undergone SHM and contain mRNAs transcribed from their pre- and post-SHM *Igh* genes. To this end, we optimised an approach for reverse transcribing mRNAs from single cells and making libraries for to assess the frequency of the second most abundant *Igh* sequence in each cell. We performed this single cell sequencing on indexed sorted cells, such that we could correlate the co-expression of pre- and post- SHM BCR mRNA with surface expression of BCR protein. We had previously found that the BCR^{low} population depends upon AID for its generation (Figure 3.4). We had also identified cells which encode destroyed BCR genes, but express an intact BCR on their surface (Figure 3.7). Therefore, if cells carrying the pre- and post-SHM BCR were restricted to the BCR^{high} population, then one could assume that the pre-SHM BCR mRNA is removed as quickly, or more quickly than the pre-SHM BCR surface protein is removed from the surface. However, if cells carrying both pre- and post-SHM BCR are present in both the BCR^{high} and BCR^{low} DZ populations, then this is consistent with a model wherein the pre-SHM is removed from the cytoplasm more slowly than the pre-SHM BCR is removed from the surface of the cell.

Libraries were sequenced using long dual end approaches on an Illumina Miseq machine and the results were interrogated for the frequency of the second most abundant read sequence for each cell. The second read must use the same V, D, and J regions as the first to ensure they both come from the same allele. *Aicda*$^{-/-}$ GC B cells were used as a control, to set a threshold for the second read which would be representative of the SHM event, and not simply of a PCR error. A problem with this technique was that we observed high background frequencies of second reads even in the *Aicda*$^{-/-}$ cells (Figure 7.5a). Therefore, we needed to set a high threshold for the frequency of cells with "real" second transcripts. However, such cells were restricted to DZs, supporting that the

"real" signal was probably genuine (Figure 7.5b). When we next looked in more detail at the phenotype of "presumed post-SHM cells", or PPSHMCs, we observed that they were similarly abundant in BCR^{high} and BCR^{low} populations, being present in both populations at a frequency of ~6% (Figure 7.5c).

Regardless of whether BCR exchange in the GC involves a surface protein "purging" event, BCR^{low} stop $codon^{+}$ populations contain cells that have removed surface BCR following SHM. Therefore, if the BCR^{low} cells still have measurable levels of pre-SHM BCR mRNA, then the pre-SHM BCR must be removed more slowly than pre-SHM BCR surface protein. This apparent slow loss of pre-SHM immunoglobulin gene transcript is seemingly inconsistent with a model in which the exchange of the BCR following SHM is facilitated by tight control of the mRNAs. Instead, these findings are more consistent with a model in which exchange of the BCR is facilitated by post-translation control of protein expression. However, the levels of background signal in the *Aicda*$^{-/-}$ GC cells were high, and this limits the conclusiveness of these results. To address this question more directly, there may be benefit labelling the individual mRNA molecules with unique molecular identifiers (UMIs), such that PCR errors could be disentangled from genuine sequence differences between different mRNA molecules[397–399]. We attempted to do so but suffered multiple technical failures that lead us to abandon this approach. However, to address the exact relationship a further examination of the relationship between co-expression of pre- and post-SHM BCR mRNA and surface protein levels is likely necessary. Recently released 10x genomics murine VDJ sequencing kits do contain UMI technology and so may represent one means of advancing these studies.

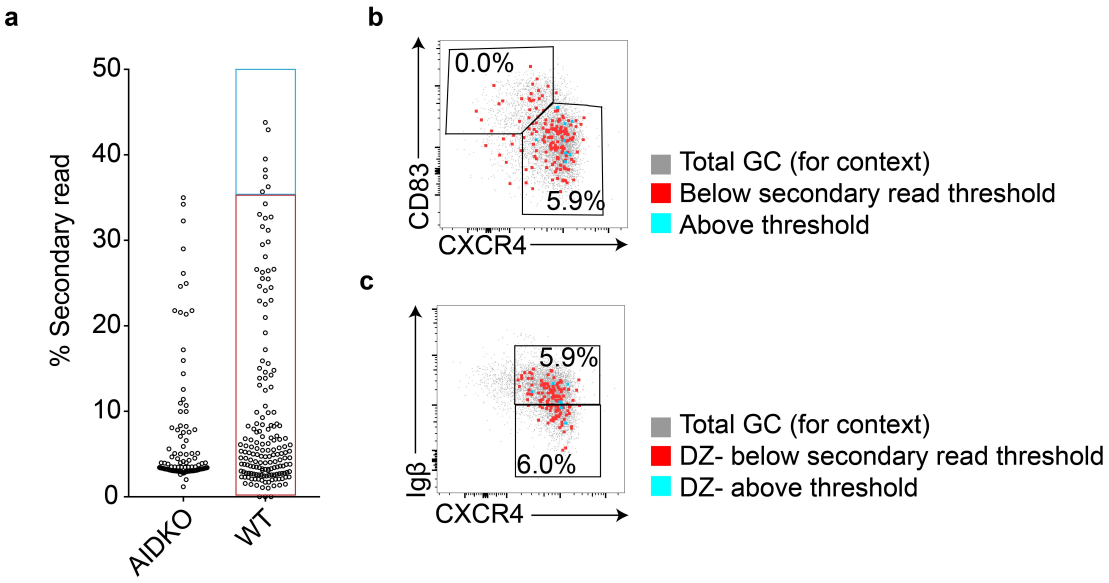


FIGURE 7.5| CELLS CONTAINING BOTH PRE- AND POST-SHM MRNA BCR ARE FOUND IN BOTH BCR^{HIGH} AND BCR^{LOW} DARK ZONE COMPARTMENTS

WT and *Aicda*-/- Mice were immunised with SRBCs and splenic IgD^{low} CD95^{+} GL7^{+} GC B cells were analysed on day 7; in WT mice BCR^{high} Pan IgG+ cell and BCR^{low} cells were selected; the IgG mRNA (in the case of the WT) and IgM mRNA (in the case of the *Aicda*$^{-/-}$) was amplified, genetically indexed, and sequenced using NGS, the frequency of the second most common read for each cell was quantified. (a) Frequency of second most common read in next generation sequencing. (b) Distribution of cells passing and not passing threshold for second most frequency read in DZ and LZ gates (c)Surface BCR fluorescent intensity of cells passing and not passing threshold for second most frequent read. (n=1)

BIBLIOGRAPHY

1. Haynes, B. F. Cardiolipin Polyspecific Autoreactivity in Two Broadly Neutralizing HIV-1 Antibodies. *Science (80-.).* **308**, 1906–1908 (2005).

2. Pinschewer, D. D. *et al.* Kinetics of protective antibodies are determined by the viral surface antigen. *J. Clin. Invest.* **114**, 988–993 (2004).

3. Wyatt, R. *et al.* The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* **393**, 705–11 (1998).

4. Gristick, H. B. *et al.* Natively glycosylated HIV-1 Env structure reveals new mode for antibody recognition of the CD4-binding site. *Nat. Struct. Mol. Biol.* **23**, 906–915 (2016).

5. Nemazee, D. & Weigert, M. Revising B Cell Receptors. *J Exp Med* **191**, 1813–1817 (2000).

6. Eisen, H. N. & Siskind, G. W. Variations in affinities of antibodies during the immune response. *Biochemistry* **3**, 996–1008 (1964).

7. Eisen, H. N. Affinity Enhancement of Antibodies: How Low-Affinity Antibodies Produced Early in Immune Responses Are Followed by High-Affinity Antibodies Later and in Memory B-Cell Responses. *Cancer Immunol. Res.* **2**, 381–392 (2014).

8. Bannard, O. & Cyster, J. G. Germinal centers: programmed for affinity maturation and antibody diversification. *Curr. Opin. Immunol.* **45**, 21–30 (2017).

9. Victora, G. D. & Nussenzweig, M. C. Germinal centers. *Annu. Rev. Immunol.* **30**, 429–57 (2012).

10. Scheid, J. F. *et al.* Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science (80-.).* **333**, 1633–7 (2011).

11. Wu, X. *et al.* Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing. *Science (80-.).* **333**, 1593–602 (2011).

12. Lingwood, D. *et al.* Structural and genetic basis for development of broadly neutralizing influenza antibodies. *Nature* **489**, 566–570 (2012).

13. Pappas, L. *et al.* Rapid development of broadly influenza neutralizing antibodies through redundant mutations. *Nature* **516**, 418–422 (2014).

14. Tan, J. *et al.* A LAIR-1 insertion generates broadly reactive antibodies against malaria variant antigens. *Nature* 1–18 (2015). doi:10.1038/nature16450

15. Verkoczy, L. *et al.* Autoreactivity in an HIV-1 broadly reactive neutralizing antibody variable region heavy chain induces immunologic tolerance. *Proc. Natl. Acad. Sci.* **107**, 181–186 (2010).

16. Bonsignori, M. *et al.* Maturation Pathway from Germline to Broad HIV-1 Neutralizer of a CD4-Mimic Antibody. *Cell* **165**, 449–463 (2016).

17. Sok, D. & Burton, D. R. HIV Broadly Neutralizing Antibodies: Taking Good Care Of The 98%. *Immunity* **45**, 958–960 (2016).

18. Pleiman, C. M., D'Ambrosio, D. & Cambier, J. C. The B-cell antigen receptor complex: structure and signal transduction. *Immunol. Today* **15**, 393–9 (1994).

19. Bretscher, P. & Cohn, M. A theory of self-nonself discrimination: paralysis and induction involve the recognition of one and two determinants on an antigen, respectively. *Science* **169**, 1042–9 (1970).

20. Liu, Y. J., Zhang, J., Lane, P. J., Chan, E. Y. & MacLennan, I. C. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur. J. Immunol.* **21**, 2951–62 (1991).

21. K., R. *et al.* Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* **416**, 94–99 (2002).

22. Okada, T. *et al.* Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol.* **3**, 1047–1061 (2005).

23. Strasser, A., Jost, P. J. & Nagata, S. The Many Roles of FAS Receptor Signaling in the Immune System. *Immunity* **30**, 180–192 (2009).

24. Rathmell, J. C., Townsend, S. E., Xu, J. C., Flavell, R. A. & Goodnow, C. C. Expansion or elimination of B cells in vivo: Dual roles for CD40- and Fas (CD95)-ligands modulated by the B cell antigen receptor. *Cell* **87**, 319–329 (1996).

25. Rathmell, J. C., Fournier, S., Weintraub, B. C., Allison, J. P. & Goodnow, C. C. Repression of B7.2 on Self-reactive B Cells Is Essential to Prevent Proliferation and Allow Fas-mediated Deletion by CD4 + T Cells . *J. Exp. Med.* **188**, 651–659 (2002).

26. Fooksman, D. R. *et al.* Development and Migration of Plasma Cells in the Mouse Lymph Node. *Immunity* **33**, 118–127 (2010).

27. Ise, W. *et al.* T Follicular Helper Cell-Germinal Center B Cell Interaction Strength Regulates Entry into Plasma Cell or Recycling Germinal Center Cell Fate. *Immunity* **48**, 702-715.e4 (2018).

28. Cyster, J. G. & Allen, C. D. C. B Cell Responses: Cell Interaction Dynamics and Decisions. *Cell* **177**, 524–540 (2019).

29. Cyster, J. G. B cell follicles and antigen encounters of the third kind. *Nat. Immunol.* **11**, 989–996 (2010).

30. Jacob, J. In situ studies of the primary immune response to (4-hydroxy-3- nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. *J. Exp. Med.* **176**, 679–687 (2004).

31. Chan, T. D. *et al.* Antigen Affinity Controls Rapid T-Dependent Antibody Production by Driving the Expansion Rather than the Differentiation or Extrafollicular Migration of Early Plasmablasts. *J. Immunol.* **183**, 3139–3149 (2009).

32. Nieuwenhuis, P. & Opstelten, D. Functional anatomy of germinal centers. *Am. J. Anat.* **170**, 421–435 (1984).

33. Jacob, J., Kelsoe, G., Rajewsky, K. & Weiss, U. Intraclonal generation of antibody mutants in germinal centres. *Nature* **354**, 389–392 (1991).

34. Hanna, M. G. An Autoradiographic and Histologic Study of Spleen White Pulp Germinal Centers During Early Intervals of the Primary Immune Response. *ORINS [reports]. U.S. Atomic Energy Commission* **34**, 1–102 (1964).

35. Park, C. S. & Choi, Y. S. How do follicular dendritic cells interact intimately with B cells in the germinal centre? *Immunology* **114**, 2–10 (2005).

36. Haberman, A. M. & Shlomchik, M. J. Reassessing the function of immune-complex retention by follicular dendritic cells. *Nat. Rev. Immunol.* **3**, 757–764 (2003).

37. Suzuki, K., Grigorova, I., Phan, T. G., Kelly, L. M. & Cyster, J. G. Visualizing B cell capture of cognate antigen from follicular dendritic cells. *J. Exp. Med.* **206**, 1485–1493 (2009).

38. Qin, D. *et al.* Fcγ Receptor IIB on Follicular Dendritic Cells Regulates the B Cell Recall Response. *J. Immunol.* **164**, 6268–6275 (2000).

39. Liu, Y. J. *et al.* Follicular dendritic cells specifically express the long CR2/CD21 isoform. *J. Exp. Med.* **185**, 165–170 (1997).

40. Allen, C. D. C. *et al.* Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat. Immunol.* **5**, 943–952 (2004).

41. Wang, X. *et al.* Follicular dendritic cells help establish follicle identity and promote B cell retention in germinal centers. *J. Exp. Med.* **208**, 2497–2510 (2011).

42. Green, J. A. & Cyster, J. G. S1PR2 links germinal center confinement and growth regulation. *Immunol. Rev.* **247**, 36–51 (2012).

43. Green, J. A. *et al.* The sphingosine 1-phosphate receptor S1P2 maintains the homeostasis of germinal center B cells and promotes niche confinement. *Nat. Immunol.* **12**, 672–80 (2011).

44. Bannard, O. *et al.* Germinal Center Centroblasts Transition to a Centrocyte Phenotype According to a Timed Program and Depend on the Dark Zone for Effective Selection. *Immunity* **39**, 912–924 (2013).

45. Gitlin, A. D., Shulman, Z. & Nussenzweig, M. C. Clonal selection in the germinal centre by regulated proliferation and hypermutation. *Nature* **509**, 637–40 (2014).

46. Caron, G., Le Gallou, S., Lamy, T., Tarte, K. & Fest, T. CXCR4 expression functionally discriminates centroblasts versus centrocytes within human germinal center B cells. *J. Immunol.* **182**, 7595–7602 (2009).

47. Victora, G. D. *et al.* Identification of human germinal center light and dark zone cells and their relationship to human B-cell lymphomas. *Blood* **120**, 2240–2248 (2012).

48. Chaudhuri, J. *et al.* Transcription-targeted DNA deamination by the AID antibody diversification enzyme. *Nature* **422**, 726–730 (2003).

49. Xu, Z. *et al.* Regulation of aicda expression and AID activity: relevance to somatic hypermutation and class switch DNA recombination. *Crit. Rev. Immunol.* **27**, 367–97 (2007).

50. Phan, R. T. & Dalla-Favera, R. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* **432**, 635–639 (2004).

51. Phan, R. T., Saito, M., Basso, K., Niu, H. & Dalla-Favera, R. BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. *Nat. Immunol.* **6**, 1054–1060 (2005).

52. Ranuncolo, S. M., Polo, J. M. & Melnick, A. BCL6 represses CHEK1 and suppresses DNA damage pathways in normal and malignant B-cells. *Blood Cells, Mol. Dis.* **41**, 95–99 (2008).

53. Basu, U. *et al.* The AID antibody diversification enzyme is regulated by protein kinase A phosphorylation. *Nature* **438**, 508–511 (2005).

54. Pasqualucci, L., Kitaura, Y., Gu, H. & Dalla-Favera, R. PKA-mediated phosphorylation regulates the function of activation-induced deaminase (AID) in B cells. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 395–400 (2006).

55. Durandy, A., Peron, S., Taubenheim, N. & Fischer, A. Activation-induced cytidine deaminase: structure-function relationship as based on the study of mutants. *Hum. Mutat.* **27**, 1185–91 (2006).

56. Ranuncolo, S. M. *et al.* Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. *Nat. Immunol.* **8**, 705–714 (2007).

57. Ito, S. *et al.* Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 1975–80 (2004).

58. Chaudhuri, J., Khuong, C. & Alt, F. W. Replication protein A interacts with AID to promote deamination of somatic hypermutation targets. *Nature* **430**, 992–998 (2004).

59. Petersen-Mahrt, S. K., Harris, R. S. & Neuberger, M. S. AID mutates E. coli suggesting a DNA deamination mechanism for antibody diversification. *Nature* **418**, 99–103 (2002).

60. Ramiro, A. R., Stavropoulos, P., Jankovic, M. & Nussenzweig, M. C. Transcription enhances AID-mediated cytidine deamination by exposing single-stranded DNA on the nontemplate strand. *Nat. Immunol.* **4**, 452–456 (2003).

61. Bransteitter, R., Pham, P., Scharfft, M. D. & Goodman, M. F. Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 4102–4107 (2003).

62. Pham, P., Bransteitter, R., Petruska, J. & Goodman, M. F. Processive AID-catalysed cytosine deamination on single-stranded DNA simulates somatic hypermutation. *Nature* **424**, 103–107 (2003).

63. Dickerson, S. K., Market, E., Besmer, E. & Papavasiliou, F. N. AID mediates hypermutation by deaminating single stranded DNA. *J. Exp. Med.* **197**, 1291–1296 (2003).

64. Sohail, A., Klapacz, J., Samaranayake, M., Ullah, A. & Bhagwat, A. S. Human activation-induced cytidine deaminase causes transcription-dependent, strand-biased C to U deaminations. *Nucleic Acids Res.* **31**, 2990–2994 (2003).

65. Peters, A. & Storb, U. Somatic hypermutation of immunoglobulin genes is linked to transcription initiation. *Immunity* **4**, 57–65 (1996).

66. Kunkel, T. A., Pavlov, Y. I. & Bebenek, K. Functions of human DNA polymerases η, κ and ι

suggested by their properties, including fidelity with undamaged DNA templates. *DNA Repair (Amst).* **2**, 135–149 (2003).

67. Pavlov, Y. I. *et al.* Correlation of somatic hypermutation specificity and A-T base pair substitution errors by DNA polymerase η during copying of a mouse immunoglobulin κ light chain transgene. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 9954–9959 (2002).

68. Rogozin, I. B., Pavlov, Y. I., Bebenek, K., Matsuda, T. & Kunkel, T. A. Somatic mutation hotspots correlate with DNA polymerase η error spectrum. *Nat. Immunol.* **2**, 530–536 (2001).

69. Wilson, T. M. *et al.* MSH2-MSH6 stimulates DNA polymerase η, suggesting a role for A:T mutations in antibody genes. *J. Exp. Med.* **201**, 637–645 (2005).

70. Diaz, M., Verkoczy, L. K., Flajnik, M. F. & Klinman, N. R. Decreased frequency of somatic hypermutation and impaired affinity maturation but intact germinal center formation in mice expressing antisense RNA to DNA polymerase zeta. *J. Immunol.* **167**, 327–35 (2001).

71. Zan, H. *et al.* The translesion DNA polymerase ζ plays a major role in Ig and bcl-6 somatic hypermutation. *Immunity* **14**, 643–653 (2001).

72. Faili, A. *et al.* Induction of somatic hypermutation in immunoglobulin genes is dependent on DNA polymerase iota. *Nature* **419**, 944–947 (2002).

73. McDonald, J. P. *et al.* 129-Derived strains of mice are deficient in DNA polymerase ι and have normal immunoglobulin hypermutation. *J. Exp. Med.* **198**, 635–643 (2003).

74. Yeap, L. *et al.* Sequence-Intrinsic Mechanisms that Target AID Mutational Outcomes on Antibody Genes. *Cell* 1–14 (2015). doi:10.1016/j.cell.2015.10.042

75. Muramatsu, M., Nagaoka, H., Shinkura, R., Begum, N. A. & Honjo, T. Discovery of activation-induced cytidine deaminase, the engraver of antibody memory. *Adv. Immunol.* **94**, 1–36 (2007).

76. Pham, P., Bransteitter, R., Petruska, J. & Goodman, M. F. Processive AID-catalysed cytosine deamination on single-stranded DNA simulates somatic hypermutation. *Nature* **424**, 103–107 (2003).

77. Yu, K., Huang, F. T. & Lieber, M. R. DNA Substrate Length and Surrounding Sequence Affect the Activation-induced Deaminase Activity at Cytidine. *J. Biol. Chem.* **279**, 6496–6500 (2004).

78. Rogozin, I. B. & Diaz, M. Cutting Edge: DGYW/WRCH Is a Better Predictor of Mutability at G:C Bases in Ig Hypermutation Than the Widely Accepted RGYW/WRCY Motif and Probably Reflects a Two-Step Activation-Induced Cytidine Deaminase-Triggered Process. *J. Immunol.* **172**, 3382–3384 (2004).

79. Zheng, N. Y., Wilson, K., Jared, M. & Wilson, P. C. Intricate targeting of immunoglobulin somatic hypermutation maximizes the efficiency of affinity maturation. *J. Exp. Med.* **201**, 1467–1478 (2005).

80. Amzel, L. M. & Poljak, R. J. Three-dimensional structure of immunoglobulins. *Annu. Rev. Biochem.* **48**, 961–97 (1979).

81. Jones, P. T., Dear, P. H., Foote, J., Neuberger, M. S. & Winter, G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* **321**, 522–525 (1986).

82. Wu, T. T. & Kabat, E. A. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J. Exp. Med.* **132**, 211–50 (1970).

83. Reynaud, C. A., Garcia, C., Hein, W. R. & Weill, J. C. Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process. *Cell* **80**, 115–125 (1995).

84. Wagner, S. D., Milstein, C. & Neuberger, M. S. Codon bias targets mutation. *Nature* **376**, 732 (1995).

85. Jolly, C. J. *et al.* The targeting of somatic hypermutation. *Semin. Immunol.* **8**, 159–168 (1996).

86. Kepler, T. B. Codon bias and plasticity in immunoglobulins. *Mol. Biol. Evol.* **14**, 637–643 (1997).

87. Kleinstein, S. H., Louzoun, Y. & Shlomchik, M. J. Estimating hypermutation rates from clonal tree data. *J. Immunol.* **171**, 4639–4649 (2003).

88. McKean, D. *et al.* Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. *Proc. Natl. Acad. Sci.* **81**, 3180–3184 (1984).

89. Heesters, B. A., Myers, R. C. & Carroll, M. C. Follicular dendritic cells: Dynamic antigen libraries. *Nat. Rev. Immunol.* **14**, 495–504 (2014).

90. Heesters, B. A. *et al.* Endocytosis and recycling of immune complexes by follicular dendritic cells enhances B cell antigen binding and activation. *Immunity* **38**, 1164–75 (2013).

91. Rothaeusler, K. & Baumgarth, N. B-cell fate decisions following influenza virus infection. *Eur. J. Immunol.* **40**, 366–377 (2010).

92. Moyron-Quiroz, J. E. *et al.* Persistence and Responsiveness of Immunologic Memory in the Absence of Secondary Lymphoid Organs. *Immunity* **25**, 643–654 (2006).

93. Cirelli, K. M. *et al.* Slow Delivery Immunization Enhances HIV Neutralizing Antibody and Germinal Center Responses via Modulation of Immunodominance. *Cell* **177**, 1153-1171.e28 (2019).

94. Krzyzak, L. *et al.* CD83 Modulates B Cell Activation and Germinal Center Responses. *J. Immunol.* (2016). doi:10.4049/jimmunol.1502163

95. Celada, F. & Seiden, P. E. Affinity maturation and hypermutation in a simulation of the humoral immune response. *Eur. J. Immunol.* **26**, 1350–1358 (1996).

96. KLEINSTEIN, S. H. & SINGH, J. P. Toward Quantitative Simulation of Germinal Center Dynamics: Biological and Modeling Insights from Experimental Validation. *J. Theor. Biol.* **211**, 253–275 (2001).

97. Nowosad, C. R., Spillane, K. M. & Tolar, P. Germinal center B cells recognize antigen through a specialized immune synapse architecture. *Nat. Immunol.* 1–11 (2016). doi:10.1038/ni.3458

98. Batista, F. D. & Neuberger, M. S. Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. *Immunity* **8**, 751–9 (1998).

99. Foote, J. & Milstein, C. Kinetic maturation of an immune response. *Nature* **352**, 530–2 (1991).

100. Victora, G. D. *et al.* Germinal Center Dynamics Revealed by Multiphoton Microscopy with a Photoactivatable Fluorescent Reporter. *Cell* **143**, 592–605 (2010).

101. Allen, C. D. C., Okada, T., Tang, H. L. & Cyster, J. G. Imaging of germinal center selection events during affinity maturation. *Science (80-.).* **315**, 528–531 (2007).

102. Liu, Y.-J. *et al.* Mechanism of antigen-driven selection in germinal centres. *Nature* **342**, 929–931 (1989).

103. Paus, D. *et al.* Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J. Exp. Med.* **203**, 1081–91 (2006).

104. Phan, T. G. *et al.* High affinity germinal center B cells are actively selected into the plasma cell compartment. *J. Exp. Med.* **203**, 2419–2424 (2006).

105. Brink, R., Phan, T. G., Paus, D. & Chan, T. D. Visualizing the effects of antigen affinity on T-dependent B-cell differentiation. *Immunol. Cell Biol.* **86**, 31–39 (2008).

106. Khalil, A. M., Cambier, J. C. & Shlomchik, M. J. B Cell Receptor Signal Transduction in the GC Is Short-Circuited by High Phosphatase Activity. *Science (80-.).* **336**, 1178–1181 (2012).

107. Luo, W. *et al.* The AKT kinase signaling network is rewired by PTEN to control proximal BCR signaling in germinal center B cells. *Nat. Immunol.* (2019). doi:10.1038/s41590-019-0376-3

108. Zhang, J., Somani, A. K. & Siminovitch, K. A. Roles of the SHP-1 tyrosine phosphatase in the negative regulation of cell signalling. *Semin. Immunol.* **12**, 361–378 (2000).

109. Zhang, Z., Shen, K., Lu, W. & Cole, P. A. The role of C-terminal tyrosine phosphorylation in the regulation of SHP-1 explored via expressed protein ligation. *J. Biol. Chem.* **278**, 4668–4674 (2003).

110. Bannard, O. M. & Cyster, J. G. When Less Signaling Is More. *Science (80-.).* **336**, 1120–1121 (2012).

111. Huntington, N. D. *et al.* CD45 links the B cell receptor with cell survival and is required for the persistence of germinal centers. *Nat. Immunol.* **7**, 190–198 (2006).

112. Shulman, Z. *et al.* Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science (80-.).* **345**, 1058–1062 (2014).

113. Watanabe, M. *et al.* Co-stimulatory function in primary germinal center responses: CD40 and B7 are required on distinct antigen-presenting cells. *J. Exp. Med.* jem.20161955 (2017). doi:10.1084/jem.20161955

114. Takahashi, Y., Dutta, P. R., Cerasoli, D. M. & Kelsoe, G. In Situ Studies of the Primary Immune Response to (4-Hydroxy-3-Nitrophenyl)Acetyl. V. Affinity Maturation Develops in Two Stages of Clonal Selection. *J. Exp. Med.* **187**, 885 LP – 895 (1998).

115. Renshaw, B. B. R. *et al.* Humoral Immune Responses in CD40 Ligand-deficient Mice Flow Cytometric Analysis of Cell Surface Ag Expression . Lym-. *Cell* **180**, (1994).

116. Kawabe, T. *et al.* The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity* **1**, 167–78 (1994).

117. Xu, J. *et al.* Mice deficient for the CD40 ligand. *Immunity* **1**, 423–31 (1994).

118. Bonifaz, L. *et al.* Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J. Exp. Med.* **196**, 1627–1638 (2002).

119. Allen, C. D. C. & Okada, T. Supplementary figuresL Imaging of Germinal Center Selection. *Science (80-.).* **315**, 528–531 (2007).

120. Gitlin, A. D. *et al.* T cell help controls the speed of the cell cycle in germinal center B cells. *Science (80-.).* **349**, 1–8 (2015).

121. Schwickert, T. a. *et al.* A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *J. Exp. Med.* **208**, 1243–1252 (2011).

122. Hou, P. *et al.* B Cell Antigen Receptor Signaling and Internalization Are Mutually Exclusive Events. *PLoS Biol.* **4**, e200 (2006).

123. Yuseff, M.-I., Pierobon, P., Reversat, A. & Lennon-Duménil, A.-M. How B cells capture, process and present antigens: a crucial role for cell polarity. *Nat. Rev. Immunol.* **13**, 475–486 (2013).

124. Mueller, J., Matloubian, M. & Zikherman, J. Cutting edge: An in vivo reporter reveals active B cell receptor signaling in the germinal center. *J. Immunol.* **194**, 2993–7 (2015).

125. Zikherman, J., Parameswaran, R. & Weiss, A. Endogenous antigen tunes the responsiveness of naive B cells but not T cells. *Nature* **489**, 160–4 (2012).

126. Minamitani, T. *et al.* Evasion of affinity-based selection in germinal centers by Epstein-Barr virus LMP2A. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 11612–7 (2015).

127. Shlomchik, M. J., Luo, W. & Weisel, F. Linking signaling and selection in the germinal center. *Immunol. Rev.* **288**, 49–63 (2019).

128. Dominguez-Sola, D. *et al.* The proto-oncogene MYC is required for selection in the germinal center and cyclic reentry. *Nat. Immunol.* **13**, 1083–91 (2012).

129. Calado, D. P. *et al.* The cell-cycle regulator c-Myc is essential for the formation and maintenance of germinal centers. *Nat. Immunol.* **13**, 1092–100 (2012).

130. Luo, W., Weisel, F. & Shlomchik, M. J. B Cell Receptor and CD40 Signaling Are Rewired for Synergistic Induction of the c-Myc Transcription Factor in Germinal Center B Cells. *Immunity* **48**, 313-326.e5 (2018).

131. Turner, J. S., Ke, F. & Grigorova, I. L. B Cell Receptor Crosslinking Augments Germinal Center B Cell Selection when T Cell Help Is Limiting. *Cell Rep.* **25**, 1395-1403.e4 (2018).

132. Dang, C. V. MYC on the path to cancer. *Cell* **149**, 22–35 (2012).

133. Bretones, G., Delgado, M. D. & León, J. Myc and cell cycle control. *Biochim. Biophys. Acta - Gene Regul. Mech.* **1849**, 506–516 (2015).

134. Finkin, S., Hartweger, H., Oliveira, T. Y., Kara, E. E. & Nussenzweig, M. C. Protein Amounts of the MYC Transcription Factor Determine Germinal Center B Cell Division Capacity. *Immunity* 1–13 (2019). doi:10.1016/j.immuni.2019.06.013

135. Sander, S. *et al.* PI3 Kinase and FOXO1 Transcription Factor Activity Differentially Control B Cells in the Germinal Center Light and Dark Zones. *Immunity* 1–12 (2015). doi:10.1016/j.immuni.2015.10.021

136. Dominguez-Sola, D. *et al.* The FOXO1 Transcription Factor Instructs the Germinal Center Dark Zone Program. *Immunity* 1–11 (2015). doi:10.1016/j.immuni.2015.10.015

137. Inoue, T. *et al.* The transcription factor Foxo1 controls germinal center B cell proliferation in response to T cell help. *J. Exp. Med.* **214**, 1181–1198 (2017).

138. Chou, C. *et al.* The Transcription Factor AP4 Mediates Resolution of Chronic Viral Infection through Amplification of Germinal Center B Cell Responses Article The Transcription Factor AP4 Mediates Resolution of Chronic Viral Infection through Amplification of Germinal Cen. *Immunity* 1–13 (2016). doi:10.1016/j.immuni.2016.07.023

139. Linterman, M. A. *et al.* IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J. Exp. Med.* **207**, 353–363 (2010).

140. Ersching, J. *et al.* Germinal Center Selection and Affinity Maturation Require Dynamic Regulation of mTORC1 Kinase Germinal Center Selection and Affinity Maturation Require Dynamic Regulation of mTORC1 Kinase. *Immunity* **46**, 1045-1058.e6 (2017).

141. Barbet, N. C. *et al.* TOR controls translation initiation and early G1 progression in yeast. *Mol. Biol. Cell* **7**, 25–42 (1996).

142. Dowling, R. J. O., Topisirovic, I., Fonseca, B. D. & Sonenberg, N. Dissecting the role of mTOR: Lessons from mTOR inhibitors. *Biochim. Biophys. Acta - Proteins Proteomics* **1804**, 433–439 (2010).

143. Reed, J. H., Jackson, J., Christ, D. & Goodnow, C. C. Clonal redemption of autoantibodies by somatic hypermutation away from self-reactivity during human immunization. *J. Exp. Med.* **213**, 1255–1265 (2016).

144. Burnett, D. L. *et al.* Germinal center antibody mutation trajectories are determined by rapid self/foreign discrimination. *Science (80-.).* **360**, 223–226 (2018).

145. Cornall, R. J., Goodnow, C. C. & Cyster, J. G. The regulation of self-reactive B cells. *Curr. Opin. Immunol.* **7**, 804–811 (1995).

146. O'Neill, S. K. *et al.* Endocytic sequestration of the B cell antigen receptor and toll-like receptor 9 in anergic cells. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 6262–7 (2009).

147. Duty, J. A. *et al.* Functional anergy in a subpopulation of naive B cells from healthy humans that express autoreactive immunoglobulin receptors. *J. Exp. Med.* **206**, 139–151 (2009).

148. Halverson, R., Torres, R. M. & Pelanda, R. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. *Nat. Immunol.* **5**, 645–650 (2004).

149. Nossal, G. J. V. Cellular Mechanisms of Immunologic Tolerance. *Annu. Rev. Immunol.* **1**, 33–62 (1983).

150. Mascola, J. R. & Haynes, B. F. HIV-1 neutralizing antibodies: understanding nature's pathways. *Immunol. Rev.* **254**, 225–44 (2013).

151. Shulman, Z. *et al.* Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science (80-.).* **345**, 1058–1062 (2014).

152. Tas, J. M. J. *et al.* Visualizing antibody affinity maturation in germinal centers. *Science (80-.).* **351**, 1048–1054 (2016).

153. Wakerley, B. R. & Yuki, N. Infectious and noninfectious triggers in Guillain-Barré syndrome. *Expert Review of Clinical Immunology* **9**, 627–639 (2013).

154. Dal Porto, J. M. Very Low Affinity B Cells Form Germinal Centers, Become Memory B Cells, and Participate in Secondary Immune Responses When Higher Affinity Competition Is Reduced. *J. Exp. Med.* **195**, 1215–1221 (2002).

155. Shih, T.-A. Y., Roederer, M. & Nussenzweig, M. C. Role of antigen receptor affinity in T cell-independent antibody responses in vivo. *Nat. Immunol.* **3**, 399–406 (2002).

156. Jacob, J., Przylepa, J., Miller, C. & Kelsoe, G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. hi. the kinetics of v region mutation and selection in germinal center b cells. *J. Exp. Med.* **178**, 1293–1307 (1993).

157. Baumgarth, N. How specific is too specific? B-cell responses to viral infections reveal the importance of breadth over depth. *Immunol. Rev.* **255**, 82–94 (2013).

158. Roost, H. P. *et al.* Early high-affinity neutralizing anti-viral IgG responses without further overall improvements of affinity. *Proc. Natl. Acad. Sci.* **92**, 1257–1261 (1995).

159. McGuire, A. T. *et al.* Specifically modified Env immunogens activate B-cell precursors of broadly neutralizing HIV-1 antibodies in transgenic mice. *Nat. Commun.* **7**, 10618 (2016).

160. Wong, R. & Bhattacharya, D. Basics of memory B-cell responses: lessons from and for the real world. *Immunology* **156**, 120–129 (2019).

161. Response, I. Respiratory virus infection of mice provokes a permanent humoral immune response. *J. Virol.* **68**, 6083–6086 (1994).

162. Jones, P. D. & Ada, G. L. Persistence of influenza virus-specific antibody-secreting cells and B-cell memory after primary murine influenza virus infection. *Cell. Immunol.* **109**, 53–64 (1987).

163. Hye, M. J., He, Y. & Sangster, M. Y. Broad dispersion and lung localization of virus-specific memory B cells induced by influenza pneumonia. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 3485–3490 (2008).

164. Takahashi, Y. Memory B cells in systemic and mucosal immune response: Implications for successful vaccination. *Biosci. Biotechnol. Biochem.* **71**, 2358–2366 (2007).

165. MacKay, F. & Schneider, P. Cracking the BAFF code. *Nat. Rev. Immunol.* **9**, 491–502 (2009).

166. Minnich, M. *et al.* Multifunctional role of the transcription factor Blimp-1 in coordinating plasma cell differentiation. *Nat. Immunol.* (2016). doi:10.1038/ni.3349

167. Angelin-Duclos, C., Cattoretti, G., Lin, K.-I. & Calame, K. Commitment of B Lymphocytes to a Plasma Cell Fate Is Associated with Blimp-1 Expression In Vivo. *J. Immunol.* **165**, 5462–5471 (2000).

168. Lau, D. *et al.* Low CD21 expression defines a population of recent germinal center graduates primed for plasma cell differentiation. *Sci. Immunol.* **2**, eaai8153 (2017).

169. Piskurich, J. F. *et al.* BLIMP-1 mediates extinction of major histocompatibility class II transactivator expression in plasma cells. *Nat. Immunol.* **1**, 526–532 (2000).

170. Lin, Y. Repression of c-myc Transcription by Blimp-1, an Inducer of Terminal B Cell Differentiation. *Science (80-.).* **276**, 596–599 (1997).

171. Lin, K.-I., Lin, Y. & Calame, K. Repression of c-myc Is Necessary but Not Sufficient for Terminal Differentiation of B Lymphocytes In Vitro. *Mol. Cell. Biol.* **20**, 8684–8695 (2000).

172. Ahmed, S., Radhakrishnan, N., Srinivasan, K. S. V. & Sanjeevi, R. Free radical graft copolymerization of poly(n-butyl methacrylate and poly(butyl acrylate) onto chlorinated rubber: Characterization and mechanical properties. *Adv. Polym. Technol.* **23**, 103–110 (2004).

173. Lin, K., Angelin-Duclos, C., Kuo, T. C. & Calame, K. Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *Mol. Cell. Biol.* **22**, 4771–80 (2002).

174. Tellier, J. *et al.* Blimp-1 controls plasma cell function through the regulation of immunoglobulin secretion and the unfolded protein response. *Nat. Immunol.* **17**, 323–330 (2016).

175. Minnich, M. *et al.* Multifunctional role of the transcription factor Blimp-1 in coordinating plasma cell differentiation. *Nat. Immunol.* **17**, 331–343 (2016).

176. Nutt, S. L., Hodgkin, P. D., Tarlinton, D. M. & Corcoran, L. M. The generation of antibody-secreting plasma cells. *Nat. Rev. Immunol.* **15**, 160–171 (2015).

177. Hibi, T. & Dosch, H. -M. Limiting dilution analysis of the B cell compartment in human bone marrow. *Eur. J. Immunol.* **16**, 139–145 (1986).

178. Iwakoshi, N. N., Lee, A. H. & Glimcher, L. H. The X-box binding protein-1 transcription factor is required for plasma cell differentiation and the unfolded protein response. *Immunol. Rev.* **194**, 29–38 (2003).

179. Reimold, A. M. *et al.* Transcription factor B cell lineage-specific activator protein regulates the gene for human X-box binding protein 1. *J. Exp. Med.* **183**, 393–401 (1996).

180. Reimold, A. M. *et al.* Plasma cell differentiation requires the transcription factor XBP-1. *Nature* **412**, 300–307 (2001).

181. Hu, C. C. A., Dougan, S. K., McGehee, A. M., Love, J. C. & Ploegh, H. L. XBP-1 regulates signal transduction, transcription factors and bone marrow colonization in B cells. *EMBO J.* **28**, 1624–1636 (2009).

182. Todd, D. J. *et al.* XBP1 governs late events in plasma cell differentiation and is not required for antigen-specific memory B cell development. *J. Exp. Med.* **206**, 2151–2159 (2009).

183. Taubenheim, N. *et al.* High Rate of Antibody Secretion Is not Integral to Plasma Cell Differentiation as Revealed by XBP-1 Deficiency. *J. Immunol.* **189**, 3328–3338 (2012).

184. Martincic, K., Alkan, S. A., Cheatle, A., Borghesi, L. & Milcarek, C. Transcription elongation factor ELL2 directs immunoglobulin secretion in plasma cells by stimulating altered RNA

processing. *Nat. Immunol.* **10**, 1102–1109 (2009).

185. Benson, M. J. *et al.* Heterogeneous nuclear ribonucleoprotein L-like (hnRNPLL) and elongation factor, RNA polymerase II, 2(ELL2) are regulators of mRNA processing in plasma cells. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 16252–16257 (2012).

186. Park, K. S. *et al.* Transcription Elongation Factor ELL2 Drives Ig Secretory-Specific mRNA Production and the Unfolded Protein Response. *J. Immunol.* **193**, 4663–4674 (2014).

187. Benhamron, S. *et al.* Regulated IRE1-dependent decay participates in curtailing immunoglobulin secretion from plasma cells. *Eur. J. Immunol.* **44**, 867–876 (2014).

188. Pengo, N. *et al.* Plasma cells require autophagy for sustainable immunoglobulin production. *Nat. Immunol.* **14**, 298–305 (2013).

189. Smith, K. G. *et al.* bcl-2 transgene expression inhibits apoptosis in the germinal center and reveals differences in the selection of memory B cells and bone marrow antibody-forming cells. *J. Exp. Med.* **191**, 475–84 (2000).

190. Kräutler, N. J. *et al.* Differentiation of germinal center B cells into plasma cells is initiated by high-affinity antigen and completed by Tfh cells. *J. Exp. Med.* **214**, 1259–1267 (2017).

191. Zhang, Y. *et al.* Plasma cell output from germinal centers is regulated by signals from Tfh and stromal cells. *J. Exp. Med.* **215**, 1227–1243 (2018).

192. Shinnakasu, R. *et al.* Regulated selection of germinal-center cells into the memory B cell compartment. *Nat. Immunol.* **17**, 861–869 (2016).

193. Suan, D. *et al.* CCR6 Defines Memory B Cell Precursors in Mouse and Human Germinal Centers, Revealing Light-Zone Location and Predominant Low Antigen Affinity. *Immunity* **47**, 1142-1153.e4 (2017).

194. Ozaki, K. *et al.* Regulation of B Cell Differentiation and Plasma Cell Generation by IL-21, a Novel Inducer of Blimp-1 and Bcl-6. *J. Immunol.* **173**, 5361–5371 (2014).

195. Hasbold, J., Corcoran, L. M., Tarlinton, D. M., Tangye, S. G. & Hodgkin, P. D. Evidence from the generation of immunoglobulin G-secreting cells that stochastic mechanisms regulate lymphocyte differentiation. *Nat. Immunol.* **5**, 55–63 (2004).

196. Radtke, D. & Bannard, O. Expression of the Plasma Cell Transcriptional Regulator Blimp-1 by Dark Zone Germinal Center B Cells During Periods of Proliferation. *Front. Immunol.* **9**, 1–16 (2019).

197. Ise, W. & Kurosaki, T. Plasma cell differentiation during the germinal center reaction. *Immunol. Rev.* **288**, 64–74 (2019).

198. Suan, D., Sundling, C. & Brink, R. Plasma cell and memory B cell differentiation from the germinal center. *Curr. Opin. Immunol.* **45**, 97–102 (2017).

199. Weisel, F. & Shlomchik, M. Memory B Cells of Mice and Humans. *Annu. Rev. Immunol.* **35**, 255–284 (2017).

200. Berkowska, M. A. *et al.* Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood* **118**, 2150–2158 (2011).

201. Good-Jacobson, K. L. & Tarlinton, D. M. Multiple routes to B-cell memory. *Int. Immunol.* **24**, 403–408 (2012).

202. Good-Jacobson, K. L. & Shlomchik, M. J. Plasticity and Heterogeneity in the Generation of Memory B Cells and Long-Lived Plasma Cells: The Influence of Germinal Center Interactions and Dynamics. *J. Immunol.* **185**, 3117–3125 (2010).

203. Takemori, T., Kaji, T., Takahashi, Y., Shimoda, M. & Rajewsky, K. Generation of memory B cells inside and outside germinal centers. *Eur. J. Immunol.* **44**, 1258–1264 (2014).

204. Zuccarino-Catania, G. V. *et al.* CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent of antibody isotype. *Nat. Immunol.* **15**, 631–637 (2014).

205. Takahashi, Y., Ohta, H. & Takemori, T. Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. *Immunity* **14**, 181–192 (2001).

206. Minici, C. *et al.* Distinct homotypic B-cell receptor interactions shape the outcome of chronic lymphocytic leukaemia. *Nat. Commun.* **8**, 15746 (2017).

207. Toyama, H. *et al.* Memory B cells without somatic hypermutation are generated from Bcl6-deficient B cells. *Immunity* **17**, 329–339 (2002).

208. Jones, D. D., Wilmore, J. R. & Allman, D. Cellular Dynamics of Memory B Cell Populations: IgM + and IgG + Memory B Cells Persist Indefinitely as Quiescent Cells . *J. Immunol.* **195**, 4753–4759 (2015).

209. Weisel, F. J., Zuccarino-Catania, G. V., Chikina, M. & Shlomchik, M. J. A Temporal Switch in the Germinal Center Determines Differential Output of Memory B and Plasma Cells. *Immunity* **44**, 116–130 (2016).

210. Crotty, S. *et al.* Cutting Edge: Long-Term B Cell Memory in Humans after Smallpox Vaccination. *J. Immunol.* **171**, 4969–4973 (2003).

211. Wheatley, A. K. *et al.* H5N1 Vaccine–Elicited Memory B Cells Are Genetically Constrained by the IGHV Locus in the Recognition of a Neutralizing Epitope in the Hemagglutinin Stem. *J. Immunol.* **195**, 602–610 (2015).

212. Krishnamurty, A. T. *et al.* Somatically Hypermutated Plasmodium-Specific IgM+ Memory B Cells Are Rapid, Plastic, Early Responders upon Malaria Rechallenge. *Immunity* 1–13 (2016). doi:10.1016/j.immuni.2016.06.014

213. Krishnamurty, A. T. *et al.* Somatically Hypermutated Plasmodium-Specific IgM+ Memory B Cells Are Rapid, Plastic, Early Responders upon Malaria Rechallenge. *Immunity* **45**, 402–414 (2016).

214. Pape, K. A., Taylor, J. J., Maul, R. W., Gearhart, P. J. & Jenkins, M. K. Different B Cell Populations Mediate Early and Late Memory During an Endogenous Immune Response. *Science (80-.).* **331**, 1203–1207 (2011).

215. Pape, K. A. *et al.* Naive B Cells with High-Avidity Germline-Encoded Antigen Receptors Produce Persistent IgM + and Transient IgG + Memory B Cells. *Immunity* **48**, 1135-1143.e4 (2018).

216. Gitlin, A. D. *et al.* Independent Roles of Switching and Hypermutation in the Development and Persistence of B Lymphocyte Memory Article Independent Roles of Switching and Hypermutation

in the Development and Persistence of B Lymphocyte Memory. *Immunity* 1–13 (2016). doi:10.1016/j.immuni.2016.01.011

217. Shlomchik, M. J. Do Memory B Cells Form Secondary Germinal Centers? Yes and No. *Cold Spring Harb. Perspect. Biol.* **10**, (2018).

218. Robbiani, D. F. *et al.* Recurrent Potent Human Neutralizing Antibodies to Zika Virus in Brazil and Mexico. *Cell* **169**, 597-609.e11 (2017).

219. Siekevitz, M., Kocks, C., Rajewsky, K. & Dildrop, R. Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. *Cell* **48**, 757–770 (1987).

220. Dogan, I. *et al.* Multiple layers of B cell memory with different effector functions. *Nat. Immunol.* **10**, 1292–1299 (2009).

221. Wrammert, J. *et al.* Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature* **453**, 667–71 (2008).

222. William, J., Euler, C. & Shlomchik, M. J. Short-Lived Plasmablasts Dominate the Early Spontaneous Rheumatoid Factor Response: Differentiation Pathways, Hypermutating Cell Types, and Affinity Maturation Outside the Germinal Center. *J. Immunol.* **174**, 6879–6887 (2005).

223. Di Niro, R. *et al.* Salmonella Infection Drives Promiscuous B Cell Activation Followed by Extrafollicular Affinity Maturation. *Immunity* **43**, 120–131 (2015).

224. Harwood, N. E. & Batista, F. D. Early events in B cell activation. *Annu. Rev. Immunol.* **28**, 185–210 (2010).

225. Depoil, D. *et al.* Early events of B cell activation by antigen. *Sci. Signal.* **2**, pt1 (2009).

226. J., W., C., E., S., C. & M.J., S. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science (80-.).* **297**, 2066–2070 (2002).

227. Marshall, J. L. *et al.* Early B blasts acquire a capacity for Ig class switch recombination that is lost as they become plasmablasts. *Eur. J. Immunol.* **41**, 3506–3512 (2011).

228. Yamanashi, Y., Kakiuchi, T., Mizuguchi, J., Yamamoto, T. & Toyoshima, K. Association of B cell antigen receptor with protein tyrosine kinase Lyn. *Science (80-.).* **251**, 192–194 (1991).

229. Burg, D. L., Furlong, M. T., Harrison, M. L. & Geahlen, R. L. Interactions of Lyn with the antigen receptor during B cell activation. *J. Biol. Chem.* **269**, 28136–28142 (1994).

230. Reth, M. Antigen receptors on B lymphocytes. *Annu. Rev. Immunol.* **10**, 97–121 (1992).

231. Sanchez, M. *et al.* Signal transduction by immunoglobulin is mediated through Iga and Igb. *J.Exp.Med.* **178**, 1049–1055 (1993).

232. Williams, G. T., Peaker, C. J., Patel, K. J. & Neuberger, M. S. The alpha/beta sheath and its cytoplasmic tyrosines are required for signaling by the B-cell antigen receptor but not for capping or for serine/threonine-kinase recruitment. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 474–8 (1994).

233. Rolli, V. *et al.* Amplification of B cell antigen receptor signaling by a Syk/ITAM positive feedback loop. *Mol. Cell* **10**, 1057–69 (2002).

234. Kurosaki, T. Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. *J. Exp. Med.* **182**, 1815–1823 (1995).

235. Unanue, E. R., Perkins, W. D. & Karnovsky, M. J. Ligand-induced movement of lymphocyte membrane macromolecules. I. Analysis by immunofluorescence and ultrastructural radioautography. *J. Exp. Med.* **136**, 885–906 (1972).

236. Reth, M. Oligomeric antigen receptors: a new view on signaling for the selection of lymphocytes. *Trends Immunol.* **22**, 356–60 (2001).

237. Kläsener, K., Maity, P. C., Hobeika, E., Yang, J. & Reth, M. B cell activation involves nanoscale receptor reorganizations and inside-out signaling by Syk. *Elife* **2014**, 1–17 (2014).

238. Gomes de Castro, M. A. *et al.* Differential organization of tonic and chronic B cell antigen receptors in the plasma membrane. *Nat. Commun.* **10**, 1–11 (2019).

239. Tolar, P., Sohn, H. W. & Pierce, S. K. The initiation of antigen-induced B cell antigen receptor signaling viewed in living cells by fluorescence resonance energy transfer. *Nat. Immunol.* **6**, 1168–1176 (2005).

240. Tolar, P., Hanna, J., Krueger, P. D. & Pierce, S. K. The Constant Region of the Membrane Immunoglobulin Mediates B Cell-Receptor Clustering and Signaling in Response to Membrane Antigens. *Immunity* **30**, 44–55 (2009).

241. Lee, J., Sengupta, P., Brzostowski, J., Lippincott-Schwartz, J. & Pierce, S. K. The nanoscale spatial organization of B-cell receptors on immunoglobulin M- and G-expressing human B-cells. *Mol. Biol. Cell* **28**, 511–523 (2017).

242. Mattila, P. K. *et al.* The Actin and Tetraspanin Networks Organize Receptor Nanoclusters to Regulate B Cell Receptor-Mediated Signaling. *Immunity* **38**, 461–474 (2013).

243. Maity, P. C. *et al.* B cell antigen receptors of the IgM and IgD classes are clustered in different protein islands that are altered during B cell activation. *Sci. Signal.* **8**, 1–16 (2015).

244. Zhu, J. W., Brdicka, T., Katsumoto, T. R., Lin, J. & Weiss, A. Structurally distinct phosphatases CD45 and CD148 both regulate B cell and macrophage immunoreceptor signaling. *Immunity* **28**, 183–96 (2008).

245. Liu, W., Sohn, H. W., Tolar, P. & Pierce, S. K. It's all about change: the antigen-driven initiation of B-cell receptor signaling. *Cold Spring Harb. Perspect. Biol.* **2**, 1–12 (2010).

246. Torres, R. M. & Hafen, K. A negative regulatory role for Ig-α during B cell development. *Immunity* **11**, 527–536 (1999).

247. Reichlin, A. *et al.* B Cell Development Is Arrested at the Immature B Cell Stage in Mice Carrying a Mutation in the Cytoplasmic Domain of Immunoglobulin β. *J. Exp. Med.* **193**, 13–24 (2002).

248. Kraus, M., Saijo, K., Torres, R. M. & Rajewsky, K. Ig-alpha cytoplasmic truncation renders immature B cells more sensitive to antigen contact. *Immunity* **11**, 537–45 (1999).

249. Gazumyan, A., Reichlin, A. & Nussenzweig, M. C. Ig beta tyrosine residues contribute to the control of B cell receptor signaling by regulating receptor internalization. *J. Exp. Med.* **203**, 1785–1794 (2006).

250. Leprince, C., Draves, K. E., Geahlen, R. L., Ledbetter, J. A. & Clark, E. A. CD22 associates with the human surface IgM-B-cell antigen receptor complex. *Proc. Natl. Acad. Sci.* **90**, 3236–3240 (2006).

251. Schulte, R. J., Campbell, M. A., Fischer, W. H. & Sefton, B. M. Tyrosine phosphorylation of CD22 during B cell activation. *Science (80-.).* **258**, 1001–1004 (1992).

252. Chan, V. W. F., Meng, F., Soriano, P., DeFranco, A. L. & Lowell, C. A. Characterization of the B lymphocyte populations in lyn-deficient mice and the role of lyn in signal initiation and down-regulation. *Immunity* **7**, 69–81 (1997).

253. Smith, K. G. C., Tarlinton, D. M., Doody, G. M., Hibbs, M. L. & Fearon, D. T. Inhibition of the B Cell by CD22: A Requirement for Lyn. *J. Exp. Med.* **187**, 807–811 (2002).

254. GM, D. *et al.* A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. *Science (80-.).* **269**, 242 (1995).

255. Rao, N. *et al.* The non-receptor tyrosine kinase Syk is a target of Cbl-mediated ubiquitylation upon B-cell receptor stimulation. *EMBO J.* **20**, 7085–95 (2001).

256. Sohn, H. W., Gu, H. & Pierce, S. K. Cbl-b Negatively Regulates B Cell Antigen Receptor Signaling in Mature B Cells through Ubiquitination of the Tyrosine Kinase Syk. *J. Exp. Med.* **197**, 1511–1524 (2003).

257. Shao, Y., Yang, C., Elly, C. & Liu, Y. C. Differential regulation of the B cell receptor-mediated signaling by the E3 ubiquitin ligase Cbl. *J. Biol. Chem.* **279**, 43646–43653 (2004).

258. Batista, F. D. & Neuberger, M. S. B cells extract and present immobilized antigen: implications for affinity discrimination. *EMBO J.* **19**, 513–520 (2000).

259. Depoil, D. *et al.* CD19 is essential for B cell activation by promoting B cell receptor-antigen microcluster formation in response to membrane-bound ligand. *Nat. Immunol.* **9**, 63–72 (2008).

260. Fleire, S. J. *et al.* B cell ligand discrimination through a spreading and contraction response. *Science (80-.).* **312**, 738–41 (2006).

261. Lee, K. *et al.* T cell receptor signaling precedes immunological synapse formation. *Science (80-.).* **295**, 1539–42 (2002).

262. Čemerski, S. *et al.* The Balance between T Cell Receptor Signaling and Degradation at the Center of the Immunological Synapse Is Determined by Antigen Quality. *Immunity* **29**, 414–422 (2008).

263. Brown, B. K. & Song, W. The actin cytoskeleton is required for the trafficking of the B cell antigen receptor to the late endosomes. *Traffic* **2**, 414–27 (2001).

264. Stoddart, A., Jackson, A. P. & Brodsky, F. M. Plasticity of B cell receptor internalization upon conditional depletion of clathrin. *Mol. Biol. Cell* **16**, 2339–48 (2005).

265. Drake, L., McGovern-Brindisi, E. M. & Drake, J. R. BCR ubiquitination controls BCR-mediated antigen processing and presentation. *Blood* **108**, 4086–4093 (2006).

266. Zhang, M. *et al.* Ubiquitinylation of Ig beta dictates the endocytic fate of the B cell antigen receptor. *J. Immunol.* **179**, 4435–4443 (2007).

267. Dragone, L. L. *et al.* Src-like adaptor protein (SLAP) regulates B cell receptor levels in a c-Cbl-dependent manner. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 18202–7 (2006).

268. Drake, L., McGovern-Brindisi, E. M. & Drake, J. R. BCR ubiquitination controls BCR-mediated antigen processing and presentation. *Blood* **108**, 4086–4093 (2006).

269. Lam, K. P., Kühn, R. & Rajewsky, K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* **90**, 1073–83 (1997).

270. Srinivasan, L. *et al.* PI3 Kinase Signals BCR-Dependent Mature B Cell Survival. *Cell* **139**, 573–586 (2009).

271. Levit-Zerdoun, E. *et al.* Survival of Ig alpha-Deficient Mature B Cells Requires BAFF-R Function. *J. Immunol.* **196**, 2348–2360 (2016).

272. Kurosaki, T. Genetic analysis of B cell antigen receptor signaling. *Annu. Rev. Immunol.* **17**, 555–92 (1999).

273. Delgado, P. *et al.* Essential function for the GTPase TC21 in homeostatic antigen receptor signaling. *Nat. Immunol.* **10**, 880–8 (2009).

274. Grande, S. M., Bannish, G., Fuentes-Panana, E. M., Katz, E. & Monroe, J. G. Tonic B-cell and viral ITAM signaling: Context is everything. *Immunol. Rev.* **218**, 214–234 (2007).

275. Monroe, J. G. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat. Rev. Immunol.* **6**, 283–294 (2006).

276. Übelhart, R. *et al.* N-linked glycosylation selectively regulates autonomous precursor BCR function. *Nat. Immunol.* **11**, 759–765 (2010).

277. Ziegler, C. G. K. *et al.* Constitutive Activation of the B Cell Receptor Underlies Dysfunctional Signaling in Chronic Lymphocytic Leukemia. *Cell Rep.* 923–937 (2019). doi:10.1016/j.celrep.2019.06.069

278. Minden, M. D. Von *et al.* Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* **489**, 309–312 (2012).

279. Gold, M. R., Matsuuchi, L., Kelly, R. B. & DeFranco, A. L. Tyrosine phosphorylation of components of the B-cell antigen receptors following receptor crosslinking. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3436–3440 (1991).

280. Yang, J. & Reth, M. The dissociation activation model of B cell antigen receptor triggering. *FEBS Lett.* **584**, 4872–4877 (2010).

281. Infantino, S. *et al.* Arginine methylation of the B cell antigen receptor promotes differentiation. *J. Exp. Med.* **207**, 711–719 (2010).

282. Hobeika, E. *et al.* CD 19 and BAFF -R can signal to promote B -cell survival in the absence of Syk . *EMBO J.* **34**, 925–939 (2015).

283. Ackermann, J. A. *et al.* Syk Tyrosine Kinase Is Critical for B Cell Antibody Responses and Memory B Cell Survival. *J. Immunol.* **194**, 4650–4656 (2015).

284. Hu, P., Han, Z., Couvillon, A. D. & Exton, J. H. Critical role of endogenous Akt/IAPs and

MEK1/ERK pathways in counteracting endoplasmic reticulum stress-induced cell death. *J. Biol. Chem.* **279**, 49420–49429 (2004).

285. Hyoda, K. *et al.* PI3K-Akt inactivation induced CHOP expression in endoplasmic reticulum-stressed cells. *Biochem. Biophys. Res. Commun.* **340**, 286–290 (2006).

286. Kraus, M., Alimzhanov, M. B., Rajewsky, N. & Rajewsky, K. Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta heterodimer. *Cell* **117**, 787–800 (2004).

287. Ohnishi, K. & Melchers, F. The nonimmunoglobulin portion of λ5 mediates cell-autonomous pre-B cell receptor signalling. *Nat. Immunol.* **4**, 849–856 (2003).

288. Bankovich, A. J. *et al.* Structural insight into pre-B cell receptor function. *Science (80-.).* **316**, 291–294 (2007).

289. Torres, R. M., Flaswinkel, H., Reth, M. & Rajewsky, K. Aberrant B Cell Development and Immune Response in Mice with a Compromised BCR Complex. *Science (80-.).* **272**, 1804–1808 (1996).

290. Zhang, M., Srivastava, G. & Lu, L. The pre-B cell receptor and its function during B cell development. *Cell. Mol. Immunol.* **1**, 89–94 (2004).

291. Luning Prak, E. T., Monestier, M. & Eisenberg, R. A. B cell receptor editing in tolerance and autoimmunity. *Ann. N. Y. Acad. Sci.* **1217**, 96–121 (2011).

292. Phan, T. G. *et al.* B Cell Receptor–independent Stimuli Trigger Immunoglobulin (Ig) Class Switch Recombination and Production of IgG Autoantibodies by Anergic Self-Reactive B Cells. *J. Exp. Med.* **197**, 845–860 (2003).

293. Goodnow, C. C. *et al.* Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**, 676–682 (1988).

294. Barnden, M. J., Allison, J., Heath, W. R. & Carbone, F. R. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* **76**, 34–40 (1998).

295. Abe, T. *et al.* Visualization of cell cycle in mouse embryos with Fucci2 reporter directed by Rosa26 promoter. *Development* **140**, 237–246 (2013).

296. Strasser, A. *et al.* Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8661–5 (1991).

297. Muramatsu, M. *et al.* Class Switch Recombination and Hypermutation Require Activation-Induced Cytidine Deaminase (AID), a Potential RNA Editing Enzyme. *Cell* **102**, 553–563 (2000).

298. Robbiani, D. F. *et al.* AID Is Required for the Chromosomal Breaks in c-myc that Lead to c-myc/IgH Translocations. *Cell* **135**, 1028–1038 (2008).

299. Itano, A. A. *et al.* Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity* **19**, 47–57 (2003).

300. Bannard, O. *et al.* Ubiquitin-mediated fluctuations in MHC class II facilitate efficient germinal center B cell responses. *J. Exp. Med.* jem.20151682 (2016). doi:10.1084/jem.20151682

301. Tiller, T. *et al.* Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J. Immunol. Methods* **329**, 112–124 (2008).

302. Wu, Y. *et al.* FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT. *Cell* **126**, 375–387 (2006).

303. Yang, Z. *et al.* Regulation of B cell fate by chronic activity of the IgE B cell receptor. *Elife* **5**, (2016).

304. Hahne, F. *et al.* flowCore: A Bioconductor package for high throughput flow cytometry. *BMC Bioinformatics* **10**, (2009).

305. Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G. & Neufeld, J. D. PANDAseq: Paired-end assembler for illumina sequences. *BMC Bioinformatics* **13**, 31 (2012).

306. Rice, P., Longden, L. & Bleasby, A. EMBOSS: The European Molecular Biology Open Software Suite. *Trends Genet.* **16**, 276–277 (2000).

307. Tiller, T., Busse, C. E. & Wardemann, H. Cloning and expression of murine Ig genes from single B cells. *J. Immunol. Methods* **350**, 183–193 (2009).

308. Yu, Y., Ceredig, R. & Seoighe, C. LymAnalyzer: A tool for comprehensive analysis of next generation sequencing data of T cell receptors and immunoglobulins. *Nucleic Acids Res.* **44**, 1–9 (2015).

309. Kuraoka, M. *et al.* Activation-induced cytidine deaminase mediates tolerance in B-cells. *Proc. Natl. Acad. Sci. USA* **108**, 11560–11565 (2011).

310. Kuraoka, M. *et al.* Complex Antigens Drive Permissive Clonal Selection in Germinal Centers. *Immunity* **44**, 542–552 (2016).

311. Thaunat, O. *et al.* Asymmetric segregation of polarized antigen on B cell division shapes presentation capacity. *Science (80-.).* **335**, 475–9 (2012).

312. Barnett, B. E. *et al.* Asymmetric B Cell Division in the Germinal Center Reaction. *Science (80-.).* **335**, 342–344 (2012).

313. Clark, M. R., Tanaka, A., Powers, S. E. & Veselits, M. Receptors, subcellular compartments and the regulation of peripheral B cell responses: the illuminating state of anergy. *Mol. Immunol.* **48**, 1281–6 (2011).

314. Brink, R. *et al.* Immunoglobulin M and D antigen receptors are both capable of mediating B lymphocyte activation, deletion, or anergy after interaction with specific antigen. *J. Exp. Med.* **176**, 991–1005 (1992).

315. Bléry, M., Tze, L., Miosge, L. a, Jun, J. E. & Goodnow, C. C. Essential role of membrane cholesterol in accelerated BCR internalization and uncoupling from NF-kappa B in B cell clonal anergy. *J. Exp. Med.* **203**, 1773–83 (2006).

316. Roth, D. B. V(D)J Recombination: Mechanism, Errors, and Fidelity. *Microbiol. Spectr.* **2**, 471–473 (2014).

317. Brogna, S. & Wen, J. Nonsense-mediated mRNA decay (NMD) mechanisms. *Nat. Struct. Mol. Biol.* **16**, 107–113 (2009).

318. Andersson, J., Lafleur, L. & Melchers, F. IgM in bone marrow-derived lymphocytes. Synthesis, surface deposition, turnover and carbohydrate composition in unstimulated mouse B cells. *Eur. J. Immunol.* **4**, 170–180 (1974).

319. Le, Q. & Maizels, N. Cell Cycle Regulates Nuclear Stability of AID and Determines the Cellular Response to AID. *PLoS Genet.* **11**, 1–24 (2015).

320. Wang, Q. *et al.* The cell cycle restricts activation-induced cytidine deaminase activity to early G1. *J. Exp. Med.* **214**, 49–58 (2017).

321. Kallies, A. *et al.* Plasma Cell Ontogeny Defined by Quantitative Changes in Blimp-1 Expression. *J. Exp. Med.* **200**, 967–977 (2004).

322. Porter, A. G. & Ja, R. U. Emerging roles of Caspase-3 in apoptosis Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.* 99–104 (2015). doi:10.1038/sj.cdd.4400476

323. Allen, C. D. C., Okada, T. & Cyster, J. G. Germinal-Center Organization and Cellular Dynamics. *Immunity* **27**, 190–202 (2007).

324. Peperzak, V., Vikstrom, I. B. & Tarlinton, D. M. Through a glass less darkly: Apoptosis and the germinal center response to antigen. *Immunol. Rev.* **247**, 93–106 (2012).

325. Takahashi, Y. *et al.* Relaxed Negative Selection in Germinal Centers and Impaired Affinity Maturation in bcl-x L Transgenic Mice . *J. Exp. Med.* **190**, 399–410 (1999).

326. Boulianne, B. *et al.* AID and caspase 8 shape the germinal center response through apoptosis. *J. Immunol.* **191**, 5840–7 (2013).

327. Mayer, C. T. *et al.* The microanatomic segregation of selection by apoptosis in the germinal center. *Science (80-.).* **358**, eaao2602 (2017).

328. Casola, S. *et al.* The B-cell receptor in control of tumor B-cell fitness: Biology and clinical relevance. *Immunol. Rev.* **288**, 198–213 (2019).

329. Varano, G. *et al.* The B-cell receptor controls fitness of MYC-driven lymphoma cells via GSK3β inhibition. *Nature* **546**, 302–306 (2017).

330. Victora, G. D. *et al.* Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* **143**, 592–605 (2010).

331. Cho, S. H. *et al.* Germinal centre hypoxia and regulation of antibody qualities by a hypoxia response system. *Nature* (2016). doi:10.1038/nature19334

332. Piccoli, C. *et al.* To breathe or not to breathe: The haematopoietic stem/progenitor cells dilemma. *Br. J. Pharmacol.* **169**, 1652–1671 (2013).

333. Panchision, D. M. He role of oxygen in regulating neural stem cells in development and disease. *J. Cell. Physiol.* **220**, 565–568 (2009).

334. Kondo, H., Shiroishi, M., Matsushima, M., Tsumoto, K. & Kumagai, I. Crystal structure of anti-Hen egg white lysozyme antibody (HyHEL-10) Fv-antigen complex. Local structural changes in the protein antigen and water-mediated interactions of Fv-antigen and light chain-heavy chain interfaces. *J. Biol. Chem.* **274**, 27623–31 (1999).

335. Mesin, L., Ersching, J. & Victora, G. D. Germinal Center B Cell Dynamics. *Immunity* **45**, 471–482 (2016).

336. Yang, J. *et al.* Tracing Donor-MHC Class II Reactive B cells in Mouse Cardiac Transplantation: Delayed CTLA4-Ig Treatment Prevents Memory Alloreactive B-Cell Generation. *Transplantation* **100**, 1683–91 (2016).

337. Silver, J. *et al.* Stochasticity enables BCR-independent germinal center initiation and antibody affinity maturation. *J. Exp. Med.* (2017). doi:10.1084/jem.20171022

338. Ramiro, A. R. *et al.* Role of genomic instability and p53 in AID-induced c-myc-Igh translocations. *Nature* **440**, 105–9 (2006).

339. Wang, Q. *et al.* Epigenetic targeting of activation-induced cytidine deaminase. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 18667–72 (2014).

340. Begum, N. A. *et al.* Further evidence for involvement of a noncanonical function of uracil DNA glycosylase in class switch recombination. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 2752–2757 (2009).

341. Lossi, L., Cocito, C., Alasia, S. & Merighi, A. Ex vivo imaging of active caspase 3 by a FRET-based molecular probe demonstrates the cellular dynamics and localization of the protease in cerebellar granule cells and its regulation by the apoptosis-inhibiting protein survivin. *Mol. Neurodegener.* **11**, 1–20 (2016).

342. Garrod, K. R. *et al.* Dissecting T Cell Contraction In Vivo Using a Genetically Encoded Reporter of Apoptosis. *Cell Rep.* **2**, 1438–1447 (2012).

343. Boulianne, B. *et al.* AID and Caspase 8 Shape the Germinal Center Response through Apoptosis. *J. Immunol.* **191**, 5840–5847 (2013).

344. Doria-Rose, N. A. *et al.* Breadth of Human Immunodeficiency Virus-Specific Neutralizing Activity in Sera: Clustering Analysis and Association with Clinical Variables. *J. Virol.* **84**, 1631–1636 (2010).

345. Simek, M. D. *et al.* Human Immunodeficiency Virus Type 1 Elite Neutralizers: Individuals with Broad and Potent Neutralizing Activity Identified by Using a High-Throughput Neutralization Assay together with an Analytical Selection Algorithm. *J. Virol.* **83**, 7337–7348 (2009).

346. Mikell, I. *et al.* Characteristics of the earliest cross-neutralizing antibody response to HIV-1. *PLoS Pathog.* **7**, (2011).

347. Kong, L. & Sattentau, Q. J. Antigenicity and immunogenicity in HIV-1 antibody-based vaccine design. *J. AIDS Clin. Res.* **3**, 1–23 (2012).

348. McCoy, L. E. & Weiss, R. A. Neutralizing antibodies to HIV-1 induced by immunization. *J. Exp. Med.* **210**, 209–223 (2013).

349. Mascola, J. R. *et al.* Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat. Med.* **6**, 207–210 (2000).

350. Moldt, B. *et al.* Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 18921–18925 (2012).

351. Shibata, R. *et al.* Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimetic virus infections of macaque monkeys. *Nat. Med.* **5**, 204–210 (1999).

352. Klein, F. *et al.* HIV therapy by a combination of broadly neutralizing antibodies in humanized mice. *Nature* **492**, 118–122 (2012).

353. Tiller, T. *et al.* Autoreactivity in Human IgG+ Memory B Cells. *Immunity* **26**, 205–213 (2007).

354. Corti, D. *et al.* Analysis of memory B cell responses and isolation of novel monoclonal antibodies with neutralizing breadth from HIV-1-infected individuals. *PLoS One* **5**, (2010).

355. Walker, L. M. *et al.* Rapid development of glycan-specific, broad, and potent anti-HIV-1 gp120 neutralizing antibodies in an R5 SIV/HIV chimeric virus infected macaque. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 20125–20129 (2011).

356. Xiao, X., Chen, W., Feng, Y. & Dimitrov, D. S. Maturation pathways of cross-reactive HIV-1 neutralizing antibodies. *Viruses* **1**, 802–817 (2009).

357. Scheid, J. F. *et al.* Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. *Nature* **458**, 636–640 (2009).

358. Sok, D. *et al.* The Effects of Somatic Hypermutation on Neutralization and Binding in the PGT121 Family of Broadly Neutralizing HIV Antibodies. *PLoS Pathog.* **9**, (2013).

359. Yang, Z. *et al.* Regulation of B cell fate by chronic activity of the IgE B cell receptor. *Elife* **5**, 1–31 (2016).

360. Diskin, R. *et al.* Increasing the Potency and Breadth of an HIV Antibody by Using Structure-Based Rational Design. *Science (80-.).* **334**, 1289–1293 (2011).

361. Zhou, T. *et al.* Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. *Science (80-.).* **329**, 811–817 (2010).

362. Ovchinnikov, V., Louveau, J. E., Barton, J. P., Karplus, M. & Chakraborty, A. K. Role of framework mutations and antibody flexibility in the evolution of broadly neutralizing antibodies. *Elife* **7**, 1–24 (2018).

363. Klein, F. *et al.* Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. *Cell* **153**, 126–138 (2013).

364. Georgiev, I. S. *et al.* Antibodies VRC01 and 10E8 Neutralize HIV-1 with High Breadth and Potency Even with Ig-Framework Regions Substantially Reverted to Germline. *J. Immunol.* **192**, 1100–1106 (2014).

365. Kepler, T. B. *et al.* Immunoglobulin gene insertions and deletions in the affinity maturation of HIV-1 broadly reactive neutralizing antibodies. *Cell Host Microbe* **16**, 304–313 (2014).

366. Wilson, P. C. *et al.* Somatic hypermutation introduces insertions and deletions into immunoglobulin V genes. *J. Exp. Med.* **187**, 59–70 (1998).

367. Briney, B. S., Willis, J. R. & Crowe, J. E. Location and length distribution of somatic hypermutation-associated DNA insertions and deletions reveals regions of antibody structural plasticity. *Genes Immun.* **13**, 523–529 (2012).

368. Krause, J. C. *et al.* An insertion mutation that distorts antibody binding site architecture enhances function of a human antibody. *MBio* **2**, 1–8 (2011).

369. Pejchal, R. *et al.* A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. *Science (80-.).* **334**, 1097–1103 (2011).

370. Pieper, K. *et al.* Public antibodies to malaria antigens generated by two LAIR1 insertion modalities. *Nature* (2017). doi:10.1038/nature23670

371. Muppidi, J. R. *et al.* Loss of signalling via G α 13 in germinal centre B-cell-derived lymphoma. *Nature* **516**, 254–258 (2014).

372. Fruehling, S. & Longnecker, R. The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction. *Virology* **235**, 241–251 (1997).

373. Pang, M. F., Lin, K. W. & Peh, S. C. The signaling pathways of Epstein-Barr virus-encoded latent membrane protein 2A (LMP2A) in latency and cancer. *Cell. Mol. Biol. Lett.* **14**, 222–247 (2009).

374. Miller, C. L., Longnecker, R. & Kieff, E. Epstein-Barr virus latent membrane protein 2A blocks calcium mobilization in B lymphocytes. *J. Virol.* **67**, 3087–94 (1993).

375. Dykstra, M. L., Longnecker, R. & Pierce, S. K. Epstein-Barr virus coopts lipid rafts to block the signaling and antigen transport functions of the BCR. *Immunity* **14**, 57–67 (2001).

376. Casola, S. *et al.* B cell receptor signal strength determines B cell fate. *Nat. Immunol.* **5**, 317–327 (2004).

377. Mancao, C., Altmann, M., Jungnickel, B. & Hammerschmidt, W. Rescue of " crippled " germinal center B cells from apoptosis by Epstein-Barr virus. *Public Health* **106**, 4339–4344 (2005).

378. Chaganti, S. Epstein-Barr virus infection in vitro can rescue germinal center B cells with inactivated immunoglobulin genes. *Blood* **106**, 4249–4252 (2005).

379. Niedobitek, G. *et al.* Immunohistochemical detection of the epstein-barr virus-encoded latent membrane protein 2A in Hodgkin's disease and infectious mononucleosis. *Blood* **90**, 1664–1672 (1997).

380. Mancao, C. & Hammerschmidt, W. Epstein-Barr virus latent membrane protein 2A is a B-cell receptor mimic and essential for B-cell survival. *Blood* **110**, 3715–3721 (2007).

381. Herzog, S. *et al.* SLP-65 regulates immunoglobulin light chain gene recombination through the PI(3)K-PKB-Foxo pathway. *Nat. Immunol.* **9**, 623–631 (2008).

382. Alkhatib, A. *et al.* FoxO1 induces Ikaros splicing to promote immunoglobulin gene recombination. *J. Exp. Med.* **209**, 395–406 (2012).

383. Dal Porto, J. M., Haberman, a M., Shlomchik, M. J. & Kelsoe, G. Antigen drives very low affinity B cells to become plasmacytes and enter germinal centers. *J. Immunol.* **161**, 5373–5381 (1998).

384. De Silva, N. S. *et al.* Transcription factors of the alternative NF-κB pathway are required for germinal center B-cell development. *Proc. Natl. Acad. Sci.* **113**, 9063–9068 (2016).

385. Pasparakis, M., Schmidt-Supprian, M. & Rajewsky, K. IκB kinase signaling is essential for maintenance of mature B cells. *J. Exp. Med.* **196**, 743–752 (2002).

386. Ruland, J. *et al.* Bcl10 is a positive regulator of antigen receptor-induced activation of NF-κB and neural tube closure. *Cell* **104**, 33–42 (2001).

387. Xue, L. *et al.* Defective developmental and function of Bcl10-deficient follicular, marginal zone and B1 B cells. *Nat. Immunol.* **4**, 857–865 (2003).

388. Heise, N. *et al.* Germinal center B cell maintenance and differentiation are controlled by distinct NF-κB transcription factor subunits. *J. Exp. Med.* **211**, 2103–2118 (2014).

389. Basso, K. *et al.* Tracking CD40 signaling during germinal center development. *Blood* **104**, 4088–4096 (2004).

390. Han, S. *et al.* Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J. Immunol.* **155**, 556–67 (1995).

391. Habuchi, S., Tsutsui, H., Kochaniak, A. B., Miyawaki, A. & van Oijen, A. M. mKikGR, a monomeric photoswitchable fluorescent protein. *PLoS One* **3**, 1–9 (2008).

392. Stewart, I., Radtke, D., Phillips, B., McGowan, S. J. & Bannard, O. Germinal Center B Cells Replace Their Antigen Receptors in Dark Zones and Fail Light Zone Entry when Immunoglobulin Gene Mutations are Damaging. *Immunity* **49**, 477-489.e7 (2018).

393. Subach, F. V *et al.* Monomeric fluorescent timers that change color from blue to red report on cellular trafficking. *Nat. Chem. Biol.* **5**, 118–26 (2009).

394. Bojkowska, K. *et al.* Measuring In Vivo Protein Half-Life. *Chem. Biol.* **18**, 805–815 (2011).

395. Mason, J. O., Williams, G. T. & Neuberger, M. S. The half-life of immunoglobulin mRNA increases during B-cell differentiation: a possible role for targeting to membrane-bound polysomes. *Genes Dev.* **2**, 1003–11 (1988).

396. Nelson, K. J., Haimovich, J. & Perry, R. P. Characterization of productive and sterile transcripts from the immunoglobulin heavy-chain locus: processing of micron and muS mRNA. *Mol. Cell. Biol.* **3**, 1317–32 (1983).

397. Kivioja, T. *et al.* Counting absolute numbers of molecules using unique molecular identifiers. *Nat Meth* **9**, 72–74 (2012).

398. Islam, S. *et al.* Quantitative single-cell RNA-seq with unique molecular identifiers. *Nat. Methods* **11**, 163–6 (2014).

399. Turchaninova, M. A. *et al.* High-quality full-length immunoglobulin profiling with unique molecular barcoding. *Nat Protoc.* **11**, 1599–1616 (2016).