Immunoglobulin isotype switching ceases in germinal centres

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Jonathan Roco, PhD scholar
Supervisor: Professor Carola Vinuesa

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Declaration

The work of this thesis has been conducted from March 2014 to September 2018 at the Department of Immunology and Infectious Disease, The John Curtin School of Medical Research (JCSMR), The Australian National University (ANU), Canberra, ACT, Australia.

Unless otherwise referenced, the results and analyses in this document represent only my original work performed under the supervision of Professor Carola Vinuesa.

This document has not been submitted for qualifications at any other academic institution.

Jonathan Roco   Prof. Carola Vinuesa

Acknowledgement

A mi Paula, porque la vida contigo es perfecta.

Y a mis padres que apoyan cada una de mis decisiones sin importar cuán lejos de casa me lleven.

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List of abbreviations

AID	Activation-induced cytidine deaminase
AIS	Adaptive immune system
AP	Apurinic/apyrimidinic
APC	Antigen presenting cell
APE1	Apurinic-apyrimidinic endonuclease 1
APE2	Apurinic-apyrimidinic endonuclease 2
BCL6	B cell lymphoma 6
BCR	B cell antigen receptor
BER	DNA base excision repair pathway
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CGG	Chicken gamma globulin
CSR	Class switch recombination
CXCR5	CXC-chemokine receptor 5
DC	Dendritic cell
dA	Deoxyadenosine
dC	Deoxycytidine
dG	Deoxyguanosine
DSB	Double-strand break (DNA)
dT	Deoxythymidine
dU	Deoxyuridine

DZ	Dark zone
EF	Extrafollicular
FACS	Fluorescent activated cells sorting
FDC	Follicular dendritic cell
FOXO	Forkhead box O
GC	Germinal centre
GLT	Germline transcript
HEL	Hen egg lysozyme
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IL	Interleukin
LN	Lymph node
LZ	Light zone
MHC	Major histocompatibility complex
MMR	DNA mismatch repair pathway
NHEJ	Non-homologous end joining repair pathway
NP	4-Hydroxy-3-nitrophenylacetyl
PAMP	Pathogen-associated molecular pattern
PC	Plasma cell
pLN	popliteal Lymph Node
PD1	Programmed cell death 1
qPCR	Quantitative polymerase chain reaction
SHM	Somatic hypermutation
SRBC	Sheep red blood cell
SSB	Single-strand break (DNA)
TCR	T cell antigen receptor
TD	T cell dependent

T_{FH}	T follicular helper cell
TI	T cell independent
T_{REG}	T regulatory cell
UNG	Uracil-DNA glycosylase
WT	Wild type

Abstract

B cell-derived antibodies are essential to protect against infection and underpin the success of most existing vaccines. Class switch recombination (CSR) is a tightly controlled DNA recombination that replaces the constant region of an antibody for the isotype that can best protect against the invading pathogen. Germinal centres (GCs) are thought to favour immunoglobulin (Ig) diversification through somatic hypermutation (SHM) and CSR at least in part because activation-induced cytidine deaminase (AID), the enzyme required for both processes, is highly expressed in GC B cells. Nevertheless, CSR also occurs in extrafollicular (EF) responses and can be initiated soon after T cell priming. Here we show that CSR is initiated prior to B cell commitment to follicular or EF differentiation and comparable proportions of switched cells are found at both sites, with little or no enrichment within GCs. Single-cell analysis of germline transcripts (GLTs) showed that CSR is triggered prior to B cell entry into the follicle and rapidly declines as B cells become GC cells. Analysis of the clonal distribution and phylogeny of individual IgM-expressing GC B cells demonstrated that CSR events largely occurred in GC B cell precursors ceasing within the first two days of differentiation into GC B cells and soon after the onset of SHM. This data also demonstrated the existence of IgM-dominated GCs. Mathematical modelling showed that IgM-dominated GCs are unlikely to occur under the assumption of ongoing switching. Lack of ongoing switching in GCs may be crucial to maintain IgM$^+$ memory B cells that can produce adequate protective isotypes

upon reinfection with antigenically-related pathogens. The chromosomal breaks required for CSR can also cause pathogenicity, gene insertions and translocations, resulting in the formation of self-reactive BCRs or giving rise to B cell lymphomas. Therefore, understanding the timing and anatomical location in which CSR occurs is a crucial aspect of B cell biology to better comprehend autoimmunity, B-cell derived lymphomas, and eventually improve the design of future vaccines.

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Chapter 1

Introduction

1.1 Preamble

During evolution mammals have developed sophisticated mechanisms to modify the B cell receptor (BCR) during the course of an immune response. Theoretically, antibody-mediated responses have evolved to recognise a nearly unlimited number of foreign antigens by the random recombination of VDJ-segments (Bassing et al. 2002). Another property of antibodies is the increment in affinity over time through the iterative introduction of point mutations by somatic hypermutation (SHM) into the immunoglobulin (Ig) variable (V) regions of both heavy (H) and light (L) chain genes (Di Noia and Neuberger 2007). This process called affinity maturation takes place in microenvironments known as germinal centres (GCs) (MacLennan 1994).

Antibodies are the secreted form of the BCR expressed by B cells. They are classified into 5 major classes: IgM, IgD, IgG, IgE and IgA, based on the constant heavy (C_H) chain they bear. Antibodies exhibit a dual function working as an effector molecule when secreted, and also as antigen-receptors when bound to the surface of B cells. Antibodies belong to the immunoglobulin superfamily (IgSF)

of proteins – one of the most common topologies found in nature – and possess a characteristic "Y-shaped" structure consisting of two variable regions that bind the antigen (the arms) and a C_H region or isotype (the stem) that determines the effector function (Schroeder and Cavacini 2010).

Mammals are probably the species with the most complex and extensive set of effector functions that distinctively vary with each isotype. Compared to their capacity to recognise antigens, the number of specialised effector functions elicited by antibodies is more restricted, falling into specific categories that include activation of the complement, opsonisation, defence of mucosal surfaces and recruitment of effector innate-cells (Vidarsson et al. 2014). Isotypes are selected in a highly dynamic manner according to the type of infectious agents guided by the help provided by T cells. The selective recombination of the IgH locus occurs through a mechanism called class switch recombination (CSR) (Coffman et al. 1993).

Class switching is a DNA recombination event that allows production of antibodies expressing isotypes different than IgM or IgD, *i.e.* IgG, IgE or IgA. CSR is part of a set of genetic alterations aimed to diversify the pool of Ig specificities and effector functions needed for pathogen clearance. Traditionally, GCs have been identified as the main places where CSR takes places *in vivo* (Honjo et al. 2002; Murphy and Weaver 2012); however, the signals underpinning this process as well as the exact spatio-temporal cues that trigger CSR still represent an intensive area of research.

CSR is the primary topic investigated in this thesis. We present empirical and modelling evidence that contribute to a better understating of the timing and anatomical location of CSR during a T dependent (TD)-immune response.

1.2 The adaptive immune system

1.2.1 The evolution of adaptive based immunity

One of the most sophisticated features of vertebrates to fight pathogens is the presence of an adaptive immune system (AIS). The AIS arose in the jawed vertebrates (gnathostomes) approximately 500 million years ago (Mya), representing the most significant and powerful strategy to achieve the large diversity of antigen-specificities found in the animal phyla (Pancer and Cooper 2006; Flajnik and Kasahara 2010).

The emergence of the AIS seems to have occurred abruptly during a brief evolutionary window, in what is called the "immunological big-bang" in the evolution of jawed vertebrates (Schluter et al. 1999). Phylogenetic studies have hypothesised that the first gnathostomes with an AIS were the placoderms (Flajnik and Kasahara 2010), which lived throughout the Devonian period[1] approximately 360 Mya (Zapata and Amemiya 2000). Although, no fossil record has been found to corroborate this idea among the extant species descending from this ancient taxon, cartilaginous fish (sharks, skates, rays, etc.) possess all the defining traits of adaptive immunity, as we defined them in humans (Zapata and Amemiya 2000). Still, the exact time at which the AIS arose remains uncertain.

From an evolutionary perspective, the key determinants that distinguish our AIS from other species resides in: i) the RAG-mediated VDJ-recombination of TCR and BCR genes; ii) the presence of MHC molecules; iii) the diversification of the effector functions of antibodies by CSR; and iv) the introduction of single

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[1]The late Devonian period is one of the five major extinction events that Earth has witnessed, where ~80% of all living species were lost. The exact reasons are unclear but changes in sea levels, ocean anoxia and presumably the impact of an asteroid are part of the leading theories (Newman and Eble 2001).

point mutations by SHM to increase the affinity of the BCR for the immunising antigen (Litman et al. 1999). These cardinal events incorporated an extra layer of complexity in the fight against pathogens compared to the AIS of agnathans (the jawless vertebrates, described below) and perhaps among other gnathostomes.

The AIS is remarkably flexible and has evolved to recognised in theory an unlimited number of antigens. The recombinatorial nature of the AIS resulted not only in a highly diverse repertoire of antigen receptors but also in an extremely specific system with the capacity to "remember" previous pathogen encounters in the form of lymphocyte-memory. Lymphocytes are at the core of the AIS composed by T and B cells. This type of circulatory cells with self-renewal and clonal expansion capacities were most likely developed early on during evolution, in the common ancestor of all vertebrates (Anderson and Rothenberg 2000).

Although, adaptive immune responses have also been reported in agnathans (Herrin and Cooper 2010), these do not depend on BCR, TCR or MHC molecules. Members of this family, lampreys and hagfish, possess a type of lymphocyte-like cells with combinatorial capacity capable of diversifying the repertoire of pathogen-detecting receptors. This alternative system generates diversity by rearranging a set of leucine-rich repeat (LRR) modules into the germline sequence of the genes encoding the variable lymphocyte receptor (VLR) (Pancer et al. 2004). To date, two single genes, *VLRA* and *VLRB*, have been described in both hagfish and lampreys (Pancer et al. 2004; Pancer et al. 2005). In the latter, 393 and 454 LRR-segments are found to be flanking the *VLRA* and *VLRB* genes, respectively (Pancer et al. 2005).

The "anticipatory" nature of these two types of adaptive immunity raises some interesting questions. Whether the LRR-based system in agnathans represents an ancestral strategy preceding the origin of the AIS in gnathostomes, or whether the recombinatorial formation of VLRs and TCR/BCR genes evolved as independent solutions to similar necessities in these two taxonomic classes are

still open to debate (Pancer and Cooper 2006; Herrin and Cooper 2010). Since only two representatives of agnathans (hagfish and lampreys) have survived to the present day, and the key evolutionary link, the placoderms, are extinct it is difficult to test this hypothesis. Despite this, there is now broad consensus that two major events – the invasion of the RAG transposon and two whole-genome duplications (WGDs) – had crucial roles in the formation of the more complex AIS in the jawed vertebrates (Anderson and Rothenberg 2000; Flajnik and Kasahara 2010).

1.2.2 VDJ-recombination

Recombinase activating gene 1 (RAG1) and RAG2 are both part of a multimeric recombination machinery that initiates the assembly of immunoglobulins and T cell receptors in a process known as VDJ-recombination (Tonegawa 1983; Lewis 1994; Schatz and Swanson 2011). The expression of RAG1/RAG2 genes is stringently restricted, both temporal and spatial, to the early stages of T and B cell development (Lewis 1994).

Structurally, the RAG-protein complex has been described consisting of a catalytic engine (RAG1) and a cofactor (RAG2) that are associated to a DNA-bending protein – high-mobility group protein 1 (HMG1) – (Swanson 2004; Teng and Schatz 2015). The recombination complex recognises short *cis*-acting elements called recombination signal sequences (RSSs). These sequences contain conserved heptamer (consensus sequence: 5'-CACAGTG-3') and nonamer (5'-ACAAAAACC-3') motifs interspaced by either 12 or 23 nucleotides called "the spacers". The sequence of the spacers can vary but the length is conserved, defining each RSS as either a 12-RSS or a 23-RSS (Gent et al. 1996; Eastman et al. 1996).

The RSSs flank the variable (V) segments on their 3'-end, diversity (D) segments on both 5' and 3' sides and joining (J) segments on their 5' sides. RAG-

mediated VDJ-recombination follows a basic requirement called the 12/23 rule (Gent et al. 1996; Eastman et al. 1996), this means that a gene segment flanked by a 12-RSS can only be joined to one flanked by a 23-RSS. This simple rule, ensures the correct recombination between the different gene segments that will encode the variable region of the TCRs and immunoglobulin molecules. Although infrequent, some degree of flexibility has also been found in studies using the *Tcrb* locus, termed the B12/23 restriction (Bassing et al. 2000; Tillman et al. 2004).

The VDJ-recombination is a carefully orchestrated "cut-and-paste" reaction that can be divided into two main steps: i) RAG-mediated DNA cleavage and ii) DNA end-processing of the cleaved genes that is carried out by a ubiquitous set of proteins belonging to the NHEJ repair pathway (Gent et al. 1995; Swanson 2004; Helmink and Sleckman 2012). The process begins with binding to the target RSSs of two genes segments, *e.g.*, D_H and J_H, by the RAG1/RAG2-complex. This complex aligns both RSSs and through its endonuclease activity generates a nick between the coding segment and its RSS creating dsDNA breaks. Then, the broken ends are repaired by NHEJ proteins, where Ku70:80, DNA-dependent protein kinase (DNA-PKcs), Atermis and DNA ligase IV play critical roles (Helmink and Sleckman 2012).

Another source of genetic variation during the BCR genesis is the random introduction of nucleotides by the enzyme terminal deoxynucleotidyl transferase (TdT) at the junction of the different gene segments before the ends are joined. Likewise, trimming of nucleotides by yet to be discovered exonucleases has also been proposed as an alternative mechanism (Murphy and Weaver 2012). In conclusion, a set of different genetic alterations along with the random recombinatorial nature of the multiple V, D and J gene segments are the basis to generate the vast number of antigen-specificities found in our immune system (Weigert et al. 1978; Lewis 1994).

1.2.3 The origins of RAG1/RAG2 proteins

In the era of high-throughput genomic techniques, the astonishing expansion of genome sequences coming from very divergent species has been crucial to unravel the origins of recombinase activating gene 1 (RAG1) and RAG2. The RAG-locus is highly conserved in jawed vertebrates (Figure 1.1), where its evolutionary acquisition can be traced from cartilaginous fish to bony fish, amphibians, reptiles, birds and mammals (Zapata and Amemiya 2000; Teng and Schatz 2015).

The structure of the RAG-locus is unusual for the genome of higher eukaryotes (Oettinger et al. 1990; Chatterji et al. 2004). The coding sequence of RAG1/RAG2 is almost completely devoid of introns and its compact organisation resembles that observed in prokaryotes and viruses (Oettinger et al. 1990). In addition to the genetic architecture, the conservation of these proteins across multiple species, and their mode of action has led to the hypothesis that RAG1/RAG2 derives from an ancient transpositional event (Kapitonov and Jurka 2005; Teng and Schatz 2015). As a matter of fact, it has been shown that purified RAG-proteins can act as a transposase *in vitro* (Agrawal et al. 1998).

How the RAG-based rearrangement system evolved from its initial transposase activity to be capable of interacting with the NHEJ-apparatus so as to join and recombine the variable-regions of TCRs and BCRs is not completely understood (Teng and Schatz 2015). Multiple rounds of insertion/deletion of the RAG-locus might have reshaped its function over time. Another possibility might rest in the C-terminal region of RAG2: this non-catalytic domain has been proposed to prevent transposition in developing lymphocytes (Elkin et al. 2003).

1.2.4 Selective pressures in the formation of the AIS

Undoubtedly, the emerge of the AIS was a unique event in the evolution of vertebrates. Notwithstanding, the development of the AIS still represents a
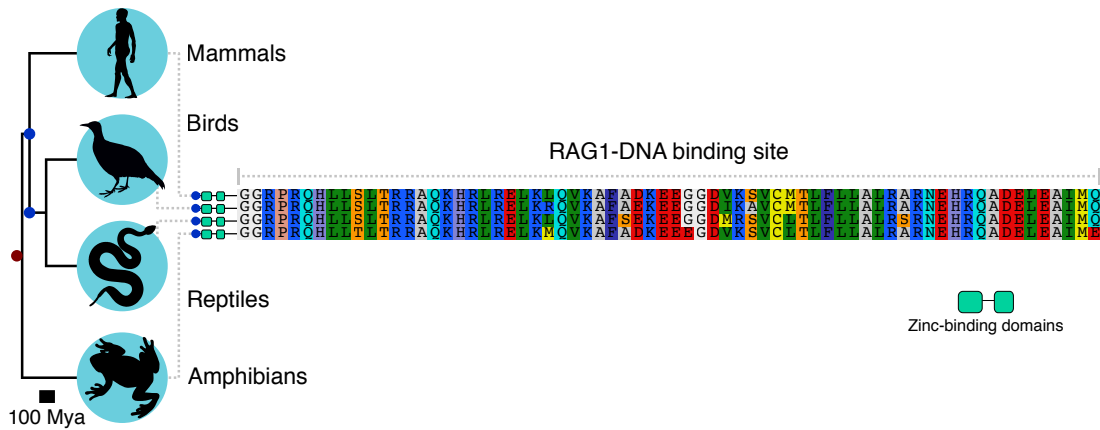
Figure 1.1. RAG1 evolution. Phylogenetic comparison of RAG1-DNA binding sites across different species. From top to bottom: *Homo sapiens* (mammals), *Gallus gallus* (birds), *Cylindrophis ruffus* (reptiles) and *Xenopus laevis* (amphibians) RAG1 sequences were used. Adapted from Flajnik and Kasahara 2010.

puzzling question for evolutionary immunologists. At first, the architecture of the AIS has an obvious advantage vs innate-based immunity. The capacity to recognise an infinite number of pathogenic-variants through the recombination of a fixed number of gene segments, provides a highly dynamic system with rapid adaptation and selection in each individual. However, this boundless plasticity comes with a price. The random formation of antigen-receptors has the associated risk of creating specificities against self, capable of causing harm to the host. To solve this problem, the AIS also needed the evolution of regulatory mechanisms (immune tolerance) running in parallel to control and eventually delete those self-reactive specificities (Hedrick 2004; Flajnik and Kasahara 2010).

The evolutionary roots of the AIS have been linked to the integration of the RAG-locus, two rounds of whole-genome duplication, and unique selective pressures during the vertebrate radiation (Streelman and Danley 2003; Flajnik and Kasahara 2010). Co-evolution of vertebrates with multiple endosymbiotic communities is probably one of the main driver forces in shaping the AIS as we know it today. The mutual benefits obtained from the close relation between host and symbiotic microorganisms demanded a readjustment in the detection based

on PAMP-recognising receptors (Pancer and Cooper 2006; Flajnik and Kasahara 2010; Kato et al. 2014). Applying too stringent rules to judge between self and foreign might have become obsolete, denying the access not only to opportunistic parasites but also to commensal microorganisms. Therefore, the formation of the AIS might have been carved by the subtle balance between commensalism and parasitism (Hedrick 2004).

Another attractive idea about the formation of the AIS has been recently proposed by Engelbrecht and colleagues. Instead of focusing on selective pressures the researchers suggest that functional innovations in vertebrates may have been critical for the generation of adaptive immunity. Vertebrates possess a low blood-to-body weight ratio; *i.e.* lower blood volumes, and have developed closed circulatory systems with blood vessels lined by a thin layer of endothelial cells. These vascular innovations resulted in a more cost-effective response leading to higher antibody titres in circulation and a more practical system for the trafficking of immune effector molecules and lymphocytes (Niekerk et al. 2015).

1.3 Immune responses

1.3.1 B cell activation and the initiation of thymus-dependent responses

Activation of B cells is a complex and highly dynamic process that is often but not exclusively initiated in the follicular areas of secondary lymphoid organs (SLOs). Follicles are mostly comprised of IgM/IgD naïve B cells within a network of follicular dendritic cells (FDCs) and stromal cells (Heesters et al. 2014). Physically, follicles provide an optimal structure for capturing and concentrating antigens that maximises the likelihood of specific BCR-antigen encounters[2](Murphy and Weaver 2012). Unlike T cells, B cells are able to recognise and bind antigen in its native conformation – without being processed by an antigen presenting cell – either in a soluble state or bound to the membrane of other cells (Yuseff et al. 2013); however, in the context of natural infections the membrane-bound form is more effectively retrieved by B cells (Batista and Harwood 2009; Yuseff et al. 2013). Marginal zone B cells are also exposed to antigen circulating in marginal zone sinuses, and follicular B cells can also capture antigen while they are circulating in the blood, as they migrate between different SLOs (Cerutti et al. 2013).

Our knowledge about the dynamics of B cell activation *in vivo* is becoming more accurate thanks to studies using intravital microscopy and *in situ* photoactivation, following the immune response against model antigens (Allen et al. 2007b; Schwickert et al. 2007; Victora et al. 2010). The compiled evidence indicates that after antigen binding, activated B cells migrate to the border between the T-cell

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[2]The estimated frequency of naïve lymphocytes specific for any given antigen is around 1 in 10,000 to 1 in 1,000,000. Therefore, the probability of a cognate interaction occurring in the periphery between T cells and B cells recognising the same antigen is approximately 1 in 10^8 to 1 in 10^{12}. This probability is greatly enhanced within SLOs (Murphy and Weaver 2012).

area and the follicle (T:B border), guided by the modulation of the chemokine receptors CXCR4 and CXCR5 (Vinuesa and Cyster 2011). At the T:B border B cells can present processed antigens in MHC class II molecules to cognate CD4$^+$ T cells (De Silva and Klein 2015). During this step, B cells that engage in contact-dependent interactions with cognate T cells receive activation signals through the CD40-CD40L axis and co-stimulatory signals in the form of cytokines, such as IL-4, IL-21 and BAFF (Klein and Dalla-Favera 2008; Vinuesa et al. 2010). This and additional interactions via the signalling lymphocyte activation molecule (SLAM)-family member of surface receptors and costimulatory molecules, such as inducible T-cell costimulator (ICOS) and its ligand (ICOSL), result in a synergistic effect that nurtures both cell types with the signals needed for survival and further differentiation (Vinuesa et al. 2010). The importance of cognate presentation has been validated by experiments with mixed bone marrow chimeras, where reconstitution with B cells lacking MHC-II protein leads to impaired clonal expansion, defective differentiation of cytokine-secreting T cells and reduced formation of the memory compartment (Crawford et al. 2006; Batista and Harwood 2009).

After activation at the T:B border, responding B cells undergo a proliferative burst at the periphery of the follicles (Jacob et al. 1991a; Jacob and Kelsoe 1992; Coffey et al. 2009) and after 2 or 3 days the same B cell clone can opt for two different pathways (Jacob et al. 1991a; Jacob and Kelsoe 1992). A fraction of B cells migrates to the extrafollicular (EF) areas – the areas between the T-zone and the red pulp in the spleen, known as bridging channels, or the medullary cords in lymph nodes – where they begin to secrete low-affinity antibodies as short-lived plasmablasts (MacLennan et al. 2003; Radbruch et al. 2006). This wave of antibody production has an early protective role against the pathogen and textbooks describe it as being IgM-dominated (Murphy and Weaver 2012). Plasmablasts growing in EF foci die within days, although direct interaction with CD11c^{high} DCs can boost their survival (García De Vinuesa et al. 1999). In some

cases, plasmablasts can also enter long-lived niches within the spleen close to collagen bonds (Sze et al. 2000)

Alternatively, a subpopulation of activated B cells along with their cognate T cells gain access to the centre of the follicle to form GCs, where B cell undergo SHM and affinity maturation to further diversify the antibody repertoire (MacLennan 1994; Victora and Nussenzweig 2012). During this step of follicular colonisation, GC B cells displace naïve B cells to the periphery of the follicle, which will form the mantle zone around the GC (MacLennan 1994; Vinuesa et al. 2010). In the case of model antigens when T-cell help is readily available, early GCs first appear around day 4 after immunisation (Jacob et al. 1991a). From days 5-7 post-immunisation, GCs become fully established and functionally polarised into DZ and LZ areas (De Silva and Klein 2015). Eventually, GC B cells differentiate into high-affinity antibody secreting plasma cells (PC) or memory B cells, which provide long-lasting immunity and rapid protection of greater magnitude during antigen re-exposure (Chan and Brink 2012; Shlomchik and Weisel 2012).

Although much has been learnt in recent years about the requirement of the transcriptional repressor B cell lymphoma 6 (BCL6) for GC formation, more research is needed to understand the molecular mechanisms instructing the migration of pre-GC B cells to the centre of the follicles (Ye et al. 1993; Dent et al. 1997; Kitano et al. 2011). In this line, certain G protein-coupled receptors (GPCRs) have been shown to contribute to the modulations of this process (Pereira et al. 2010). For instance, Ebi2 (Epstein-Barr virus induced molecule-2), which is expressed in naïve B cells and increases early after antigen activation, attracts B cells to the periphery of the follicle. However, Ebi2 is downregulated in GC B cells re-routing their course back to the centre of the follicle to become GC B cells (Gatto et al. 2009a; Pereira et al. 2009). Of note, deficiency of Ebi2 in mice leads to a reduction in the early antibody response to a TD-antigen (Pereira et al. 2009). In addition to Ebi2, $S1P_2$ (sphingosine 1-phosphate type 2) receptor is

upregulated by GC B cells, promoting their clustering towards the centre of the follicle (Pereira et al. 2010). This migration has been shown to be guided to those areas with low levels of its ligand S1P (Green et al. 2011).

Commitment to EF foci or GCs are the most well-known cell fates during B cell activation. A third B cell differentiation program into early memory B cells has also been described (Taylor et al. 2012; Kaji et al. 2012). Work conducted by the Jenkins group identified a pool of precursor cells that became memory B cells independently of GCs. This subset is characterised as non-class switched $CD73^-$ $GL7^+$ $CD38^{hi}$, with minimal if any BCR mutations that rapidly respond to antigen stimulation during a primary response (Taylor et al. 2012). These cells can form equally well from $Bcl6^{-/-}$ and wild type (WT) precursors; however, only WT B cells can join existing GC reactions and later give rise to class-switched memory B cells (Taylor et al. 2012).

Summarising, this set of complex activation processes is the basis for TD-immune responses against protein antigens. However, B cell activation has also been noted in responses that do not depended on help provided by T cells, also known as thymus or T cell independent (TI)-responses. Formation of GCs with TI-antigens, *e.g.*, NP-Ficoll, requires extensive cross-linking of the BCR as well as higher frequencies of high-affinity B cells (Vinuesa et al. 2000). Although normal in appearance, GCs generates under these conditions are short-lived and neither undergo affinity maturation nor produce plasma cells nor memory B cells, and synchronously abort at the time centrocytes should seek T_{FH} help within GCs (Vinuesa et al. 2000).

1.3.2 Germinal centres

Germinal centres are specialised micro-environments critical for the formation of long-lived plasma cells and memory B cells. In the spleen and other SLOs,

GCs are formed upon immunisation in the centre of primary follicles (MacLennan 1994; Chan and Brink 2012; Victora and Nussenzweig 2012). Within GCs, B cells undergo SHM and clonal selection based on the affinity of the BCR for the immunising antigen. This process called affinity maturation leads to the formation of high-affinity antibodies needed for adequate host protection against invading pathogens (Jacob et al. 1991b; Chan and Brink 2012; Di Noia and Neuberger 2007).

GCs are heterogenous compartments where multiple cell subsets coexist. FDCs are one the main populations that reside within GCs (Heesters et al. 2014). They develop from vascular mural cells and are fundamental for the correct formation of GCs (Krautler et al. 2012). Among their functions, FDCs deliver survival signals to B cells and chemoattractant molecules, such as CXCL13, needed for follicular migration of T and B cells (Aguzzi et al. 2014). They also act as organisational stroma maintaining the right architecture of the follicle, allowing B cells to aggregate in the right conformation (Wang et al. 2011). However, the main role of FDCs is undoubtedly the capture of antigen in the form of immune complexes. These complexes are stable over time providing long-term retention of antigens for B cell activation and correct affinity maturation (Aguzzi et al. 2014; Heesters et al. 2014).

Initiation of the GC reaction is a highly coordinated cascade of events that primarily depends on the master transcription factor BCL6 (reviewed below) (Dent et al. 1997; Ye et al. 1997). Micro-anatomically mature GCs are divided into two compartments or zones (Allen et al. 2007a; Victora and Mesin 2014). A dense area where B cells proliferate intensively and somatically mutate their BCRs, known as the dark zone (DZ), and a selection zone, called the light zone (LZ), where B cells can find and retrieve antigen from FDCs to seek help from T follicular helper (T_{FH}) cells to undergo affinity-based selection (Allen et al. 2007a; Chan and Brink 2012; Meyer-Hermann et al. 2012; Mesin et al. 2016). T_{FH} cells

are a subpopulation of effector CD4$^+$ T cells specialised in helping B cells within GCs (Crotty 2011; Vinuesa et al. 2016).

GC B cells in the DZ are also known as centroblasts, whereas LZ B cells are known as centrocytes (MacLennan 1994). GCs are mainly composed of proliferating cells, approximately 2/3 of the GC B cells are centroblasts, which are among the fastest proliferating cells with cell cycles between 6 to 8h (Liu et al. 1991). Interzonal movement across DZ and LZ is needed for correct affinity-maturation to occur (Kepler and Perelson 1993; Allen et al. 2007b). This migration is guided by a gradient of chemokines, which are secreted by specific stromal cells located in each zone (Ansel et al. 2000; Allen et al. 2004). GC B cells positively selected in the LZ can re-enter the DZ to further diversify their BCR, those clones counter-selected die *in situ* and are engulfed by tingible body macrophages (Fliedner 1967). Alternatively, a subset of LZ cells leave the GC reaction as either plasma cells or memory B cells.

Plasma cells are terminally differentiated B cells specialised in antibody secretion. Generation of PCs depends on B-lymphocyte-induced maturation protein 1 (BLIMP1) along with interferon-regulatory factor 4 (IRF4) and X-box-binding protein 1 (XBP1) (Angelin-Duclos et al. 2000; Iwakoshi et al. 2003b; Nutt et al. 2015). Together, these transcription factors shape the genetic program of PCs towards secretion of Igs, repression of other lineage-determining transcription factors and expression of genes required for migration to long-lived niches in the bone marrow (Radbruch et al. 2006; Nutt et al. 2015). For instance, to maintain PC identity BLIMP1 represses *Bcl6* and *Pax5* (Lin et al. 2002; Shaffer et al. 2002). On the other hand, BLIMP1 also upregulates XBP1, which is a mediator of the unfolded protein response (UPR), needed to cope with the metabolic stress caused by the high rate of antibody production (Iwakoshi et al. 2003a).

In the case of memory B cells, they represent a heterogenous subset of antigen-experienced cells that provides rapid antibody protection upon re-challenge with

previously encountered pathogens (McHeyzer-Williams et al. 2011; Shlomchik and Weisel 2012; Kurosaki et al. 2015). To date, no specific transcription factor has been described for memory B cells. Instead, their identification relies on functional properties, such as expression of class-switched (IgM$^-$ IgD$^-$) antibodies, rapid expansion after antigen re-exposure and evidence of somatic hypermutation in the IgV region (indicative of a GC origin) (Anderson et al. 2007; Weisel and Shlomchik 2017). However, several studies have noted the existence of a diverse pool of memory B cells without any of these attributes (Anderson et al. 2007; Dogan et al. 2009; Taylor et al. 2012). The lack of a definitive memory B cell marker has been a major limitation to study this population. However, correlation of some markers has been found under different settings. Changes in expression of BACH2, CD21, CD80, PD-L2, CD73 and CD27 (in humans) are widely used to discriminate memory B cells from other subsets (Zuccarino-Catania et al. 2014; Shinnakasu et al. 2016; Weisel and Shlomchik 2017).

In summary, GCs provide a unique environment where BCR diversification through SHM takes place accompanied by clonal selection. In this selection process, clones bearing low-affinity or autoreactive BCRs are outcompeted by high-affinity clones. In this manner, GCs drive the evolution of the antibody repertoire to the next level, allowing the generation of highly specific long-lived plasma cells and memory B cells (Radbruch et al. 2006; Shlomchik and Weisel 2012). These two subsets constitute a pool of experienced antigen-specificities that can rapidly react during reinfection, establishing a sophisticated arm of long-lasting protection in the host (Zhang et al. 2016; Inoue et al. 2018).

1.3.3 Phenotype of GC B cells

Histologically, mouse GCs have classically been characterised as regions that are enriched for binding to peanut agglutinin (PNA) (Rose et al. 1980),

surrounded by IgD$^+$ follicular mantles and adjacent to the T zones. Likewise, in both mouse and human, they can be recognised as sites of intensive proliferation using BrdU stain or expression of cell cycle molecules, such as Ki67 (Liu et al. 1991; Liu and Arpin 1997). The dense network of FCDs is also used to localised GCs by formation of antigen depots or direct staining with CD23 and intense CD21 labelling (Hardie et al. 1993; Fischer et al. 1998). The presence of a specific subset of phagocytic cells – the tingible body macrophages – are also used to delimit GCs (Fliedner 1967).

By flow cytometry, mouse GC B cells are identified as B220$^+$ cells expressing high levels of Fas and GL-7, with downregulation of CD38 and loss of surface IgD (Smith et al. 1995; Liu and Arpin 1997; Oliver et al. 1997; Naito et al. 2007). In humans, GC B cells are CD19$^+$ B cells that have lost IgD, have upregulated CD38 and lack CD27 expression. In general, the level of any surface Ig is lower in GC B cells compared to naïve, follicular mantle or marginal zone B cells (MacLennan 1994). Another unique feature is the vast amount of the protein activation-induced deaminase (AID) produced by GC B cells. AID deaminates deoxycytidine residues in the IgV- and IgH-regions during SHM and CSR of Ig genes (Vaidyanathan et al. 2014). However, formation of BCL6 is the key hallmark to identify the GC phenotye in both mouse and human GC B cells (Dent et al. 1997; Ye et al. 1997; Basso and Dalla-Favera 2010).

Dissection of human GC B cells into centroblasts (DZ) and centrocytes (LZ) was traditionally performed using surface expression of CD77. These subsets were primarily identified in humans as CD38$^+$ IgD$^-$ CD77$^+$ DZ cells or CD38$^+$ IgD$^-$ CD77$^-$ LZ cells (Pascual et al. 1994). However, different studies have reported poor resolution to discriminate these subpopulations when CD77 is used (Klein et al. 2003; Högerkorp and Borrebaeck 2006), as CD77$^-$ cells have been shown to contained a mixture of centroblasts, centrocytes and even plasma cells (Högerkorp and Borrebaeck 2006). Furthermore, human naïve B cells become CD38$^+$ IgD$^-$

CD77$^-$ soon after activation *in vitro* (Gagro et al. 2003), suggesting that this marker combination does not reliably identify LZ B cells.

These and other studies were critical in leading the field to search and validate new molecular markers to identify GC B cells in both mouse and human (Klein et al. 2003; Victora and Nussenzweig 2012). Using mouse models and chemical approaches, CXCR4 was shown to be important in the structural organisation of GCs (Allen et al. 2004). Then, studies using *in situ* photoactivation of GC B cells coupled to transcriptional profiling of these cells identified CD83 and CD86 as two new centroblast/centrocytes markers (Victora et al. 2010). Today, the consensus is that DZ B cells can be identified by flow cytometry as CXCR4^{hi} CD83^{lo} CD86^{lo}, and LZ B cells as CXCR4^{lo} CD83^{hi} CD86^{hi} (Allen et al. 2004; Victora et al. 2010).

The phenotypical description of DZ and LZ cells bears important consequences for the correct interpretation of the different processes occurring in GCs. With regard to the content of this thesis, it is important to point out that some of the key studies aimed to decipher the molecular network behind mechanisms associated with GCs, such as CSR, were conceived in experiments using cell subsets discriminated by CD77 (Liu et al. 1996).

1.3.4 GCs are polarised structures: DZ and LZ

The functional polarisation between two anatomically distinct areas (DZ and LZ) is the basis for the dramatic increment in affinity observed in the antibody titre over time. This phenomenon called affinity maturation is a unique feature of GCs. The classical model, and its regulation in GCs, was first conceived by Ian MacLennan (MacLennan 1994). GC B cells positioned in the DZ (centroblasts) undergo clonal expansion and SHM; then, these cells move to the LZ (centrocytes) where BCR affinity for the immunising antigen is tested. Those clones with the highest affinity can retrieve more antigen from FCDs and eventually present

more antigen peptides to T_{FH} cells. Competition for limiting T_{FH} cell help results ultimately in selection of the clones with the highest-affinity (Victora and Nussenzweig 2012).

Iterative rounds of proliferation and selection between the DZ and LZ are required for correct affinity maturation (Kepler and Perelson 1993). However, the clues and signals needed for proper transit between these two zones still remain partially understood. Studies conducted by the Cyster group have highlighted the relevance of the chemokine receptor CXCR4 to retained GC B cells in the DZ (Allen et al. 2004). Centroblasts expressing CXCR4 are attracted chemotactically to the DZ following a gradient of CXCL12, where a dense network of resident reticular cells is likely to be the main source of this chemokine (Allen et al. 2004; Bannard et al. 2013). Genetic or chemical disruption of CXCR4 is sufficient to alter the DZ/LZ organisation, impairing the correct segregation of GC B cells across these two areas (Allen et al. 2004).

On the other hand, centrocytes respond to the chemokine CXCL13, which binds to the receptor CXCR5. CXCL13 is produced in large quantities by FCDs in the LZ, guiding the migration of centrocytes to this area (Allen et al. 2004). Interestingly, the expression of CXCR5 is very similar in centroblats and centrocytes (Allen et al. 2004). Therefore, it has been proposed that as centroblasts begin to differentiate into centrocytes, CXCR4 expression is downregulated favouring the responsiveness towards CXCL13 (Allen et al. 2004). Although small, GCs still can be found in mice deficient for CXCR5 or CXCL13 (Ansel et al. 2000; Voigt et al. 2000); however, with atypical architectures. For instance, CXCL13$^{-/-}$ mice possess GCs with LZ areas in random orientations, located near the centre of the GC or close to the T-zone regions (Allen et al. 2004).

As mentioned before, despite the key roles of CXC4 and CXCR5, functional GCs capable of undergoing affinity maturation can still be found even in the absence of these receptors (Allen et al. 2004; Voigt et al. 2000), emphasising

the presence of additional or redundant mechanisms controlling the formation of GCs and the interzonal migration across the DZ/LZ (Mesin et al. 2016). Other contributors involved in the initiation and establishment of GCs are: IRF4, MEF2B, MEF2C, Ebi2, and SPR1 (De Silva and Klein 2015).

More recently, two studies have addressed the role of the transcription factor FOXO1 in instructing GC polarisation. In Foxo1$^{\text{flox/flox}}$:Cγ1Cre mice, where FOXO1 is selectively deleted in activated B cells induced to switch to IgG1, GCs were characterised as LZ-only structures after immunisation with sheep red blood cells (SRBCs) (Dominguez-Sola et al. 2015; Sander et al. 2015). In wild type GCs, formation of FOXO1 is restricted to the DZ, suggesting that this transcription factor is needed for the correct organisation and segregation of centroblasts. Besides, mice lacking FOXO1 possess impaired SHM and reduced CSR to IgG1 (Dominguez-Sola et al. 2015; Sander et al. 2015).

In summary, the functional polarisation of GCs relies primarily in the balance between CXCR4 and CXCR5 through the gradient of the chemokines CXCL12 and CXCL13, including the help of different proteins and transcription factors.

1.3.5 BCL6 drives the GC transcriptional program

In 1993, genetic studies identified the transcriptional repressor BCL6 as a pathogenic gene frequently translocated in diffuse large B-cell lymphoma (DLBCL) cell lines. Chromosomal breakpoints in BCL6 lead to its juxtaposition onto the IgH and IgL chain loci (Baron et al. 1993; Ye et al. 1993). Soon after its discovery, it was shown that BCL6 is required for B cells to adopt a GC-program, since Bcl6$^{-/-}$ mice failed to develop GCs and high-affinity antibodies (Dent et al. 1997; Ye et al. 1997). In 2009, this functional description was extended by the work of three different groups (including our own), showing that BCL6 is also essential for a specific group of CD4$^+$ T cells, namely T$_{\text{FH}}$ cells, to follow the GC pathway. In

this manner, T_{FH} cells were accepted as an entirely new T cell lineage (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009).

BCL6 belongs to the BTB/POZ/ZincFinger (ZF) family of transcription factors. In humans, the BCL6 gene is located on chromosome 3 and encodes a 95 kDa protein. Structurally, BCL6 consists of a N-terminal BTB/POZ domain, a central region containing PEST motifs and six C-terminal ZF DNA-binding motifs (Chang et al. 1996; Huang and Melnick 2015). The C-terminal region recognises specific BCL6-bindings motifs in the promoter region of target genes, whereas the transcriptional repressor activity of BCL6 is exerted by its BTB domain (Huang and Melnick 2015). The BTB region acts as a scaffold domain that allows the interaction with different co-repressor proteins (Parekh et al. 2007). These co-repressor partners include: NCOR1, NCOR2, BCOR, CTBP1 and MTA3, which are thought to be important in fine-tuning the regulatory activity of BCL6 in different pathways and cell types (Huynh and Bardwell 1998).

Chromatin Immunoprecipitation (ChIP)-on-chip studies have revealed the complexity of the gene network regulated by BCL6. With over 3000 genes being physically bound in primary GC B cells and DLBCL cell lines (Ci et al. 2009), BCL6 is involved is multiple cellular processes including GC-development, DNA damage response, cell cycle arrest, and apoptosis (Basso and Dalla-Favera 2010). Although, it is still uncertain the precise number of genes regulated by BCL6 (Basso et al. 2010), its transcriptional program under normal physiological conditions and disease is becoming more clear. BCL6-mediated regulation is likely to be influenced not only by its co-factors, but also by the action of other transcription factors. Indeed, several studies have also identified the presence of DNA-binding domains for other transcription factors near the BCL6-binding domain of many genes. This includes binding sites for MYB and IRF8 (both involved in GC-development) and antagonist of BCL6, such as IRF4 and different STAT-members (Basso et al. 2010). These findings might represent a new layer of

regulation to modulate the activity of BCL6 (Basso and Dalla-Favera 2012).

BCL6 plays many key roles in the development and function of GC B cells. Some of the most relevant functions of BCL6 are: i) BCL6 increases the tolerance to DNA damage needed for adequate Ig somatic mutation driven by AID and facilitates the proliferation of DZ cells. This state is maintained through the negative regulation of key members of the DNA damage response, such as TP53, CDKN1A, ATR, and CHEK1 (Phan et al. 2005; Parekh et al. 2007; Ranuncolo et al. 2007; Ranuncolo et al. 2008); ii) BCL6 downregulates BCL2 expression. This proapoptotic state of GC B cells represents a safeguard against the loss of reactivity or acquisition of self-reactivity. Thus, GC B cells will die by default if not rescued by positive BCR-stimulation, or T cell-derived signals during the LZ selection process (Saito et al. 2009); iii) BCL6 inhibits the plasma cell differentiation program by direct repression of *Blimp1* (Shaffer et al. 2000; Tunyaplin et al. 2004). This last point may be important to block the premature exit of B cells from the GC reaction as plasma cells (Shaffer et al. 2000; Basso and Dalla-Favera 2012).

Although BCL6 production is restricted to GC B cells and T_{FH} cells, naïve B cells also show a basal level of mRNA expression without protein formation (Basso and Dalla-Favera 2012). This raises some interesting questions regarding induction and regulation of BCL6 in B cells. Surprisingly, despite the large body of evidence supporting BCL6 activity in GC formation, the signals needed for its induction *in vivo* still remain largely unknown. ChIP analyses have shown that IRF8 binds the BCL6-promoter and is able to induce *Bcl6* expression; however, this effect is only partial as unimmunised IRF8$^{-/-}$ mice still possess a small population of BCL6^{+} PNA^{+} cells and disorganised GC structures (Lee et al. 2006). Downregulation of Ebi-2 has also been shown to facilitate BCL6 formation (Gatto et al. 2009b; Pereira et al. 2010). Regarding post-translational regulation, several mechanisms have been described affecting BCL6 stability and degradation including acetylation of the PEST domain along with different pathways inducing the ubiquitin-proteasome

system. These pathways involve BCR-MAPK signalling, DNA damage inducing ATM-activation and FBXO11-signalling through the SKP1-CUL1-SCF complex (Niu et al. 1998; Phan et al. 2007; Duan et al. 2012).

1.3.6 Affinity maturation

The antibody responses elicited via GCs are characterised by extremely high affinity and specificity (Shlomchik 2003). Compared to late or secondary immune responses it's well documented that early antibody responses are of lower affinity. By contrast, increments of 10,000-folds in the affinity against hapten-conjugates have been reported after the GC reaction has been established (Eisen and Siskind 1964). So, how can this microenvironment shape immune responses in such an astonishing manner?

The clonal selection theory proposed by Burnet in 1957 and the VDJ recombination of BCR genes partially explain the vast number of antigenic-specificities found in the immune system. However, these do not resolve the increment in affinity and specificity observed over time after antigen exposure (Victora and Nussenzweig 2012). In 1993, Perelson and colleagues theorised the cyclic re-entry model (Kepler and Perelson 1993), which proposed that GC B cells undergo iterative cycles of selection and mutation migrating between the DZ and LZ. Thus, positively selected B cells in the LZ – those clones bearing the BCRs with the highest affinity – would move back to the DZ to undergo further rounds of proliferation/mutation and then return to the LZ in a cyclic manner. This system of Darwinian-like selection coupled to SHM explained the remarkable increment in Ig affinity observed during a GC response (Kepler and Perelson 1993; Chan and Brink 2012; Meyer-Hermann et al. 2012)

Despite the elegance of this model, scepticism was found among scholars. Experimental data supporting the cyclic re-entry model came later by three

independent groups using *in vivo* imaging of GC B cells (Allen et al. 2007b; Hauser et al. 2007; Schwickert et al. 2007). However, further validation was needed due to technical limitations, such as the low number of events analysed and the short window of time the cells were imaged (Victora and Nussenzweig 2012). Definitive data supporting these studies were provided in 2010 by the Nussenzweig group. Using *in situ* photoactivation along with mathematical simulations of the data, it was determined a net vector of interzone migration from DZ to LZ, with migration rates of 15%/hr for DZ to LZ and 3%/hr for LZ to DZ (Victora et al. 2010). This implies approximately 10-30% of GC B cells re-entering the DZ after each round of selection in the LZ (Victora et al. 2010; Meyer-Hermann et al. 2012; Mesin et al. 2016).

1.3.7 Role of T_{FH} cell help in clonal selection

An interesting question arising from the previous studies is how clonal selection occurs in the context of a cyclic re-entry model. Different theories have been postulated, such as competition for limiting amount of antigen on the surface of FDCs, antigen masking by secreted antibodies, or a refractory time for centrocytes to retrieve antigen from FDCs (Iber and Maini 2002; Meyer-Hermann et al. 2006; Meyer-Hermann et al. 2009). However, mathematical modelling predicted that the dominant factor driving affinity-based selection in the LZ is the competition between B cell clones for limited T cell help (Meyer-Hermann et al. 2012). This hypothesis was tested using anti-DEC-205 antibodies loaded with protein antigens[3]. In this system antigen is not limiting and its capture and presentation does not depend on BCR-affinity or cross-linking (Victora et al. 2010). As expected, in mice transferred with both $DEC205^{+/+}$ and $DEC205^{-/-}$ cells, GCs were mainly composed of $DEC205^{+/+}$ cells. These cells were able to survive, proliferate and re-cycle within the DZ/LZ, whereas $DEC205^{-/-}$ cells were

counter-selected (Victora et al. 2010). Moreover, the number of cell divisions occurring in the DZ were directly proportional to the amount of antigen captured and presented by DEC205$^{+/+}$ to T$_{FH}$ cells in the LZ (Gitlin et al. 2014).

These findings have led to a new understating of selection in GCs. It is hypothesised that cognate interaction with T$_{FH}$ cells not only provides the survival signals needed to rescue B cells from apoptosis, but it also sets an internal "timer" in positively selected LZ B cells that determines the extent of clonal expansion and SHM in the DZ (Gitlin et al. 2014; Victora and Mesin 2014). The molecular mechanism controlling this "timer" has been associated to the regulation of different transcription factors. In this line, Hodgkin and colleagues have proposed that proliferation and death are cell-intrinsic timed fates in lymphocytes (Heinzel et al. 2017). In the case of proliferation, cells are timing divisions rather than "counting" them modulated by c-MYC. When cell cultures were treated with drugs that slowed down the rate of proliferation the "time-to-divide" was unaffected, whereas the total number of cell divisions was reduced. These results are explained by timed changes in the production rate of c-MYC, which control the size of the proliferative response in T and B cells (Heinzel et al. 2017).

1.3.8 Role of c-MYC in GC B cell proliferation and maintenance

In GC B cells, c-MYC has critical roles for the initiation and maintenance of GCs (Calado et al. 2012; Dominguez-Sola et al. 2012). Transgenic c-Myc-GFP-reporter mice identified a small subpopulation of GC B cells ($\sim$5-10%) expressing c-MYC in mature GCs (Dominguez-Sola et al. 2012). Induction of

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[3]DEC-205 is a surface lectin expressed by GC B cells that can uptake and deliver antigens to endosomal vesicles rich in MHC-II molecules. This pathway allows antigen presentation in a BCR-independent manner (Victora et al. 2010).

c-MYC occurs transiently in positively selected LZ cells, preparing these cells to re-enter cell cycle in the DZ. Then, c-MYC is repressed by Bcl6 in the DZ, allowing these cells to return to the LZ (Dominguez-Sola et al. 2012). More recently, it has been shown that c-MYC$^+$ GC B cells possess low rate of apoptosis. This protection from dead was associated with positive selection within GCs (Mayer et al. 2017). Downregulation of c-MYC in the DZ seems odd considering the high rate of proliferation of centroblast; however, this is consistent with the "time-to-divide" model and the idea of an intrinsic timer modulated by c-MYC formation (Dominguez-Sola et al. 2012; Heinzel et al. 2017).

c-MYC contributes to sustain proliferation of DZ B cells, in part, through its ability to induce the transcription factor activating enhancer-binding protein (AP4). APA4 is induced by c-MYC in LZ cells that have received cognate stimulation (Chou et al. 2016). In addition, AP4-null mice possess small GCs with impaired proliferation of centroblasts, leading to defects in affinity maturation of antibodies against LCMV (Chou et al. 2016). Recent reports show evidence that polypyrimidine tract-binding protein 1 (PTBP1), and RNA-binding protein, is also needed for correct expression of c-MYC in LZ cells as well as proper alternative splicing of transcripts needed for proliferation in the DZ (Monzón-Casanova et al. 2018). In summary, the biphasic regulation of c-MYC aided by APA4 and PTBP1 might be vital to regulate the "timer" that switches the DZ/LZ phenotypes during selection in the GC (Victora and Mesin 2014; Heinzel et al. 2017).

The idea of intrinsic timers controlling proliferation within GCs might resemble circadian clocks found in many organisms and cells types, where genes are expressed rhythmically in cycles of 24h or other time intervals modulated by environmental factors, such as light, temperature or nutrients (Scheiermann et al. 2018). In the filamentous fungus *Neurospora crassa*, the FRQ/WC oscillator regulates cell cycle via *skt-9* gene expression (Hong et al. 2014), a homolog of the CDK1 inhibitor WEE1 kinase previously showed to modulate proliferation of liver

cells driven by a circadian clock in mouse (Matsuo et al. 2003).

The presence of intrinsic clocks has also been demonstrated in different types of immune cells (Scheiermann et al. 2018). For instance, the number of lymphocytes in circulation oscillates during the course of the day, with a peak during the resting phase of an organism (daytime for rodents) (Scheiermann et al. 2018). In T cells, this has been linked to rhythmic expression of *Ccr7* and *S1pr1* genes, which oscillate in a time-of-day-dependent manner. These two genes showed opposite phases, being *Ccr7* (lymph node retention) expressed mainly at night and *S1pr1* (lymph node egress) during the day (Druzd et al. 2017). Additionally, targeted disruption of the brain and muscle ARNT-like 1 (BMAL1) protein – a transcription factor critical to modulate the mammalian circadian oscillator as part of the CLOCK-BMAL1 protein complex – severely impaired the rhythmic homing and egress of T cells (Druzd et al. 2017).

1.3.9 Role of BCR-signalling in c-MYC induction and GC B cell selection

Recent studies have shown that BCR-signalling plays a novel role in achieving maximal c-MYC expression in GC B cells, thereby inducing proper LZ/DZ recycling and adequate selection within GCs (Luo et al. 2018). Shlomchik and colleagues reported that GC B cells can reprogram the effector pathways downstream of the BCR and CD40 compared to naïve B cells.

In normal conditions, either CD40 or BCR-signalling are equally capable of activating both PI3K-AKT and NF-κB pathways leading to c-MYC formation in naïve B cells (Luo et al. 2018). In contrast, GC B cells treated with anti-CD40 failed to induce PI3K and AKT kinases, and therefore there was no upregulation of c-MYC or increased activation of FOXO1. Nevertheless NF-κB activation remained unaffected. However, this did not result in c-MYC upregulation or

increased activation of FOXO1. Conversely, BCR stimulation alone did not induce NF-κB translocation to the nucleus, and this was independent of the isotype expressed, IgM or IgG. Although attenuated, signalling through the BCR led to transient and weak phosphorylation of Syk and Btk kinases. Surprisingly, this effect was sufficient to phosphorylate and inactivate FOXO1 in DZ cells by activation of the Syk-PI3K-AKT pathway (Khalil et al. 2012; Luo et al. 2018). More importantly, upregulation of c-MYC in GC B cells was only observed when the BCR and CD40 were both stimulated (Luo et al. 2018).

Taken together, these observations provide an interesting re-interpretation of how DZ/LZ cyclic reentry and selection might work in GCs, where the contribution of BCR engagement is not just relegated to capturing and processing antigens to compete for limiting survival signals derived from T$_{FH}$ cells (Luo et al. 2018). In this new scenario, survival, selection and the correct migration of GC B cells is directly linked to BCR-activation, which when coupled to CD40 stimulation during cognate interaction with T$_{FH}$ cells results in a synergistic cooperation that modulates the transcriptional program of GC B cells (Luo et al. 2018). In this manner, rewiring the signals delivered via BCR and CD40 receptor adds an additional layer of control within GCs, as the need for two signals to converge in time and location impose more stringent conditions for correct clonal selection (Luo et al. 2018).

1.3.10   T$_{FH}$ cells

A crucial step for adequate humoral responses is the activation of antigen-specific naïve T cells. CD4$^+$ T cells are first primed in the T-cell area by DCs, where stable contacts are formed through the TCR with peptide:MHC-II complexes (Deenick and Ma 2011; DuPage and Bluestone 2016). DCs also provide accessory signals, such as cytokines and costimulation via CD28:B7.1/B7.2 interactions,

needed for correct T-cell activation (Choi et al. 2011; Goenka et al. 2011; DuPage and Bluestone 2016). Among activated CD4$^+$ T cells, a distinct subset, namely T_{FH} cells, becomes specialised in supporting B cells in their differentiation, formation of GCs and selection within GCs (Crotty 2011; Vinuesa et al. 2016).

In order to promote B cell responses, T_{FH} cells must access the T:B border after being primed by DCs. This process is chemotactically orchestrated by downregulation of the C-C chemokine receptor type 7 (CCR7) and up-regulation of the follicular homing receptor CXCR5 (Hardtke et al. 2005), which responds to the chemokines CCL19/21 and CXCL13, respectively. Expression of these receptors is intimately ligated to the formation of BCL6 and more specifically to the interplay between the transcription factors achaete-scute homologue 2 (ASCL2) and Krüppel-like factor 2 (KLF2) (Deenick and Ma 2011). For decades, it was known that T cells expressing CXCR5 were preferentially recruited to GCs and found to be engaged with cognate B cells (Breitfeld et al. 2000; Schaerli et al. 2000). However, the presence of a transcription factor driving a T_{FH} differentiation program remained controversial; hence denying the lineage status to T_{FH} cells (Crotty 2014; Vinuesa et al. 2016).

Doubts about the nature of T_{FH} cells as an independent T cell subset were resolved in 2009 by three separate groups (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009). Similar to B cells, commitment to the GC pathway by T_{FH} cells depends on the master transcription factor BCL6 (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009). In the absence of BCL6, formation of T_{FH} cells is severely impaired, whereas its overexpression drives normal T_{FH} differentiation *in vivo* (Johnston et al. 2009; Crotty et al. 2010). Upon formation in T_{FH} cells, BCL6 antagonises all the classical T cell effector subsets: T_{H1}, T_{H2} and T_{H17}, by negatively regulating Prdm1 (encoding BLIMP1) and the transcription factors that genetically program these populations: T-BET, GATA3 and RORγt, respectively (Hatzi et al. 2015). However, this effect is partial in many situations, as T_{FH} cells

are able to express the signature cytokines of other T cell populations, *e.g.*, INF-γ, IL-4 or IL-17, although this is modulated as they become GC T_{FH} cells. This incredible plasticity allows T_{FH} cells to adapt to a variety of needs and pathogens, but it was a major criticism that also questioned the nature of these cells as a unique subset (Zhu et al. 2010).

T_{FH} cell formation is a multistep process driven primarily by BCL6. As revealed by Bcl6$^{YFP/+}$ reporter mice, formation of BCL6 follows waves during T_{FH} cell development (Baumjohann et al. 2011; Choi et al. 2011; Kitano et al. 2011). Early upregulation of this transcriptional repressor depends on inducible costimulator (ICOS) signalling during the initial priming stage by DCs in the T-cell area (Choi et al. 2011). Consistent with this observation, T cells lacking ICOS molecules fail to develop T_{FH} cells after lymphocytic choriomeningitis virus (LCMV) infection (Choi et al. 2011). A second wave of Bcl6 coincides with the induction of CXCR5 expression, reinforcing and sustaining the T_{FH} transcriptional program prior to cognate interaction with B cells at the T:B border (Baumjohann et al. 2011). A third and final wave takes place within GCs through direct interaction with GC B cells (Baumjohann et al. 2011). Thus, the T_{FH} gene program is established early on during immune activation and maintained at different stages before and during GC formation (Crotty 2014; Vinuesa et al. 2016).

In addition to BCL6, immunophenotyping of mouse and human identified T_{FH} cells as a distinctive CD44^{hi} CXCR5^{hi} ICOS^{hi} PD1^{hi} subset (Dorfman et al. 2006), being CXCR5 and PD1 the most commonly surface markers used experimentally. T_{FH} formation is cell contact-dependent and location-sensitive (Qi 2016). Its development in mice depends on IL6- and IL21-signalling through STAT3 activation (Nurieva et al. 2008; Eddahri et al. 2009), which further enhances Bcl6 expression (Nurieva et al. 2008; Nurieva et al. 2009). DCs are thought to be the main source of IL-6 during immune activation, once secreted

IL-6 induces IL-21 production, reinforcing the autocrine production of IL-21 by T_{FH} cells. Consistently with this role, loss of IL-6 and IL-21 has been shown to be detrimental for the correct formation of T_{FH} cells (Eto et al. 2011; Qi 2016). In humans, IL-12, TGF-β and IL-23 have been shown to be important for T_{FH} formation (Schmitt et al. 2014).

Today, it is well accepted that T_{FH} cells shape humoral responses. BCL6 expression in T cells is required during the first T:B interaction to produce both EF- and GC-responses against protein antigens, and to instruct B cells to class switch to the appropriate isotype for optimal and adequate effector functions against pathogens (Toellner et al. 1996; Pape et al. 2003; Lee et al. 2011). In the absence of BCL6-expressing $CD4^+$ T cell, responses against HEL-SRBC or NP-CGG are characterised by a lack of extrafollicular IgM and IgG antibody responses, as well as defective GC responses (Lee et al. 2011). Absence of extrafollicular IgG responses is also observed in models using Salmonella infection (Lee et al. 2011). An exception to the requirement of T_{FH} cells for class-switched extrafollicular antibody responses was reported in the context of influenza, in which IgG2a responses where observed in mice lacking BCL6 in T cells (Miyauchi et al. 2016). Accessory signals provided by T_{FH} also induce expression of AID in activated B cells, most likely at the T:B border, and during the GC reaction to sustain SHM (Cattoretti et al. 2006).

There are some suggestions that help provided by T_{FH} cells might influence cell fate decision in B cells. A long-standing question in the field is how B cells are able to choose between an EF- or GC-pathway. A number of ideas has been proposed over the years (Vinuesa et al. 2010); with evidence that BCR affinity plays an important role (Paus et al. 2006). Also T:B interactions stabilised by the SLAM -familly members CD84 and Ly108 have been shown to be important (Cannons et al. 2010). Both receptors signal through SLAM-associated protein (SAP), which, when absent, prevent the formation of T_{FH} cells and thus GCs

but has less profound effects on the EF response (Qi et al. 2008; Linterman et al. 2009). The strength and duration of the signals transmitted via SAP in T cells, *i.e.* prolonged T:B interaction times, is likely to increase the probability of B cells entering the GC differentiation pathway (Vinuesa et al. 2010).

Although much is known about the processes regulated by T_{FH} cells, the clues and signals needed for their correct development and function are just beginning to be unravelled. For instance, our lab has recently published that human T_{FH} cells produce large amounts of the neurotransmitter dopamine. When T cells and B cells interact, dopamine is released inducing translocation of inducible T-cell co-stimulator ligand (ICOSL) within minutes to the surface of B cells. T_{FH} cells rapidly respond to ICOSL stimulation externalising more CD40L to the synaptic clef, which potentiates the immune-synapse between T cells and B cells (Papa et al. 2017). These novel role of dopamine highlights not only the complexity of T_{FH} cell helper functions to fine tune the activation of B cells, but also shows the delicate interconnection between the nervous- and the immune-system.

1.4 Class Switch Recombination

1.4.1 Overview: SHM and CSR

Germinal centres are critical in remodelling the antibody repertoire after immunisation by making it more tailored to the characteristic of the invading pathogen (Berek et al. 1991; Jacob et al. 1991b). In this process, the generation of high-affinity antibodies arises through the accumulation of point mutations in the IgV regions of both H and L chain genes. This mechanism called somatic hypermutation (SHM) was conceptualised by Lederberg in 1959 and further refined by Brenner and Milstein in 1966 (Lederberg 1959; Brenner and Milstein 1966). Today is known that SHM is initiated by the enzyme AID and occurs iteratively in DZ B cells introducing mutations at rates $\sim 10^6$ times higher than the background mutation level observed in other cell types (Pascual et al. 1994; Muramatsu et al. 2000; Di Noia and Neuberger 2007).

Although SHM preferentially targets the VDJ-coding regions – sparing the Ig promoter, other non-coding regulatory regions and even surrounding genes – this process and thus the generation of high-affinity antibodies is random in nature (Di Noia and Neuberger 2007; Maizels and Scharff 2003). This means that the introduction of mutations can equally lead to changes that maintain, improve or reduce the affinity of the antibody for the immunising antigen (Martin et al. 2015). These changes can also result in formation of non-functional antibodies by substitutions affecting the topology of the antibody or premature stop codons arising in the Ig coding sequence (Martin et al. 2015). Therefore, expansion of high-affinity clones can only occur when SHM is coupled to affinity-based selection. In these circumstances, clones bearing those mutations that result in stronger BCR:antigen binding will have a competitive advantage against low affinity clones, and thus can be positively selected (Peled et al. 2008; Chan and Brink 2012;

Victora and Mesin 2014).

A second genetic alteration aimed to modify the effector function of antibodies after antigen encounter is class switch recombination (CSR) (Coffman et al. 1993; Stavnezer 1996). CSR is a genetic rearrangement that allows IgM/IgD mature B cells to express antibodies of the IgA, IgG or IgE classes that differ in effector functions, without altering the specificity for the immunising antigen (Coffman et al. 1993; Stavnezer 1996). Initial characterisation of CSR came from studies where the serum antibody titre of immunised animals shifted from being predominately IgM to be dominated by the IgG classes over time (Bauer et al. 1963). Unlike SHM, where the bulk of mutations are concentrated in the VDJ-segments – more specifically the complementarity determining regions (CDRs) – the CSR machinery can target the IgH locus anywhere within long intronic areas called switch (S) regions (Stavnezer et al. 2004).

Although CSR can be initiated soon after T cell priming, it is still generally accepted that CSR is a unique feature of GCs (Honjo et al. 2002; Crotty 2011). One likely reason for this (and further discuss below), may be the shared events between SHM and CSR, such as the requirement for AID protein (Muramatsu et al. 2000). In this regard, GCs provide a unique environment to withstand the high rate of mutations initiated by AID activity by modulating the DNA-damage response (Victora and Nussenzweig 2012). Other similarities are the pathways needed to repair the DNA lesions induced by AID, such as the DNA base excision repair (BER) pathway and the mismatch repair(MMR) pathway (Chaudhuri and Alt 2004; Stavnezer et al. 2008). However, growing evidence supports that SHM and CSR are two independent events (Rudikoff et al. 1984; Honjo et al. 2002; Shinkura et al. 2004).

Regardless of the differences of when and where SHM and CSR are activated. These two processes represent remarkable features of the adaptive immune system that provide a second chance to further diversify the BCR repertoire and develop

more specific antibody responses against foreign antigens (Honjo et al. 2002; Stavnezer et al. 2004).

1.4.2   CSR: molecular mechanism

During the last decades great efforts have been made in order to understand the molecular basis of Ig class switching, having mouse models a pivotal role on it. The murine IgH locus consists of eight constant heavy (C_H) genes arranged as: 5'–VDJ-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-C γ2a-Cε-Cα–3' (the human IgH locus is similarly organised but not identical) (Shimizu et al. 1981; Shimizu et al. 1982). Each C_H gene – except for Cδ, which is produced by alternative splicing from IgM transcripts – is organised into a germline transcription unit that includes a promoter, a non-coding exon (referred to as "intervening" or I exon) and a S region followed by a cluster of C_H exons (Lorenz and Radbruch 1996; Chaudhuri et al. 2007).

Isotype switching involves recombination between two S regions, highly repetitive intronic areas of variable size (∼1-12kb) and G-rich in the non-coding strand (Lorenz and Radbruch 1996; Stavnezer et al. 2004). Induction of CSR can be broadly divided into four steps (Figure 1.2): i) transcription across a donor and an acceptor S region starting from the respective I_H promoters; ii) introduction of DNA breaks anywhere within the exposed S regions. Generation of these breaks depends primarily on AID activity together with BER enzymes; iii) double-strand breaks (DSBs) in the S regions induce activation of the non-homologous end joining (NHEJ) repair system; iv) recognition and repair of DSBs deletes the intervening DNA between the two S region as an episome (switch circle), leading to juxtaposition of the VDJ-segment with a new set of C_H exons (Stavnezer et al. 2004; Cerutti 2008). As a result, CSR allows expression of antibodies with different effector and biological properties with no modifications on the specificity

for antigen (Lorenz and Radbruch 1996).

1.4.3 CSR: germline transcripts

Induction of CSR in activated B cells is driven by cytokines and T-cell derived signals, such as CD40, that are sensed by responsive elements present in the I_H promoters (Stavnezer et al. 2004). It has been observed that these regions are essential to instruct B cells to switch to particular isotypes when the right co-stimulatory signals are delivered; *e.g.*, IL-4 induces specifically IgG1 and IgE switching, while IFN-γ modules IgG3 and IgG2a formation (Isakson et al. 1982; Snapper et al. 1988; Coffman et al. 1993). In fact, targeted mutagenesis or complete replacements of the I exon promoters severely impairs CSR, demonstrating that transcriptional control over specific promoter regions can direct switching to the isotype best suited for optimal host protection (Zhang et al. 1993; Jung et al. 1993).

As depicted in Figure 1.2, switch transcription starts at the specific I_H promoter, spans the I exon, S region and terminates 3' of the respective C_H gene (Stavnezer 1996). A byproduct of this transcriptional event is the synthesis of germline transcripts (GLTs). GLTs are polyadenylated non-coding mRNAs that can undergo splicing of the intronic S region. After splicing the I exon is directly connected to the downstream C_H exons: I_H-C_H, becoming a mature GLT (Lennon and Perry 1985; Lutzker and Alt 1988). Although GLT does not contain open reading frames, deletion of the donor splice site in the $I_{\gamma 1}$ exon inhibits CSR to IgG1 (Lorenz et al. 1995). Additional studies have shown that the primary unspliced GLT can interact with the exposed S region leading to formation of R-loops. This RNA-DNA hybrids might act as substrates guiding the recruitment and assembly of the protein complexes needed to initiate isotype switching (Tian and Alt 2000; Yu et al. 2003; Chaudhuri et al. 2003). Despite these observations,
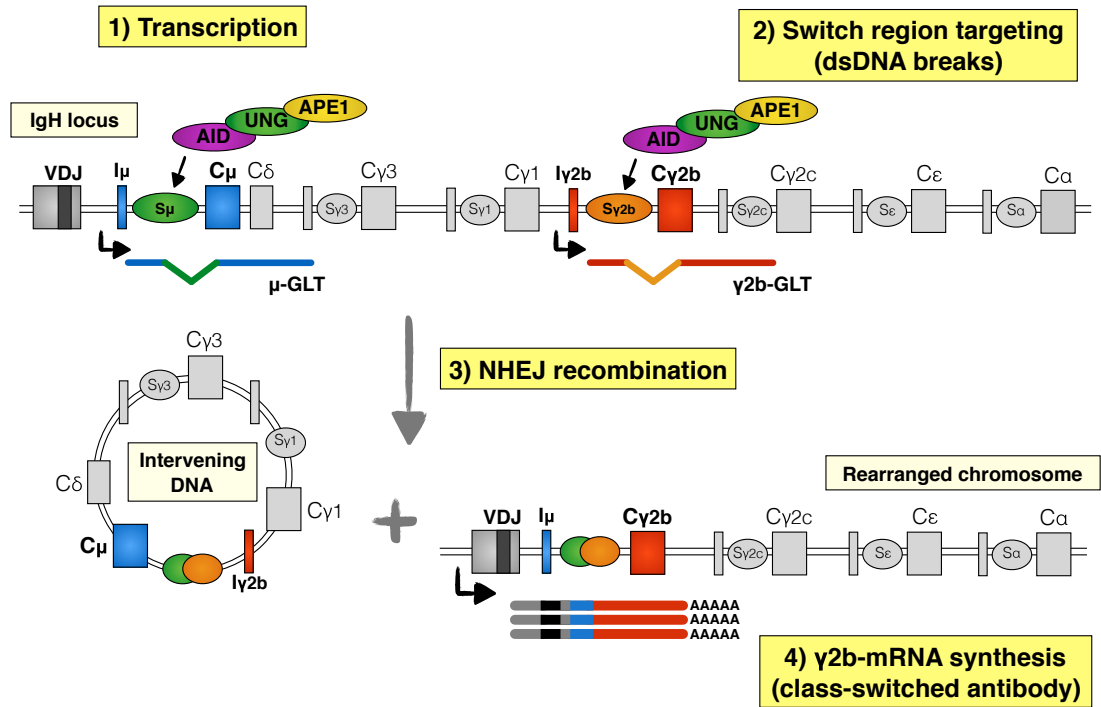
Figure 1.2. Diagram of isotype switching from IgM to IgG2b. Mature naïve B cells express IgM/IgD isotypes. CSR is triggered when antigen-activated B cells sense cytokines and T-cell help. 1) Integration of these signals leads to transcription from specific promoter upstream of the C_μ and the $C_{\gamma 2b}$ genes (black arrows), with production of μ- and γ2b-germline transcripts (GLTs); 2) AID, UNG and APE1 target the specific S regions introducing DNA-breaks; 3) these breaks are repaired by members of the NHEJ-recombination pathway resulting in recombination between S_μ and $S_{\gamma 2b}$ regions; 4) following this rearrangement switch circles are released and the VDJ-coding regions is linked to the downstream isotype leading to production of IgG2b.

the precise role of GLTs in CSR still represents a puzzling question in the field (Cogné and Birshtein 2003).

GLT appearance always precedes DNA recombination of the C_H genes in B cells primed to undergo CSR. For this reason, GLT formation has long been used as a readout to study the onset of CSR (Lorenz et al. 1995; Cogné and Birshtein 2003). Another molecular marker used to identify cells that have successfully completed recombination is the episome released after the genomic rearrangement of the IgH locus (Chaudhuri et al. 2007). This episome known as switch circle, retains the GLT-promoter of the recently switched isotype, which remains active for a short period of time producing non-coding mRNAs, namely, switch circle transcripts (SWCTs): I_H-C_μ (Kinoshita et al. 2001).

Honjo and colleagues discovered in 2001 the product of switch circles (SWCT) using the murine lymphoma cell line CH12F3-2A (Kinoshita et al. 2001). This cell line can normally switch *in vitro* from IgM to IgA after stimulation with CD40L, IL-4 and TGF-β. Since its discovery, SWCTs have been used and characterised in different systems including primary cells and human samples (Fagarasan et al. 2001; Litinskiy et al. 2002; McHeyzer-Williams et al. 2015). As a result of the transient nature of SWCTs, they are considered the gold standard to identify ongoing CSR. However, most of the work conducted has been done using cell lines or in primary cells re-stimulated *in vitro*, whereas detection of SWCTs on freshly isolated *ex vivo* samples is still challenging to assess.

1.4.4 Role of AID, UNG and APE1 in CSR

The fundamental role of AID in CSR has been examined using animal models and in human patients with type II hyper-IgM syndrome (HIGM-2), where a mutation in the AID-coding gene results in a rare immunodeficiency characterised by elevated levels of serum IgM and absence of other isotypes (Muramatsu et al.

2000; Revy et al. 2000). In general, AID deficiencies result in loss of CSR and SHM in mouse and human, and eliminate gene conversion in chickens – a process related to SHM (Arakawa et al. 2002; Muramatsu et al. 2000; Revy et al. 2000)

AID was identified via subtractive cloning in the cell line CH12F3-2A as an RNA-editing enzyme due to its homology with the RNA-editing cytidine deaminase APOBEC1 (Muramatsu et al. 1999; Stavnezer and Amemiya 2004). Subsequent studies confirmed that AID specifically targets single-stranded DNA (ssDNA) as primary substrate, catalysing the deamination of deoxycitosine (dC) into deoxyuridine (dU) (Dickerson et al. 2003; Pham et al. 2003). The dU residues left by AID are removed by uracil DNA glycosylase (UNG), forming abasic sites. Processing of these abasic sites by apurinic/apyrimidinic endonuclease 1 (APE1) leads to the generation of single-strand breaks (SSBs) in the donor and acceptor S regions. Proximal SSBs in opposite strands form DSBs that induce activation of the NHEJ repair system with subsequent deletion and recombination of the IgH locus (Chaudhuri and Alt 2004; Chaudhuri et al. 2007; Stavnezer et al. 2008; Xu et al. 2012).

To date, AID is the only enzyme found to be exclusively activated during CSR (and SHM), whereas repair of the nucleotides targeted by AID relies on ubiquitous components present in essentially all cells to cope with DNA damage (Chaudhuri et al. 2007). Different mouse models have been critical to elucidate the enzymes and pathways activated downstream of AID. For instance, out of the four uracil DNA glycosylases found in mammals, UNG is the main protein for CSR in this family (Stavnezer et al. 2008). UNG-deficient mice have a $\sim$95% reduction in CSR, and human patients with deleterious mutations in the gene encoding UNG present similar defects (Rada et al. 2002; Imai et al. 2003). After excision of the dU nucleotides the DNA backbone is targeted and cleaved by APE proteins creating SSBs (Chaudhuri et al. 2007; Stavnezer et al. 2008).

In mammals there are two main apurinic/apyrimidinic endonuclease proteins,

APE1 and APE2 (Hadi et al. 2002). Biochemical evidence suggests that APE1 accounts for >95% of the endonuclease activity of a cell, being as well the main APE protein in CSR (Wilson and Barsky 2001). Compared to APE1, APE2 has weaker endonuclase activity and its function has been associated with SHM (Burkovics et al. 2006a; Sabouri et al. 2009). Understanding the role of APE1 in CSR has been difficult to assess, in part, due to the embryonic lethality of APE1 knockout (KO) mice (Xanthoudakis et al. 1996). Despite these limitations, activated splenic B cells from haploinsufficient APE1$^{+/-}$ mice present a 20-40% reduction in CSR compared to wild type mice (Guikema et al. 2007; Schrader et al. 2009); however, this phenotye is probably less severe than the complete KO mouse (Guikema et al. 2007). The problem of lethality was addressed using the CH12F3 cell lines, where complete deletion of APE1 reduces class switching to IgA by ∼80% (Masani et al. 2013).

The lack of complete inhibition of class switching in APE1$^{-/-}$ CH12F3 cells suggests that other processes might be acting either in conjunction to or separate from APE1 (Stavnezer et al. 2008; Masani et al. 2013). In this line, the MMR pathway can contribute to CSR in certain circumstances. The MMR is a long-patch DNA repair system, where not only the damaged base is removed but also surrounding nucleotides (Kunkel and Erie 2005). MMR-deficient mice for MSH2, MSH6 or EXO1 present reduced CSR; although the defects are less severe than APE1-deficient models (Ehrenstein and Neuberger 1999; Bardwell et al. 2004; Martomo et al. 2004). It is hypothesis that the MMR pathway may be important in situations where SSBs are too far apart to constitute DSBs (needed to induce the NHEJ pathway). In such cases, excision of larger DNA segments will lead to DSBs formation, and thus activation of the recombination machinery (Stavnezer et al. 2008).

Elucidating the molecular network needed to repair the DNA lesions induced by AID is still an active area of research. In addition to the examples already

discussed, proteins, such as Ku70, Ku80, 53BP1 and H2AX among others; are also involved at some extent in CSR (Chaudhuri and Alt 2004).

1.4.5 Is CSR a germinal centre process?

Traditionally, GCs have been identified as the main areas where CSR takes place *in vivo* (Kraal et al. 1982; Liu et al. 1996). Evidence supporting this idea came from studies comparing the expression of GLTs in different B cell subsets purified from human tonsils (Liu et al. 1996). It was reported that GLT expression was restricted to GC B cells displaying a centrocyte phenotype (Liu et al. 1996). This in addition to enrichment of switch circles in GC B cells, which were also found in naïve B cells, provided the molecular framework to support CSR as a GC process. However, identification of GC B cells was done using CD77, which poorly discriminates centrocytes and centroblasts between them, and from other subsets, such as plasmablasts and activated B cells (Högerkorp and Borrebaeck 2006).

The requirement of GCs for CSR has also been supported by studies in alymphoplasia (aly) mutant mice, where a point mutation in the coding sequence of the NF-κB inducing kinase (NIK) causes severe defects in SHM and CSR (Shinkura et al. 1996; Shinkura et al. 1999). In this model, mice are also characterised by absence of lymph nodes (LNs), Peyer's Patches and defective follicular structure in the spleen (Miyawaki et al. 1994). In this regards, defects in CSR are probably the result of impaired T:B interactions during early follicular B cell activation, rather than lack of normal GC structures. Likewise, detrimental mutations in BCL6, lymphotoxin-α, CD40 and some subunits of NF-κB, all present defects in early T:B interactions, GC formation and CSR (Kawabe et al. 1994; Ye et al. 1997; Fu et al. 1997; Zelazowski et al. 1997)

On the other hand, induction of CSR prior to GC formation has also been reported by different groups. For instance, a primary response against CGG

in the endogenous repertoire of C57BL/6 mice was shown to elicit induction of γ1-GLT at day 4 after immunisation in the absence of GCs (Toellner et al. 1996). Moreover, a lack of correlation between GC size and GLT expression was also noted (Toellner et al. 1996). Similarly, EF foci of IgG class-switched B cells have also been observed as early as day 2 in the spleen of mice after a primary immunisation with NP-CGG (Jacob et al. 1991a).

Monitoring early stages of B cell activation *in vivo* using adoptive cell transfers has also supported the notion of CSR before establishment of GCs. Transfer of transgenic 3-83 KIH B cells along with DO11.10 T cells showed that antigen-specific IgG2a$^+$ B cells first appeared at the T:B border of splenic follicles at day 2 after transfer. IgG2a$^+$ B cells reached a peak of expansion between day 3-4 after prime, without distinct formation of GCs (Pape et al. 2003). However, a main criticism to this study was the increased cognate T-cell help delivered along with an also elevated number of antigen-specific B cells (Pape et al. 2003). Thus, it was argued that these conditions may not be reflective of a physiological response.

CSR has also been detected outside GCs in the absence of T-cell help, particularly in the lamina propria (LP) of the intestine. In the LP switching from IgM to IgA has been predicted to occur in a T-cell independent manner through local interaction of B cells with LP stromal cells. B cells co-cultured with LP stromal cells – which do not express CD40L – in the presence of LPS, IL-5 and TGF-β showed enhanced class switching to IgA (Fagarasan et al. 2001). Similarly, upon interaction with natural killer (NK) cells, germline transcription to γ2a was induced in B cells (Gao et al. 2001). Although the amount of functional IgG2a generated was marginal, it is hypothesised that NK-interaction might reprogram B cells skewing their transcriptional program to preferentially switch to IgG2a when additional signals are delivered (Gao et al. 2001). In human B cells, CSR can also occur via interaction with B lymphocyte stimulator protein (BlyS) and

proliferation-inducing ligand (APRIL), both upregulated by activated DC cells (Litinskiy et al. 2002).

1.5 Hypothesis and aims

Despite the significant body of data and tools available, to date no definitive report has described exactly the time and location at which CSR is triggered in B cells during the course of an immune response. Presumably in the past, this might have been due to the lack of animal models and technical resources needed to study early stages of B cell activation *in vivo*. Indeed, most of the studies published on this subject have been done using *in vitro* culture systems, mouse models with severe defect in the architecture of SLOs, adoptive transfers with increased cell numbers, or after clonal expansion of antigen specific B cells when GCs are already formed (Shinkura et al. 1996; Liu et al. 1996; Toellner et al. 1996; Pape et al. 2003).

Using different transgenic mouse models, transcriptomic analyses at the single cell level, mathematical models, microscopy and photoactivation to analyse the clonal diversity and phylogeny of individual GCs, we aim to characterise the induction, kinetics and microanatomical location of CSR during a primary immune response. Based on the evidence discussed in this chapter, we hypothesise that CSR is a process that occurs prior to GC formation; most likely, in the follicles during the first cognate interaction at the T:B border or during reactivation of memory B cells.

CSR is critical to develop appropriate and specific immune responses against invading pathogens. Thus, providing new insights and a re-evaluation to our knowledge about this process is not only an intellectual challenge, but also a step forward to better understand the molecular basis of the immune response, autoantibody mediated-diseases and to improve the design of future vaccines, just to mention some direct applications.

Chapter 2

Materials and Methods

2.1   Human samples

Human tonsils were obtained from consenting donors at The Canberra Hospital and Calvary John James Hospital (Canberra, ACT, Australia), following routine tonsillectomy. Tonsils were processed by mechanical disruption of the tissue and cells were isolated using Ficoll Hypaque (GE Healthcare Life Sciences) gradient centrifugation (Papa et al. 2017). All experiments with humans were approved by the Australian National University's Human Experimentation Ethics Committee and the University Hospitals Institutional Review Board.

2.2   Animals

C57BL/6, SW_{HEL} (Phan et al. 2003), B1-8^{hi} tdTomato^{+} (Shih et al. 2002), Cγ1Cre:mT/mG (Casola et al. 2006; Muzumdar et al. 2007) and photoactivatable (PA)-GFP mice (Victora et al. 2010) were bred and maintained in specific-pathogen-free conditions at the Australian National University (ANU), Canberra, Australia; The University of Birmingham, Birmingham UK; and The Rockefeller University,

New York, USA. All experiments were performed according to the regulations approved by the local institution ethics committee, including the Australian National University's Animal and human Experimentation Ethics Committees.

2.2.1 Adoptive transfer of SW$_{HEL}$ B cells

SW$_{HEL}$ mice heterozygous for both V_L and V_H chain alleles of the anti-HEL BCR were sacrificed by cervical dislocation and splenocytes were collected. Single cell suspensions were obtained by mechanically disrupting the tissue through 70 μm nylon mesh filters (BD Bioscience) using complete RPMI 1640 media (Sigma-Aldrich). The exact frequency of SW$_{HEL}$ B cells was determined by flow cytometry prior to adoptive transfer using HEL protein conjugated to Alexa Fluor 647 (A647). SW$_{HEL}$ B cells (CD45.1$^+$) were resuspended in PBS 1x and adoptively transferred by intravenous injection (*i.v.*) into congenic C57BL/6 recipient mice (CD45.2$^+$) along with $2x10^8$ sheep red blood cells (SRBCs) conjugated to a mutated form of hen egg lysozyme (HEL2x) (Paus et al. 2006; Chan et al. 2009). For experiments analysing the early stages of the immune response (days 1.0 – 2.5) $1.5x10^5$ HEL-binding cells were transferred; whereas for analysis of late phases (days 3.0 – 8.5) $3x10^4$ cells were given as previously described (Paus et al. 2006; Chan et al. 2009).

2.2.2 Adoptive transfer of B1-8^{hi} tdT$^+$ B cells

C57BL/6 recipient mice (8-10 weeks old) were pre-immunised by intraperitoneal (*i.p.*) injection of 100 μg of chicken gamma globulin (CGG; # C-1000-10, Biosearch Techonolgies) emulsified in Complete Freund's adjuvant (CFA; # F5881, Sigma-Aldrich). Three days later, mice were *i.v.* transferred with B1-8^{hi} tdT$^+$ cells along with 50 μg of 4-hydroxy-3-nitrophenyl acetyl (NP) conjugated to CGG (ratio 30-39, # N-5055D-5, Biosearch Technologies). This mixture was delivered in 200 μL of PBS 1x. Single cell suspensions from B1-8^{hi} tdT$^+$ donor splenocytes

were prepared similar to the procedure for SW$_{HEL}$ B cells. To investigate early stages (17h – day 1.5) of the immune response 1.8×10^5 B1-8^{hi} tdT$^+$ cells were transferred into recipients. For late stages (days 2 – 18) recipient mice received 6×10^4 B1-8^{hi} tdT$^+$ cells. The exact frequency of B1-8^{hi} tdT$^+$ cells was determined by flow cytometry prior to transfer, as measured by binding of the hapten NP conjugated to APC.

2.3 Flow cytometry analyses and FACS sorting

Single cells suspensions were prepared from mouse spleens, lymph nodes and human tonsils as previously described (Tas et al. 2016; Papa et al. 2017). After processing, cell subsets were examined using flow cytometry using the antibodies listed on Table 2.1. Antibody cocktails were prepared in FACS buffer: PBS 1x (Sigma-Aldrich) containing 2% fetal bovine serum (FBS, Gibco) and 2mM EDTA (Sigma-Aldrich). For detection of HEL-binding cells, HEL protein (Sigma-Aldrich) was conjugated to A647 using a protein labelling conjugation kit (Thermo Fisher). Dead cells were excluded using either 7-Aminoactinomycin D (7-AAD, Thermo Fisher) or Zombie aqua dye (# 423102, BioLengend). Cells were stained with primary antibodies followed by secondary reagents for 30 min at 4°C in the dark. Intracellular staining was performed using the FOXP3/Transcription Factor Staining Buffer Set (# 00552300, eBioscience) according to the manufacturer's instructions. Samples were acquired on a LSRII or Fortessa cytometer (BD) and analysed using FlowJo software v10.3 (Tree Star).

2.4 Immunofluorescence

Frozen tissue sections were fixed in cold acetone for 10-20 min. Donor-derived SW$_{HEL}$ B cells were detected in the spleen of recipient mice as previously

Target	Catalog No.	Source
Mouse		
CD11b-A700	#101222	BioLegend
CD11b-FITC	#553310	BD Bioscience
CD16/32 (Fc-block)	#553152	BD Bioscience
CD3-A700	#100216	BioLegend
CD3-biotin	#100303	BioLegend
CD3-FITC	#553062	BD Bioscience
CD38-A700	#56-0381-82	eBioscience
CD38-BV421	#562768	BD Bioscience
CD38-PE	#120707	BioLegend
CD38-A700	#56-0381-82	eBioscience
CD38-BV421	#562768	BD Bioscience
CD38-PE	#120707	BioLegend
CD45.1-A700	#110724	BioLegend
CD45.1-PB	#110722	BioLegend
CD95-BV421	#562633	BD Bioscience
CD95-PE	#554258	BD Bioscience
CXCR5-biotin	#551960	BD Bioscience
IgD-FITC	#11-5993-85	eBioscience
IgG1-biotin	#553441	BD Bioscience
IgG2a-biotin	#550332	BD Bioscience
IgG2b-biotin	#406704	BioLegend
IgG2c-biotin	#553504	BD Bioscience
IgG3-biotin	#553401	BD Bioscience
IgM-FITC	#553437	BD Bioscience
IgM-PECy7	#25-5790-82	eBioscience
Streptavidin-APC	#S868	Thermo Fisher
Streptavidin-BV605	#405229	BioLegend
B220-APCCy7	#103224	BioLegend
Human		
CD19-PECy7	#557835	BD Bioscience
CD27-FITC	#555440	BD Bioscience
CD38-PE	#347687	BD Bioscience
CD4-APCCy7	#557871	BD Bioscience
CD86-A421	#562432	BD Bioscience
CXCR4-APC	#306510	BioLegend
Human TruStain FcX (Fc-blocking solution)	#422302	BioLegend

Table 2.1. Flow cytometry antibodies and reagents

described (Chan et al. 2009). T-cell areas were identified with anti-CD3-biotin antibody (# 100303, BioLegend) followed by streptavidin conjugated to Alexa 350 (# S11249, Thermo Fisher). B cell follicles were visualised by staining with anti-IgD FITC (# 11-5993-85, eBioscience). Cγ1-Cre:mT/mG spleen sections were stained with anti-CD3 (# 550275, BD Pharmingen) and anti-IgD (# 553438, BD Pharmingen) followed by biotin conjugated goat anti-hamster antibody (Jackson ImmunoResearch), streptavidin Alexa 405 (# S32351, Thermo Fisher) and donkey anti-rat Alexa-647 antibody (Jackson ImmunoResearch). For human samples, tonsil sections were blocked and permeabilised with 3% BSA (Sigma-Aldrich) and 0.5% Triton X-100 (Sigma-Aldrich). APE1 protein was detected using anti-APE1 antibody (sc-17774, Santa Cruz Biotechnology) followed by donkey anti-mouse Alexa 568 antibody (# A10037, Thermo Fisher). Follicles were identified using anti-human IgD-FITC antibody (# 555778, BD Bioscience). Cell nuclei were counterstained using DAPI (Sigma-Aldrich). Stained sections were mounted using Vectashield (Vector Laboratories, # H-1200) and visualised using an Olympus IX71 inverted fluorescence microscope or a Zeiss Axio ScanZ1. Images were compiled using Adobe Photoshop CS6 software.

2.5 *In vitro* B cell cultures

Human tonsil B cells were FACS-purified and then cultured in complete media: RPMI 1640 media (Sigma-Aldrich) supplemented with 2 mM L-Glutamine (Gibco), 100 U penicillin-streptomycin (Gibco), 0.1 mM nonessential amino acids (Gibco), 100 mM Hepes (Gibco), 55 mM beta-mercaptoethanol (Gibco) and 10% FBS (Gibco). Cells were maintained for 72h in an incubator at 37°C with 5% CO_2.

2.6 Western blot

Naïve, DZ and LZ B cells were isolated from human tonsils by flow cytometry. Activated B cells were obtained by *in vitro* stimulation of purified naïve B cells (2.5×10^6 cells/mL) with IL-21 and CD40L (10 ng/mL and 1 µg/mL, respectively) for 72h. Total protein extraction was performed using RIPA buffer (Thermo Fisher) supplemented with protease inhibitor (Roche). 10 µg of whole-cell extracts from each cell subset were separated by SDS-PAGE (12% w/v), blotted onto nitrocellulose membranes and incubated with anti-APE1 antibody (sc-17774, Santa Cruz Biotechnology). β-actin was used as a loading control (# A5441, Sigma-Aldrich). Enhanced chemiluminescence (ECL) development was performed after incubation with secondary antibodies conjugated to horseradish peroxidase (HRP) using Pierce ECL Western Blotting Substrate reagent (Thermo Fisher) according to manufacturer's instructions. Images were acquired on an Image Quant LAS 4000 machine (GE Healthcare Life Sciences). Densitometry analysis was performed using Image Studio software version 5.2.5 (LI-COR Biosciences).

2.7 *In vitro* analysis of switch circle transcripts

Splenic naïve B cells were stimulated *in vitro* with IL-4 and CD40L (10 ng/mL and 1 µg/mL, respectively) for 24h, 48h and 72h to induce class-switching to IgG1. Total RNA was extracted using a PicoPure RNA Isolation Kit (# KIT0204, Thermo Fisher) according to the manufacturer's instructions. DNase treatment was performed on-column using RNase-Free DNase Set (# 79254, Qiagen). RNA quality and concentration were determined using an Agilent 2100 Bioanalyzer instrument. 300 ng of total RNA was transcribed into cDNA using SuperScript IV Reverse Transcriptase (# 18090050, Thermo Fisher). Switch circle transcripts (SWCTs) were pre-amplified using 5 µL of Power SYBR Green Master

Mix (# 4367659, Thermo Fisher) with 50 nM of forward (F) and reverse (R) primers. $F1_{\gamma1\text{-SWCT}}$: 5'-TCGAGAAGCCTGAGGAATGTG-3'; and $R1_{\gamma1\text{-SWCT}}$: 5'-GGTACTTGCCCCCTGTCCTCAG-3' (Pone et al. 2015); or $F2_{\gamma1\text{-SWCT}}$: 5'-GGCCCTTCCAGATCTTTGAG-3'; and $R2_{\gamma1\text{-SWCT}}$: 5'-AATGGTGCTGGGC AGGAAGT-3' (Kinoshita et al. 2001). The pre-amplification was performed at 50°C for 2 min, 95°C for 2 min, followed by 22 cycles of 95°C for 15s and 60°C for 4 min. Pre-amplification products were diluted 1:5 in ultra-pure DNase/RNase-Free distilled water (# 10977023, Thermo Fisher), and 1 µL was used for qPCR analysis of *Actb* and γ1-SWCT. qPCR reactions were performed using 5 µL of Power SYBR Green Master Mix (# 4367659, Thermo Fisher) with 250 nM of F and R primers (for γ1-SWCT assays see above, for *Actb* see Table 2.2). Samples were measured in triplicate using an Applied Biosystems 7900HT Fast Real-Time machine (Thermo Fisher). qPCR thermal profile: 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 30s at 95°C followed by 1 min at 60°C, and melting curve analysis from 60°C to 95°C. Amplified product for γ1-SWCT were resolved by electrophoresis in a 1.5% (w/v) agarose gel. Expected fragment sizes: F1-R1 = 310bp; F2-R2 = 408bp.

2.8 qPCR analysis

Total RNA was extracted from mouse samples with Trizol (# 15596026, Thermo Fisher). RNA quality and concentration were determined with an Agilent 2100 Bioanalyzer instrument (Agilent Technologies). Only samples with a RIN score ≥ 8 were selected for digestion of genomic DNA and cDNA synthesis using RQ1 RNase-Free DNase (# M6101, Promega) and SuperScript III Reverse Transcriptase (# 18080093, Thermo Fisher), respectively. Duplex qPCR analyses were conducted for each target gene (FAM-labeled probe sets) using *Ubc* (VIC-labeled, Taqman assay # Mm01198158_m1, Thermo Fisher) or *Actb* (HEX-labeled

probe set, Biosearch Technologies) as reference genes. Samples were measured in triplicate using an Applied Biosystems 7900HT Fast Real-Time machine (Thermo Fisher). GLTs were assessed using the primers and dual-labeled BHQ probes listed in Table 2.2. These assays have been previously described and validated (Marshall et al. 2011). Primers and probes were manufactured by Biosearch Technologies. Data is expressed as a fold-change using the $\Delta\Delta C_T$ method.

2.9 One step qPCR analysis

Total RNA from 2,000 - 15,000 cells was purified using a PicoPure RNA Isolation Kit (# KIT0204, Thermo Fisher) according to the manufacturer's instructions. DNase treatment was performed on-column using RNase-Free DNase Set (# 79254, Qiagen). RNA quality and concentration were determined using an Agilent 2100 Bioanalyzer instrument. Real-time one-step RT-PCR quantification was performed using the QuantiTect Multiplex RT-PCR Kit (# 204643, Qiagen) in a final volume of 6μL. Duplex reactions with limiting primer concentrations were conducted in the same well for γ1-GLT (FAM-labeled probe set) and *Actb* (HEX-labeled probe set). Reactions were run for 40 cycles in triplicate using an Applied Biosystems 7900HT Fast Real-Time machine. Expression levels of the target gene were normalised to *Actb* levels and presented as a fold-change using the $\Delta\Delta C_T$ method. Assays were optimised and validated using RNA from *in vitro* activated naïve B cells (data not shown).

2.10 Single cell qPCR

Single-cell qPCR was performed as previously described (Nefzger et al. 2016). In brief, cells were FACS deposited into qPCR 96-well plates filled with 10 μL of lysis buffer and processed with the Single Cell to C_T kit (Life Technologies).

Target	Forward primer	Reverse primer	Probe
γ1-GLT	5'-CGAGAAGCCTGAGGAATGTGT-3'	5'-GGAGTTAGTTTGGGCAGCAGAT-3'	5'-FAM-TGGTTCTCTCAACCTGTGTAGTCCATGCCA-3'
γ2b-GLT	5'-CGCACACCTACAGACAACCAG-3'	5'-GTCACAGAGGAACCAGTTGTATC-3'	5'-FAM-CCAGGGGCCAGTGGATAGACTGAT-3'
γ2c-GLT	5'-GGACCACTAAAGCTGCTGACACAT-3'	5'-AACCCTTGACCAGGCATCCT-3'	5'-FAM-AGCCCCATCGGTCTATCCACTGGC-3'
γ2c-GLT	5'-GGACCACTAAAGCTGCTGACACAT-3'	5'-AACCCTTGACCAGGCATCCT-3'	5'-FAM-AGCCCCATCGGTCTATCCACTGGC-3'
γ2c-GLT	5'-GGACCACTAAAGCTGCTGACACAT-3'	5'-AACCCTTGACCAGGCATCCT-3'	5'-FAM-AGCCCCATCGGTCTATCCACTGGC-3'
γ3-GLT	5'-GACCAAATTCGCTGAGTCATCA-3'	5'-ACCGAGGATCCAGATGTGTCA-3'	5'-FAM-CTGTCTATCCCTTGGTCCCTGGCTGC-3'
Actb	5'-CGTGAAAAGATGACCCAGATCA-3'	5'-TGGTACGACCAGAGGCATACAG-3'	5'-HEX-TCAACACCCCAGCCATGTACGTAGCC-3'

Table 2.2. Sequences of primers and probes used to detect GLT and *Actb* expression in single cell qPCR studies.

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cDNA was produced from the lysate as per kit's instructions. Samples were submitted to 18 cycles of pre-amplification using the following TaqMan assays (Life Technologies): *Actb* (# Mm00607939_s1), *Foxo1* (# Mm00490672_m1), *Apex1* (Mm01319526_g1), *Apex2* (# Mm00518685_m1), *Prdm1* (# Mm00476128_m1), *Bcl6* (# Mm00477633_m1) and *Aicda* (# Mm01184115_m1). GLTs were detected using the probe sets described in Table 2.2. Single-cell qPCR data collection was performed with a Biomark instrument (Fluidigm) on pre-amplified templates that were positive screened for the housekeeper *Actb* (manually tested by qPCR). Reactions were run for 40 cycles. Data was analysed using the Biomark software package "Real-Time PCR analysis" (Fluidigm). Data cleaning and normalisation were done using custom R code (R version 3.3.3, R Core Team). The limit of detection was set to 40 cycles. Undetermined C_T were given a value of 40. Heatmaps and violin plots of the resulting data were generated using the ggplot2 package (version 2.2.1) in the R environment.

2.11   GC B cell clonal trees: PA-GFP mice

PA-GFP-transgenic mice (Victora et al. 2010) were immunised with CGG-alum and draining popliteal lymph nodes (pLNs) were harvested 15 and 20 days later. Two individual GCs per pLN were photoactivated and single-cell sorted independently. V_H genes from individual GC B cells were amplified and sequenced. Data for day 15 was re-analysed from sequences previously published (Tas et al. 2016) using a similar methodology. Data for day 20 was generated *de novo* for this study. Only clones containing ≥ 4 IgM$^+$ cells, or at least one IgM$^+$ and one IgG$^+$ cell were considered informative and are presented in this paper.

2.12 Mathematical modelling

All simulations are based on a previously published agent-based model of B and T cell dynamics within the GC (Meyer-Hermann et al. 2012), which lacks an isotype switching model (see below). Briefly, the model describes dynamics of B and T cells in discrete three-dimensional space including diffusion of chemotactic signals that influence cell motility. The GC reaction starts with founder B cells migrating into the virtual GC area within the first four days at a rate of 2 cells per hour. Each B cell divides six times before it is allowed to differentiate to a LZ phenotype that depends on antigen collection for survival beyond a critical time period. Antigen is collected by B cells in an affinity-dependent manner, where affinity of a B cell for an antigen is represented by Hamming distance in four-dimensional shape space. Furthermore, B cells depend on T cell help for survival. For competing B cells, T_{FH} cells polarise towards the cell with the higher amounts of collected and processed antigen. B cells collect T_{FH} signals and require a sufficient total amount of collected signal for survival. Each selected B cell returns to the DZ and divides a number of times that depends on the amount of collected antigen, a mechanism termed dynamic number of divisions (Meyer-Hermann 2014) and supported by experimental data (Gitlin et al. 2014). Differentiation to GC output cells is induced in a probabilistic manner (LEDAX model) (Meyer-Hermann et al. 2012). A full description of the modelling framework and its compatibility with recent experimental data has been recently published (Binder and Meyer-Hermann 2016).

For the present context of B cell isotype switching, a novel model of GC dynamics needed to be developed, which explicitly represents the different isotypes and allows for different models of how isotype switching happens. Newly arrived cells are assumed to predominantly express IgM. In accordance with

early measurements of the amount of IgG$^+$ B cells (Fig. 3.2 and Fig. 3.6), we assume that 35% of the founder cells are already expressing IgG. At each division event, the daughter cells switch from IgM to IgG with a defined probability. This switching probability p can be either constant or decrease over time according to an exponential decay model with a half-life corresponding to the observed decrease in GLTs (Fig. 3.5), $p(t) = p_0\, e^{-yt}$, where p_0 denotes the switching probability at the beginning and y is the decay of the switching probability over time.

The initial switching probability is calibrated by data on the relative amount of either isotype at later time points of the reaction for both the constant and the dynamic switching model separately. To account for a possible preferential output for the IgG isotype, we introduced a bias factor, η, that increases the probability of IgG$^+$ cells to become plasma cells while decreasing the output probability for IgM$^+$ cells by the same amount, keeping the total amount of output cells comparable. The different conditions were simulated in 400 *in silico* GCs and the distribution of the fraction of IgG$^+$ B cells at day 21 after onset of the GC reaction was evaluated among these 400 GCs.

To test for the impact of the timing of CSR, we combined the dynamic switching model with a delay in switching, leading to a limited time interval for switching at different times of the GC reaction. We tested a delay of different time intervals, t_{switch}, (Fig. 3.20D). Each delay was simulated in 400 GCs and the diversity of the IgG fraction at day 21 within these 400 simulations was assessed using the difference between the upper and the lower quartile (interquartile range, IQR).

All simulations were performed using custom C++ code. Simulation output was analysed using the R statistics language; plots of the simulation output were created using the ggplot2 library.

2.13 Statistical Analysis

Datasets were analysed using Mann-Whitney test (U test, two-tailed), except for quantification of western blots, in which a paired t-test (two-tailed) was used. To compare more than two groups or sets of data a Kruskal-Wallis test was performed followed by Dunn's post-test. The test employed to analyse the different experiments is indicated in each figure legend. Statistical tests were selected based on the distribution and the variance characteristics of the data. Normality was assessed with Shapiro-Wilk test. All statistical analyses were performed with Prism software version 7 (GraphPad Software) and R software version 3.3.3 (R Core Team). The exact p-values are shown in each figure.

Chapter 3

Results

3.1 Evaluation of CSR in the early phases of a TD-response

In order to study CSR *in vivo* we took advantage of the switch-HEL (SW$_{HEL}$) system. The SW$_{HEL}$ model is a genetically modified mouse where $\sim$5-15% of the B cells carry a high-affinity BCR against hen egg lysozyme (HEL) (Phan et al. 2003). SW$_{HEL}$ mice were developed by Brink and colleagues as an IgH knock-in line, using two independent constructs (Phan et al. 2003). The first one was randomly inserted, and it is the same Vκ-light chain specificity found in MD4 mice (Goodnow et al. 1988). The second transgene is the V$_H$ region of the HyHEL10 hybridoma targeted specifically to the endogenous IgH locus (Phan et al. 2003). This heavy and light chain combination results in a BCR that binds HEL with high affinity. Unlike other HEL-transgenic models (Goodnow et al. 1988), SW$_{HEL}$ mice can switch to all Ig-isotypes and undergo normal SHM after challenge with the appropriate HEL protein or HEL conjugate (Phan et al. 2003; Paus et al. 2006; Chan et al. 2009).

We used a mutated form of HEL protein that carries two amino-acid substitutions. This mutant, termed HEL2x, binds to the BCR expressed by SW_{HEL} B cells with lower affinity than native HEL protein for the BCR expressed by SW_{HEL} B cells ($8x10^7$ M^{-1} and $2x10^{10}$ M^{-1}, respectively) (Paus et al. 2006). This reduced affinity resembles more closely the affinity range found in a physiological context (Paus et al. 2006; Chan et al. 2009). SW_{HEL} mice also express the congenic marker CD45.1 (Ly5a), which allows us to track these cells after transfer into CD45.2$^+$ (Ly5b) recipient mice (Phan et al. 2003; Paus et al. 2006). Appropriate numbers of SW_{HEL} B cells (CD45.1$^+$) were *i.v.* transferred into congenic C57BL/6 recipient mice (CD45.2$^+$) along with $2x10^8$ SRBCs conjugated to HEL2x protein (HEL2x-SRBC).

For visualisation of the earliest phases of the immune response (days 1.0 – 2.5) $1.5x10^5$ SW_{HEL} B cells were transferred; whereas for late phases (days 3.0 – 8.5) $3x10^4$ HEL-binder B cells were delivered (Brink et al. 2008). The exact frequency of SW_{HEL} B cells was determined by flow cytometry prior to adoptive transfer as described in Chapter 2.

Spleens from recipient mice were harvested at different time points after immunisation and analysed by FACS, qPCR, and immunofluorescence (IF) histology. The results obtained from these experiments will be discussed in the next section.

3.1.1 Characterisation of the immune response of anti-HEL specific B cells using the SW_{HEL} system

We first characterised the dynamics of SW_{HEL} B cell activation during a primary immune response against HEL2x-SRBCs. To do this, spleens from adoptively transferred recipient mice were collected every 12h or 24h from day 1.5 to day 8.5 after challenge (Fig. 3.1A). Figure 3.1B shows the gating strategy employed to identify donor-derived HEL-binding B cells. FACS analysis was used

to investigate clonal expansion, class switching and differentiation of responding antigen-specific SW_{HEL} B cells during the time course.

As shown in Figure 3.2, from day 1.5 to day 3.0 donor-derived SW_{HEL} B cells remained as a single population of activated B cells. Previous reports have shown that these cells possess a high rate of proliferation, with most of the transferred cells being recruited to the immune response (Chan et al. 2009). By day 3.5 the extrafollicular (EF) vs germinal centre (GC) fate decision has already been made by HEL-binders. Complete differentiation towards one of these two compartments was clearly distinguished over the next two days (day 4.5 and day 5.5) (Fig. 3.2). During this period, responding SW_{HEL} B cells differentiated as either $CXCR5^{lo}$ $B220^{lo}$ extrafollicular plasmablasts (EFPBs) or $CXCR5^{hi}$ $B220^{hi}$ GC B cells. HEL^+ EFPBs were also identified by expression of the plasma cell markers CD138 and BLIMP1 (Fig. 3.3), whereas HEL^+ GC B cells were Fas^{hi} and expressed the GC transcription factor BCL6 (Fig. 3.1B and Fig. 3.3). Finally, by day 8.5 the EF compartment was lost, whilst the GC reaction persisted (Fig. 3.2).

Similar kinetics of activation and migration were observed when spleen sections were subjected to histologic analysis (Fig. 3.4). Upon HEL2x-SRBC immunisation adoptively-transferred SW_{HEL} B cells first appeared at the T:B border on day 1.5, at the periphery of the follicles on day 2.5, and within primary follicles on day 3.0 (Fig. 3.4). On day 3.5, HEL-binding B cells were found forming nascent GCs and HEL-binding EFPBs were also seen (Fig. 3.4). GCs were fully established on day 4.5 and day 5.5 after challenge (Fig. 3.4).
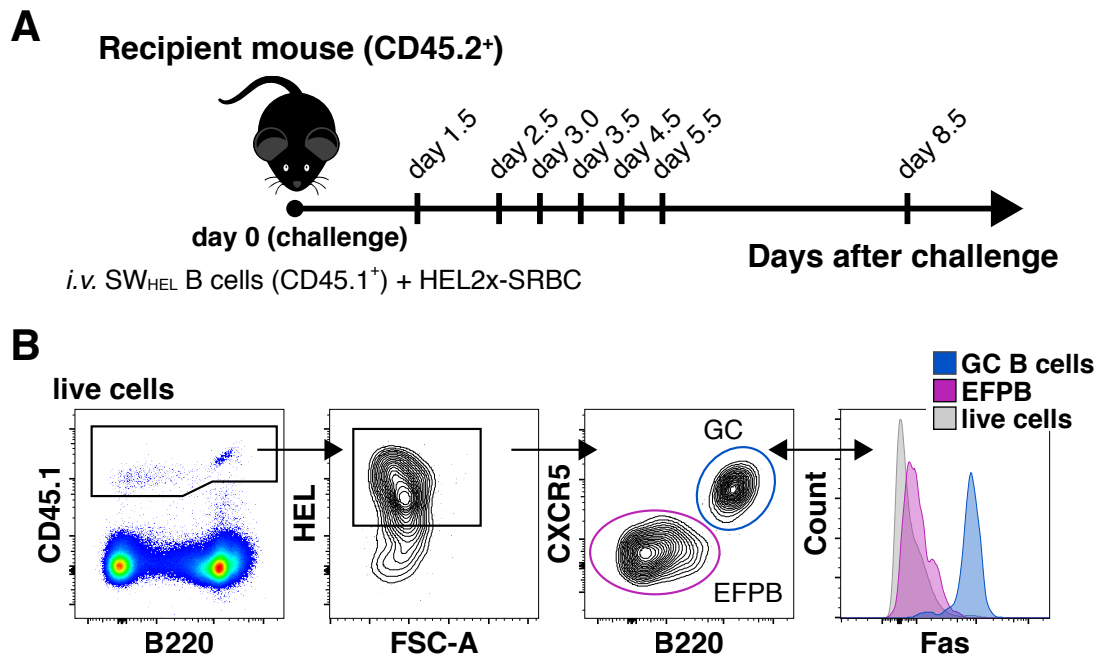
Figure 3.1. Adoptive transfer protocol and gating strategy used to identify SW$_{HEL}$ B cells in recipient mice. A) Adoptive transfer protocol of SW$_{HEL}$ B cells. In brief, CD45.2$^+$ recipient mice were adoptively transferred with CD45.1$^+$ SW$_{HEL}$ B cells ($3x10^4$ or $1.5x10^5$ cells) and simultaneously *i.v.* challenged with $2x10^8$ SRBCs conjugated to HEL2x protein. **B)** Representative FACS plots of responding SW$_{HEL}$ B cells recovered from congenic recipient mice challenged as shown in **(A)**. Donor-derived SW$_{HEL}$ B cells were gated as CD45.1$^+$ HEL$^+$ B220$^{hi/lo}$ live lymphocytes (7AAD$^-$). Extrafollicular plasmablasts (EFPBs) and germinal centre (GC) B cells were characterised as CXCR5^{lo} B220^{lo} Fas^{lo} and CXCR5^{hi} B220^{hi} Fas^{hi}, respectively. Data is representative from one mouse at day 5.5 after immunisation.
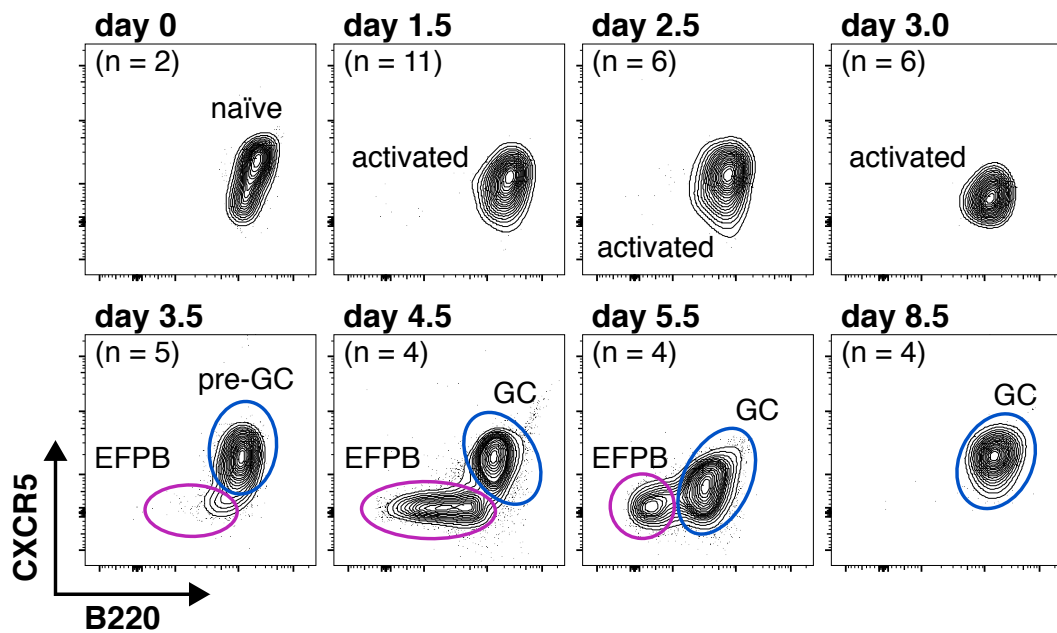
Figure 3.2. Kinetics of the immune response of transferred SW$_{\text{HEL}}$ B-cells against HEL2x-SRBCs. Flow cytometric profiles of splenocytes harvested from recipient mice at the specified time points after immunisation (days 1.5 – 8.5) as shown in Fig. 3.1. Responding SW$_{\text{HEL}}$ B cells were identified as naïve HEL$^+$ B cells (day 0, prior to injection), activated HEL$^+$ B cells (days 1.5 – 3.0), and extrafollicular plasmablast (EFPB) or germinal centre (GC) HEL-binding B cells (days 3.5 – 8.5). n = number of recipient mice used in each time point. Data is representative of two independent experiments.
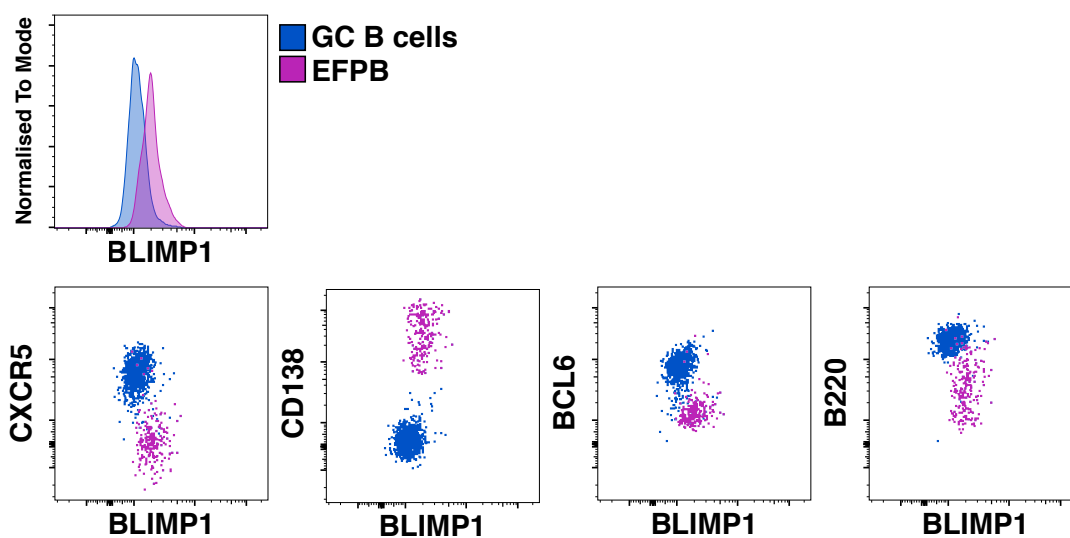
Figure 3.3. Phenotypic characterisation of HEL-binding EFPBs and GC B cells after adoptive transfer. Representative flow cytometric plots showing surface expression of BLIMP1 vs CXCR5, CD138, BCL6 or B220 in HEL-binding B cells recovered 5 days after challenge as shown in Fig. 3.1. Donor-derived SW_{HEL} B cells were pre-gated as EFPBs or GC B cells as shown in Fig. 3.2.
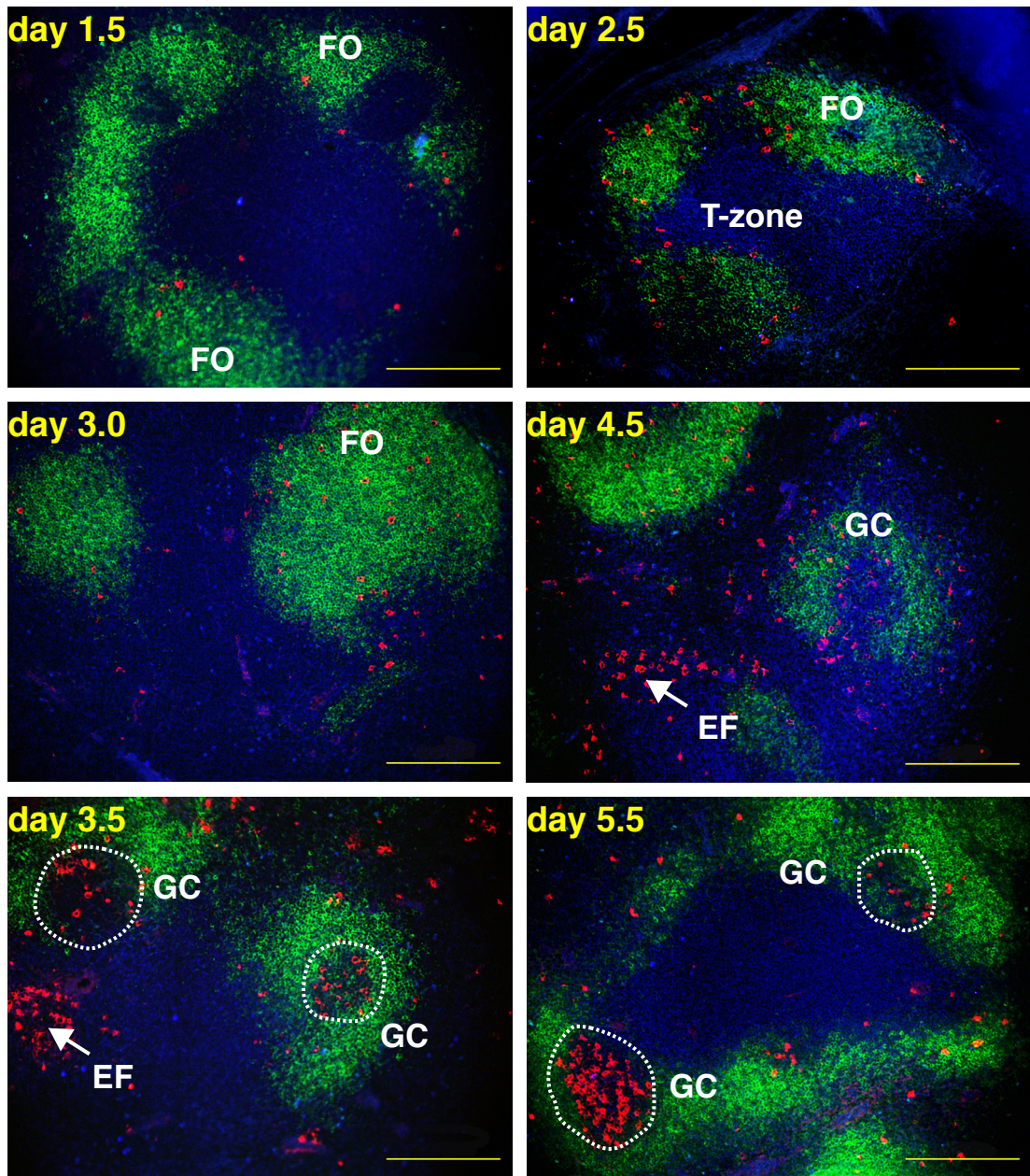
Figure 3.4. Migration of responding SW$_{\text{HEL}}$ B cells in the spleen of recipient mice after HEL2x-SRBC immunisation. Immunofluorescence images of frozen spleen sections collected at the specified time point (days $1.5 - 5.5$) from congenic recipient mice transferred as shown in Fig. 3.1. Samples were stained for HEL-binding B cells (red), IgD (green), and CD3 (blue). The dynamics of activation underwent by HEL$^+$ B cells are described: **A)** SW$_{\text{HEL}}$ B cells were observed at the T-zone areas 1.5 days after challenge; **B)** 24h later HEL-responders localised to the T:B border; **C)** by day 3.0 HEL$^+$ B cells have increased in numbers and were found close to the centre of follicles; **D)** on day 3.5 follicles containing GCs were first seen and some cells had already departed to form EF foci; fully established GCs and foci of EFPBs were best observed on days 4.5 and 5.5 **(E-F)**. Scale bars $= 200$ μm. Data is representative of two independent experiments.

3.1.2 Germline transcript expression is induced in early activated B cells and is downregulated in GC B cells

Having determined the kinetics of activation of SW_{HEL} B cells, we next examined the ability of these cells to undergo CSR *in vivo* by measuring the expression of germline transcripts (GLTs). GLTs (also known as switch-transcripts) are spliced polyadenylated non-coding mRNAs transcribed from specific promoters located upstream of each set of C_H genes (except for IgD) (Stavnezer 1996; Chaudhuri and Alt 2004). Expression of GLTs precedes DNA recombination of the IgH locus in B cells primed to undergo CSR; therefore, GLT production is a reliable marker to study induction of CSR *in vivo*.

Production of GLTs was initially linked to a centrocyte stage of GC B cell differentiation by *ex vivo* studies (Liu et al. 1996). As mentioned in Chapter 1, this report relied on markers no longer considered centrocyte-specific (IgD^- $CD38^+$ $CD77^lo$), as they also identify recently-activated B cells and plasmablasts. We next sought to test whether the formation of GLTs was predominantly associated with GC B cells during the course of an immune response. The SW_{HEL} system allows precise assessment of the stage in B cell differentiation in which GLTs are produced. This is due to the very synchronous response of responding B cells and the clear phenotypic changes of antigen-specific B cells as they transit from an early activated stage to the EF or GC phases. It is well documented, that responses against HEL-SRBC in the SW_{HEL} model elicit an IgG1- and IgG2b-dominated antibody response (Brink et al. 2008). Thus, expression of γ1-GLT and γ2b-GLT, as the main isotypes induced in this system, was assessed by qPCR in bulk populations of donor-derived SW_{HEL} B cells purified as described in Figure 3.2.

As shown in Figure 3.5A, γ1-GLT was first detected as early as day 1.5 in a subset of early activated SW_{HEL} B cells. In contrast to previous studies that have

linked GLT-expression to LZ GC B cells (Liu et al. 1996), we found that γ1-GLT reached its maximal expression at day 3.0 after immunisation, a time point that precedes the appearance of the first GC B cells, which were first detectable on day 3.5. Surprisingly, GC B cells purified on day 3.5 expressed lower levels of γ1-GLT than day 3.0 HEL$^+$ B cells. Additionally, this transcript was further downregulated over the next days in GC B cells. By day 8.5, γ1-GLT expression was almost undetectable. Thus, we found that induction of γ1-transcription is associated with the early stages of B cell activation, before GCs have been established (Fig. 3.5A). Similar kinetics were observed for γ2b-GLT (Fig. 3.5B). Of note, total RNA amounts used for PCR amplification as determined by the raw C_T values of the house-keeping gene *Ubc*, and RNA quality were comparable throughout the time-course (data not shown).

Commitment to the GC pathway by antigen-activated B cells is driven by the master transcription factor BCL6 (Dent et al. 1997; Basso and Dalla-Favera 2010). As expected in this system, *Bcl6* expression was detected exactly at day 3.5, correlating precisely with the appearance of GC B cells by both FACS and microscopy (Fig. 3.2 and Fig. 3.4). *Bcl6* was not induced in any of the early activated HEL$^+$ B cell subsets (Fig. 3.5C).

As discussed in Chapter 1, a critical step for initiation of CSR is the generation of double-strand breaks (DSBs) in the intronic S regions. DSBs are created through deamination of dC residues into dU mediated by the enzyme AID, encoded by the *Aicda* gene (Muramatsu et al. 1999; Muramatsu et al. 2000; Stavnezer et al. 2008). *Aicda* is highly expressed in GCs, and it is considered a signature gene of the GC-transcriptional program. These observations, in addition to its dual role as an activator of CSR and SHM might have contributed to the widespread belief that CSR is a process that if not exclusive, is greatly favoured in GCs (Muramatsu et al. 2000). We observed clear upregulation of *Aicda* at day 2.5 after challenge in antigen-specific B cells, 24 hours before the onset of GC

formation. *Aicda* expression increased gradually thereafter, reaching a plateau from day 4.5 to day 8.5 in GC B cells (Fig. 3.5D).

Taken together, these observations indicate that the signals needed to induce CSR *in vivo* are delivered long-before GC formation. Thus, isotype-switching is triggered at the early stages of the immune response, in B cells that have not yet differentiated nor committed to the EF- or GC-pathway.

3.1.3 Formation of surface class-switched antibodies occurs early during B cell activation

As GLTs were expressed as early as day 1.5, we next sought to compare the production of class-switched antibodies expressed on the surface of GC B cells and EFPBs by FACS (Fig. 3.6A-D). Jenkins and colleagues reported that after transfer of 3-83 KIH B cells with DO11.10 T cells, the first antigen-specific IgG2a$^+$ B cells appeared at day 2 after immunisation (Pape et al. 2003). Similarly, we found that IgG1$^+$ SW$_{HEL}$ B cells first appeared at day 2.5, one day after detection of the first GLT (Fig. 3.6B), coinciding with the earliest detection of *Aicda* mRNA (Fig. 3.5D). IgG$^+$ cells increased exponentially over the following two days, reaching a plateau from days 4.5 to 6.5 with approximately 70% of GC B cells and 90% of EFPBs having switched (Fig. 3.6C and 3.6D). No increase in the percentage of total IgG$^+$ cells or IgG1$^+$ or IgG2b$^+$ cells was observed in GCs compared to EFPBs over time (Fig. 3.6D). Moreover, a slightly greater fraction of IgG$^+$ cells was observed within EFPBs compared to GCs on day 6.5 (Fig. 3.6D).
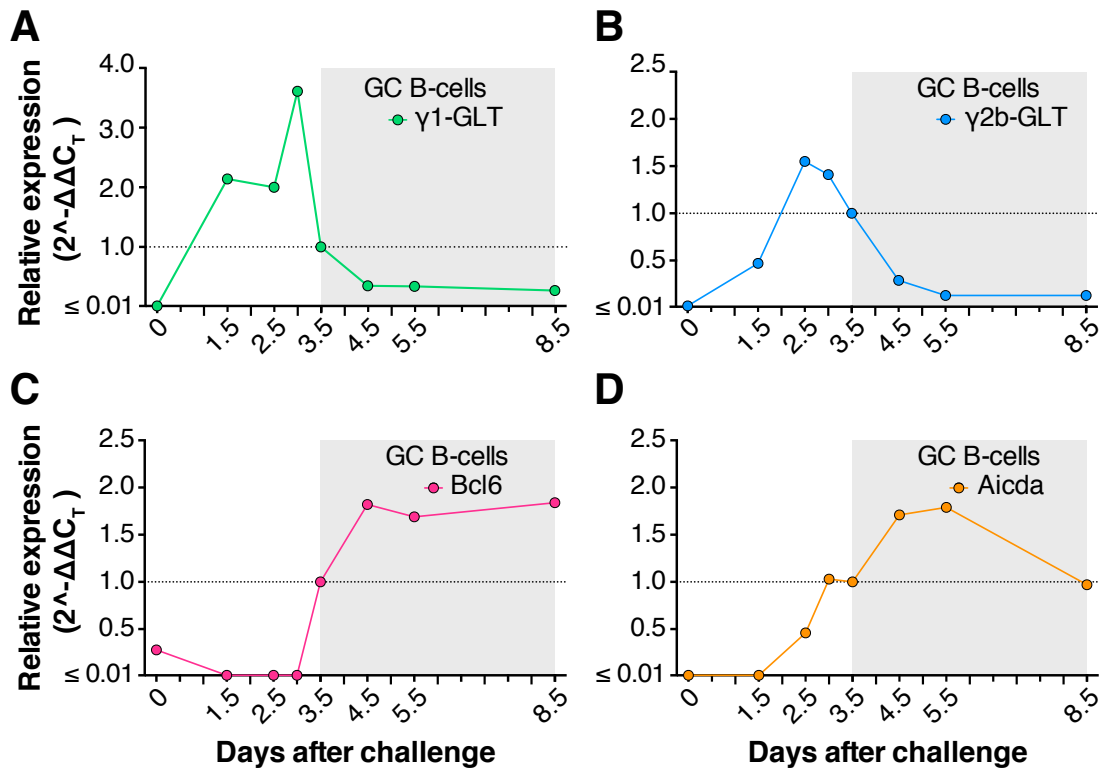
Figure 3.5. Isotype switching commences prior to germinal centre onset.
qPCR gene expression profile of donor-derived SW_{HEL} B cells purified as shown in
Figure 3.2. Plots for γ1-GLT **(A)**, γ2b-GLT **(B)**, *Bcl6* **(C)** and *Aicda* **(D)** genes
are presented. Duplex qPCR analyses were conducted for each target using *Ubc* as a
reference gene in IgM$^+$ HEL$^+$ naïve (day 0), activated (days 1.5 – 3.0), and GC B cells
(days 3.5 – 8.5; grey background). Samples were measured in triplicate, and the average
cycle threshold (C_T) value was normalised to the average C_T value of *Ubc* at each time
point. Data is presented as a fold-change compared to day 3.5 values using the $\Delta\Delta C_T$
method. Dots represent the mean of pooled biological replicates as in Figure 3.2. Data
is representative of two independent experiments.
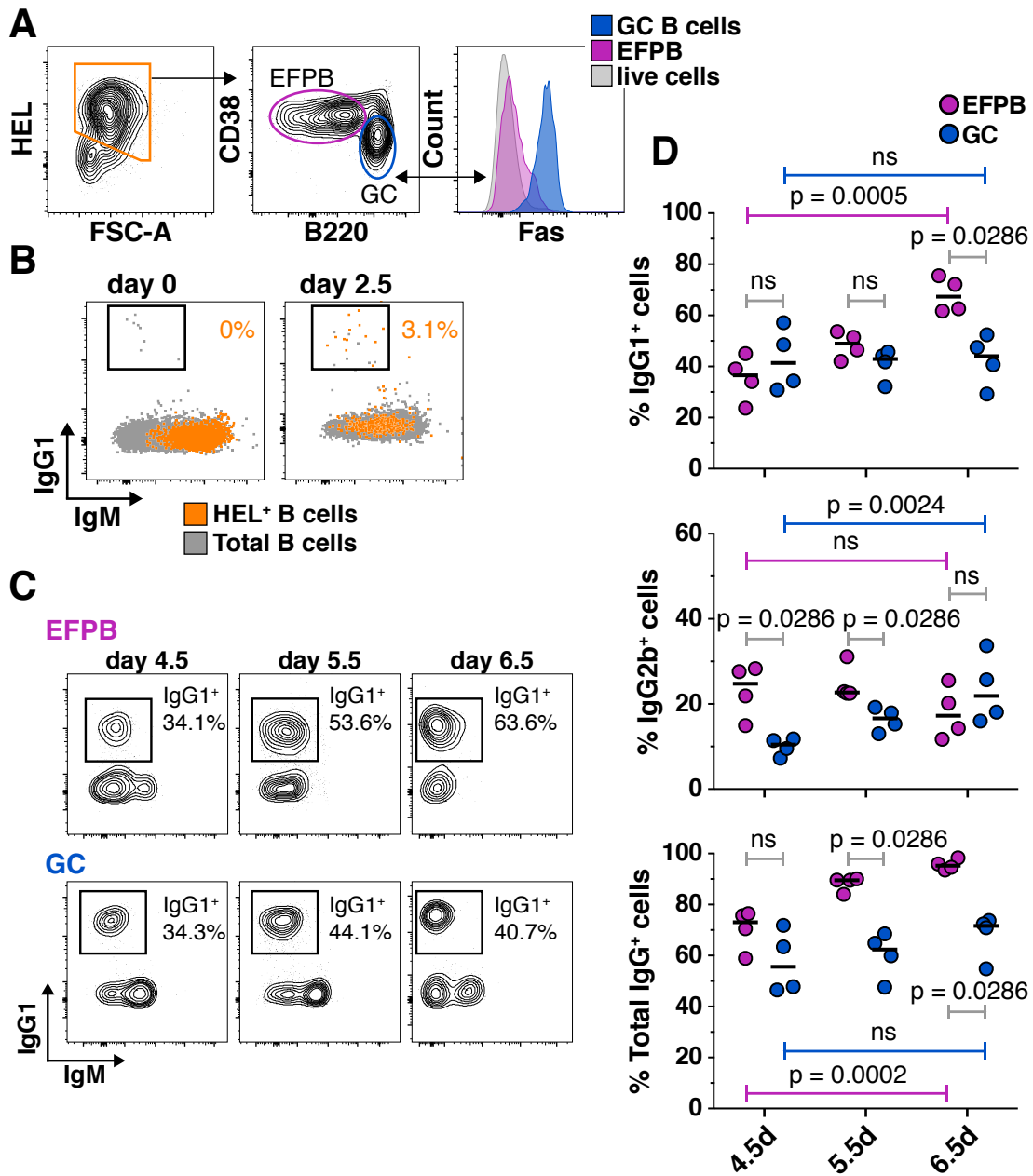
Figure 3.6. Class switching proceeds at comparable rates in germinal centres and extrafollicular sites.

3.1.4 Single cell transcriptomic analysis revealed that GCs are not favourable for CSR

Gene expression analysis in bulk populations of donor-derived SW_{HEL} B cells suggested that GCs were not the places where CSR took place. However, these results could not exclude the possibility that CSR might be a rare event induced in a small fraction of GC B cells. If this hypothetical scenario were true, then CSR would be induced in GC B cells receiving T-cell help and undergoing affinity-based selection in the LZ (centrocytes). Indeed, it has been suggested that CSR may occur in positively selected LZ B cells that have accessed T_{FH} cell help and upregulated the oncogene c-MYC (Sander et al. 2015), which promotes survival, clonal expansion and cyclic reentry within the GC (Dominguez-Sola et al. 2012). c-MYC$^+$ LZ B cells accounts for $\sim$8% of the total population of GC B cells (Dominguez-Sola et al. 2012). Therefore, bulk-cell analysis may lack the sensitivity needed to examine these rare events. To overcome this limitation, we employed a similar adoptive transfer approach using SW_{HEL} B cells, but this time we assessed GLT formation at the single-cell level by qPCR.

Figure 3.6. Class switching proceeds at comparable rates in germinal centres and extrafollicular sites. A) Representative flow cytometric plots showing the gating strategy employed to identify donor-derived cells after immunisation as shown in Fig. 3.1. HEL-binding B cells were gated from live splenocytes (7AAD$^-$) and then subdivided as HEL$^+$ CD45.1$^+$ B220^{lo} CD38^{hi} EFPBs and HEL$^+$ CD45.1$^+$ B220^{hi} CD38^{lo} GC B cells. **B)** Flow cytometric analysis for surface expression of IgG1 and IgM in naïve (day 0) and activated (day 2.5) SW_{HEL} B cells. The numbers in orange indicate the percentage of IgG1$^+$ CD45.1$^+$ SW_{HEL} B cells. **C)** Flow cytometric plots showing gating strategy to quantify surface expression of IgG1 in EFPBs and GC B cells (day 4.5 – 6.5) as summarised in **(D)**. Numbers indicate percentages of donor-derived HEL$^+$ IgG1$^+$ cells. **D)** Quantification of IgG1, IgG2b and total IgG in EFPBs or GC B cells. Bars represent medians and dots individual mice (n=4). Horizontal grey bars show comparisons between EFPB and GC subsets at the same time point (Mann-Whitney U test). Horizontal purple and blue bars show comparisons between EFPBs or GC B cells (Kruskal-Wallis test), respectively. Numbers on top of bars indicate the respective p-value. ns = not significant. Data is representative of two independent experiments.

Spleens from recipient mice were harvested after HEL2x-SRBC immunisation as already described (Fig. 3.1A). IgM^+ HEL^+ SW_{HEL} B cells were single-cell purified by FACS on day 3.0 as activated B cells (Fig. 3.7A), and on day 6.5 as GC B cells recovered at the peak of the reaction (Fig. 3.7B). To ensure we were not underestimating GLT production in GC B cells ($CD38^{lo}$ Fas^+), we further subdivided these cells into DZ ($CXCR4^{hi}$ $CD86^{lo}$) and LZ ($CXCR4^{hi}$ $CD86^{lo}$) B cells (Fig. 3.7B).

Single-cell gene expression analyses were conducted at The Australian Regenerative Medicine Institute, Monash University (Melbourne, Australia), supervised by Dr Christian Nefzger and Dr Jose Polo. We used the Biomark platform (Fluidigm) to analyse 42 IgM^+ activated B cells (day 3), 42 IgM^+ DZ B cells and 100 IgM^+ LZ B cells (day 6.5). In these subsets, we measured the expression of μ-GLT, γ1-GLT, γ2b-GLT, γ2c-GLT, γ3-GLT, *Aicda* and *Bcl6* (Fig. 3.8). We also assessed the transcription factors *Foxo1* and *c-Myc* (suggested to be important for GC-switching), as well as *Apex1* and *Apex2* (enzymes important for CSR and SHM, respectively) (Fig. 3.8). Quality control of the cDNA-preparation was done using *Actb* levels (C_T range: 12-20). All qPCR assays were robustly quantitative and linear (data not shown).

Compared to 52% of day 3 IgM^+ activated B cells expressing γ1-GLTs, less than 3% of IgM^+ DZ or IgM^+ LZ B cells expressed this transcript at day 6.5 (Fig. 3.9A-B). Similar results were observed for γ2b-GLT, with 45% of day 3 B cells positive but only 4.8% and 1% of DZ and LZ B cells respectively expressing γ2b-GLT (Fig. 3.9A-B). Of note, 38% of GLT-positive B cells expressed more than one GLT.

Similar to bulk-cell measurements (Fig. 3.5), *Aicda* was also found in activated B cells, with 45% of day 3 B cells positive for this transcript. The frequency of *Aicda*-expressing cells increased substantially by day 6.5 in DZ B cells (Fig. 3.9A-B). Likewise, *Bcl6* was enriched in day 6.5 GC B cells, with 76%

of DZ B cells and 60% of LZ B cells expressing it (Fig. 3.9A-B). Assessment of *Cxcr4* and *Cd86* in DZ vs LZ GC B cells coincided with the phenotypes observed by flow cytometry (Fig. 3.11).

It has been suggested that CSR requires FOXO1 and c-MYC co-expression, as mice lacking functional FOXO1 present reduced class-switching to IgG1 after SRBC immunisation (Dominguez-Sola et al. 2015; Sander et al. 2015). Consistent with this idea, ∼70% of day 3 B cells were double positive for *Foxo1* and *c-Myc*, and ∼80% of these double positive cells expressed GLTs (Fig. 3.10A-B). By contrast, despite 41% of LZ cells expressing *Foxo1*, and 9% co-expressing *Foxo1* and *c-Myc* (Fig. 3.10A-B), none of the *Foxo1* and *c-Myc* double positive LZ cells expressed GLTs (Fig. 3.10B). This was also true for DZ B cells, with no *Foxo1*$^+$ *c-Myc*$^+$ cells expressing GLTs (Fig. 3.10B).

Collectively, the results obtained by single-cell qPCR support the notion that GLT production (leading to activation of CSR) is not a feature of mature GCs.
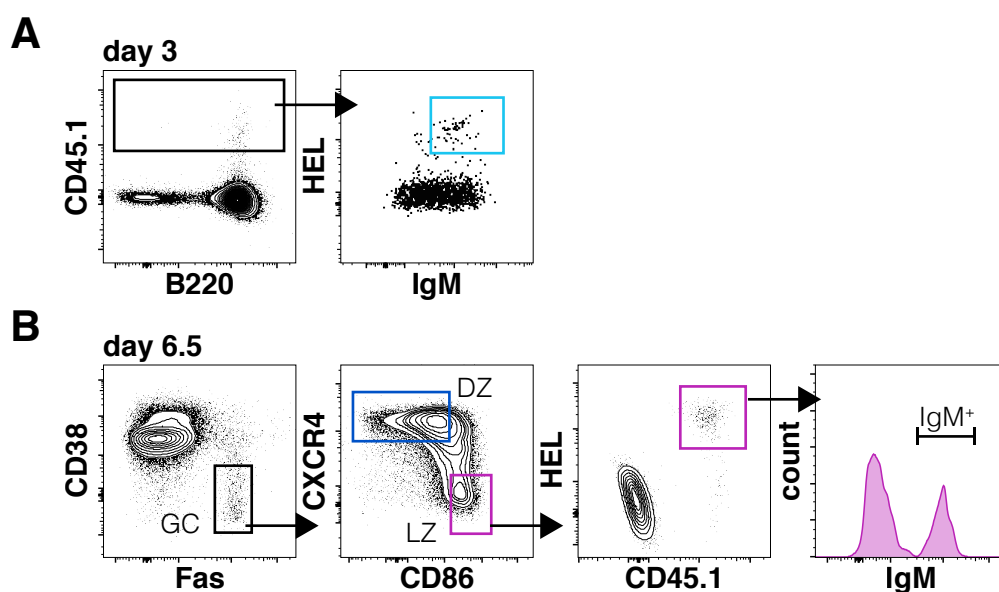
Figure 3.7. Gating strategy employed to FACS-purified responding SW$_{\text{HEL}}$ B cells for single cell qPCR studies. A-B) Flow cytometry plots showing gating strategy to purify HEL-binding B cells as described in Fig. 3.1. Responding SW$_{\text{HEL}}$ B cells were single cell FACS sorted on day 3 **(A)** and day 6.5 **(B)** after HEL2x-SRBC immunisation. Donor-derived cells were purified as IgM$^+$ HEL$^+$ CD45.1$^+$ activated B cells (day 3) **(A)** and IgM$^+$ HEL$^+$ CD45.1$^+$ GC B cells (day 6.5) **(B)**. DZ and LZ B cells were sorted based on CXCR4 and CD86 expression as depicted.
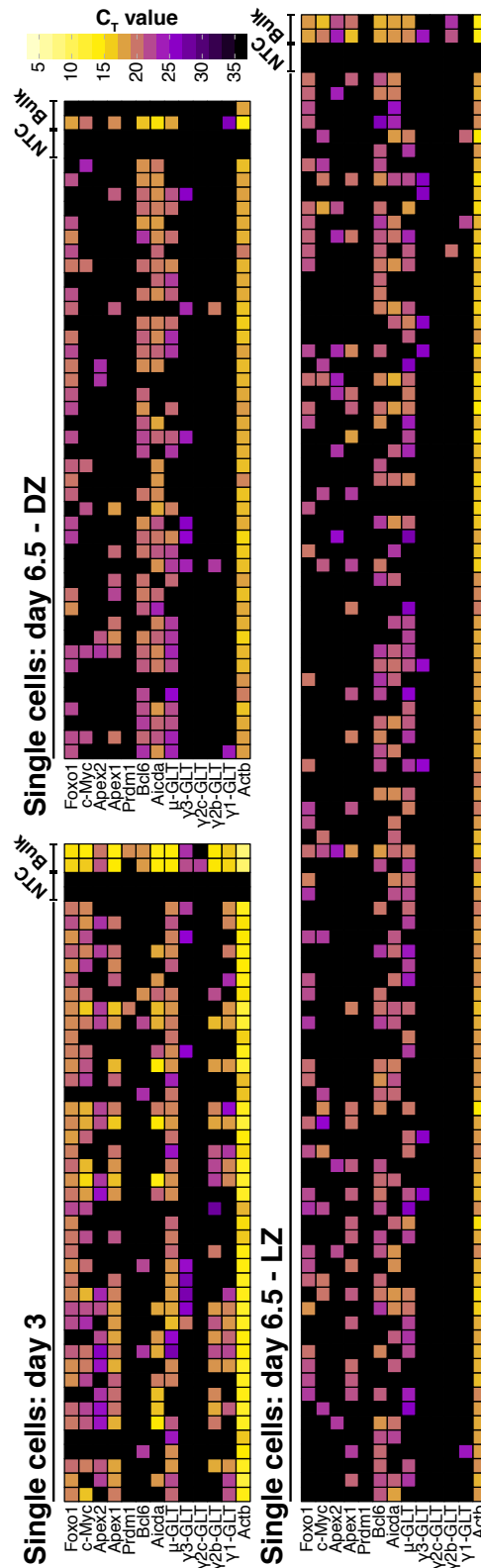
Figure 3.8. Single cell qPCR analysis of selected targets in early activated and GC B cells. Heatmap showing single cell qPCR expression profile of selected targets in B blasts, DZ and LZ SW$_{HEL}$ B cells purified as described in Fig. 3.7. NTC = no template control. Bulk = bulk population control of 20 cells. Data is representative of two independent experiments. Performed in collaboration with Dr Christian Nefzger and Dr Jose Polo.
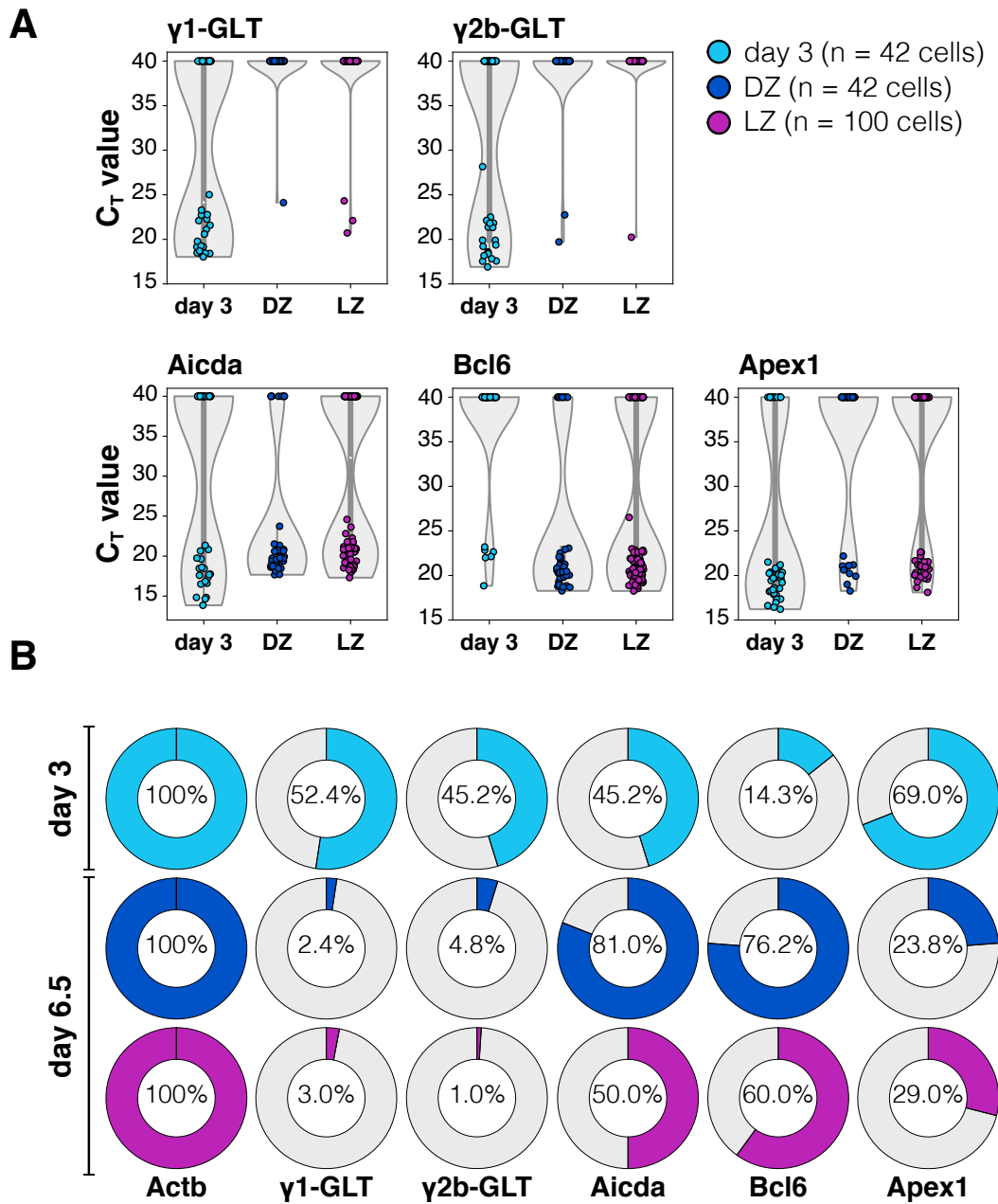
Figure 3.9. GLTs are not predominantly expressed in mature GCs. A) Quantification of raw C_T values for γ1-GLT, γ2b-GLT, *Aicda*, *Bcl6* and *Apex1*. Samples were obtained as shown in Figure 3.8. Violin plots depict data distribution; each dot represents an individual cell. The limit of detection for analysis was set to 40 cycles. **B)** Pie charts showing quantification of target genes as shown in **(D)**. Numbers indicate the percentage of cells expressing the indicated target. Cells with a C_T value < 40 were considered positive events.

A

Foxo1⁺ cMyc⁺

B
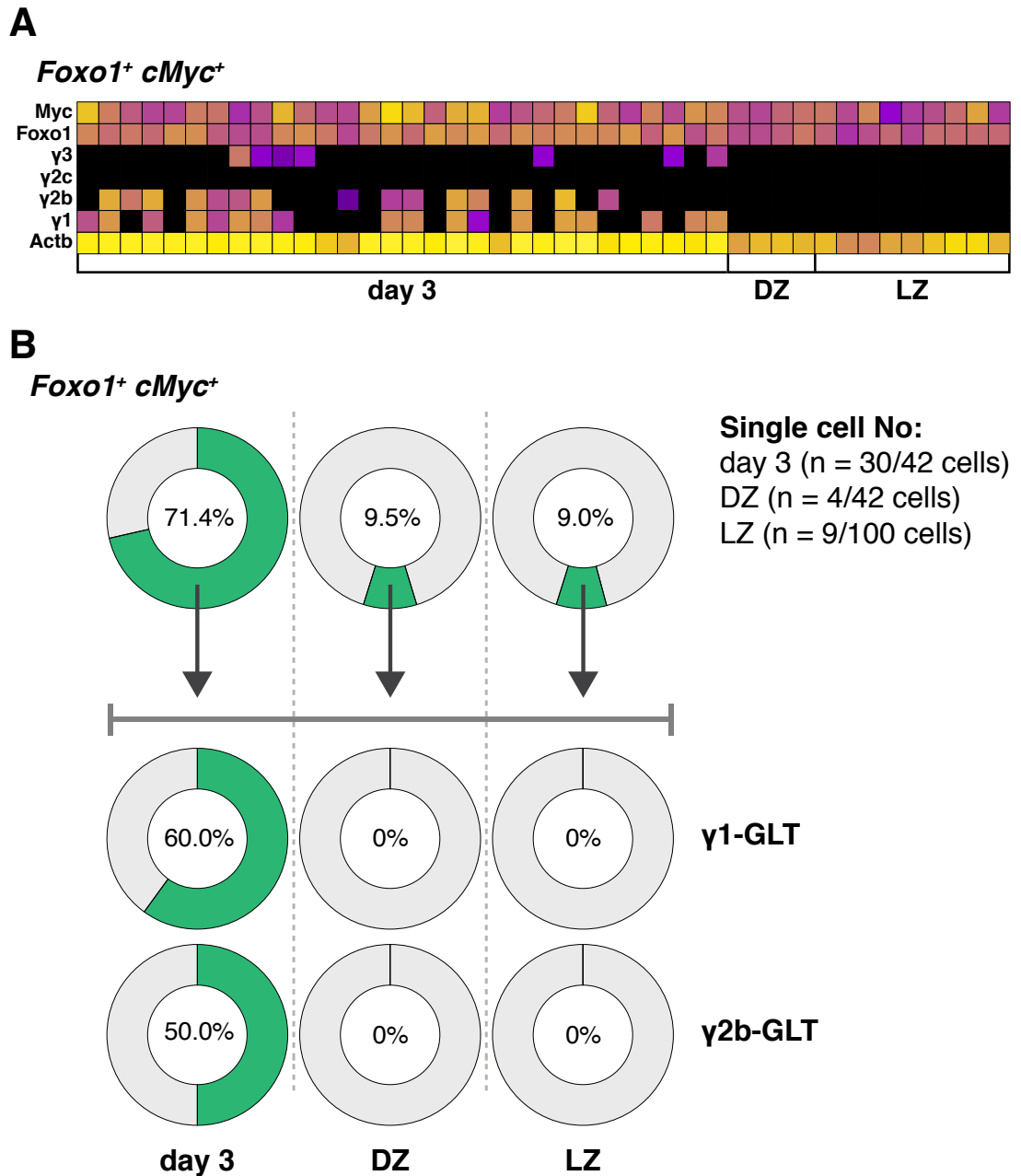
Foxo1⁺ cMyc⁺

Figure 3.10. *Foxo1⁺ c-Myc⁺* **GC B cells do not express GLTs. A)** Heatmap showing single cell expression of γ1, γ2b, γ2c and γ3-GLTs by qPCR in double positive *Foxo1⁺ c-Myc⁺* B blast, DZ and LZ GC B cells. Activated B blasts were purified on day 3.0, whereas GC B cells (both DZ and LZ subsets) were isolated on day 6.5 (see Fig. 3.7 for details). **B)** Pie charts showing quantification of γ1- and γ2b-GLT in double positive *Foxo1⁺ c-Myc⁺* cells as for **(A)**. Numbers indicate the percentage of cells expressing the indicated target. Cells with a C_T value < 40 were considered positive events. Performed in collaboration with Dr Christian Nefzger and Dr Jose Polo.
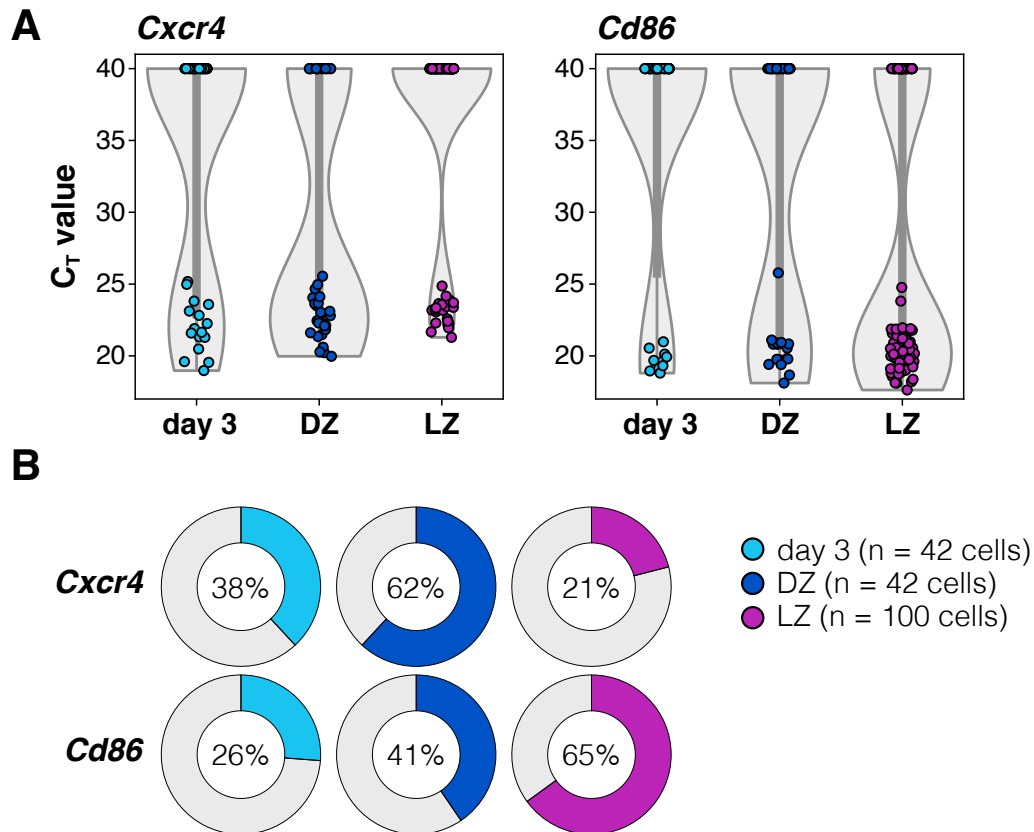
Figure 3.11. Expression of *Cxcr4* and *Cd86* in donor-derived SW$_{\text{HEL}}$ B cells. A) Violin plots showing expression levels for the genes *Cxcr4* and *Cd86* by single cell qPCR in activated B cells (day 3) and GC B cells (day 6.5), subdivided as LZ and DZ cells. **B)** Pie charts showing quantification of target genes shown in **(A)**. Numbers indicate the percentage of cells expressing the indicated target. Cells with a C$_T$ value < 40 were considered positive events. Sample were obtained as described in Fig. 3.7.

3.2 Evaluation of CSR in the late phases of a TD-response

3.2.1 Assessment of CSR in late stages of GC reactions

We next sought to investigate responses to a different combination of antigen/adjuvant that induces longer-lived GCs to exclude the possibility that short-lived GCs, such as those induced by SRBC (Chan et al. 2009), may not be favourable to ongoing switching. To do this, we used B1-8^{hi} tdTomato$^+$ (tdT$^+$) mice. In this Ig-transgenic mouse, B1-8^{hi} B cells represent $\sim$10%-20% of the total B cell compartment, and have a high affinity for the hapten 4-Hydroxy-3-nitrophenylacetyl (NP) (Reth et al. 1978; Allen et al. 1988; Shih et al. 2002). The canonical B1-8 clone was isolated by hybridisation of splenic B cells to myeloma cells after NP-immunisation (Reth et al. 1978). These clones carry a BCR with an affinity for NP of 5x10^5 M^{-1} and is assembled through pairing of the V$_H$186.2 segment with a λ1 light chain (Allen et al. 1988). The B1-8^{hi} BCR was developed by targeted mutagenesis, where a point mutation from TGG$\rightarrow$TTG (Trp$\rightarrow$Leu, codon 33) in the V$_H$186.2 segment results in a 10-fold increment in the affinity for NP (5x10^6 M^{-1}) compared to normal B1-8 cells (Allen et al. 1988; Shih et al. 2002).

Similar to the SW$_{HEL}$ model, the immunisation regime with B1-8^{hi} tdT$^+$ cells requires a higher number of cells transferred for correct visualisation of the early stages of B cell activation. Thus, 1.8x10^5 were injected to analyse the first days of the immune response (17h – day 1.5), whereas 6x10^4 were administered to investigate late phases (day 2 – 18). After transfer, donor-derived NP-reactive B cells were followed by expression of the fluorescence protein tdTomato (Shih et al. 2002).

NP-reactive B1-8^{hi} tdT$^+$ cells were transferred together with 50 μg of NP-CGG into C57BL/6 recipient mice that had been primed with 100 μg of CGG in complete Freund's adjuvant (CFA) 3 days earlier (Fig. 3.12A-B). This priming strategy makes the kinetics of the first few days comparable to those shown for SW$_{HEL}$ responses (Fig. 3.2B), but with GCs persisting longer. Similar to the HEL2x-SRBC response, γ1-GLT peaked between day 2 – 2.5 (Fig. 3.13); at this time point, GLTs were found in cells with an intermediate phenotype (CD38^{int} Fas^{int}) between EFPB and GC B cells (Fig. 3.12B and Fig. 3.13). We also found that GLTs had significantly declined in GC B cells isolated on day 6.5 (Fig. 3.13).

Late stages of the immune response against NP were analysed up to day 18 (Fig. 3.14A-B). GC B cells isolated on days 14 and 18 remained with low levels of γ1-GLT similar to day 6.5 (Fig. 3.13), and more importantly, did not show a second wave of GLT production (Fig. 3.15A). Consistent with early induction of CSR, surface expression of IgG antibodies was first seen at day 2 in NP-binding B cells (Fig. 3.15B). IgG$^+$ GC B cells peaked and reached a plateau between day 4 and day 8, and remained at constant levels through day 18 (Fig. 3.15B), a period in which GCs were sustained (Fig. 3.15C). Thus, there was no evidence of reactivation of GLTs or increased rates of Ig-switching in the late stages of the GC response.

Figure 3.12. Adoptive transfer protocol and gating strategy used to identify B1-8^{hi} tdT$^+$ B cells at early stages of the immune response against NP-CGG. A) Adoptive transfer protocol of B1-8^{hi} tdT$^+$ B cells employed to investigate the early phases of the immune response to NP-CGG. C57BL/6 mice were immunised subcutaneously *(s.c.)* with 100 µg of CGG in complete Freund's adjuvant (CFA). Three days later, mice were adoptively transferred with total splenocytes containing either 1.8x10^5 (17h – day 1.5) or 6x10^4 (days 2.5 – 6.5) B1-8^{hi} tdT$^+$ B cells and simultaneously challenged with 50 µg of NP-CGG. Spleens were harvested at the indicated time points. **B)** Flow cytometric plots showing gating strategy used to identify the transferred cells. Top panel shows representative plots of CD38 vs Fas for donor-derived B1-8^{hi} tdT$^+$ B cells. Bottom panel shows the same FACS-profile for recipient cells in the same animal. Data is representative of two independent experiments.
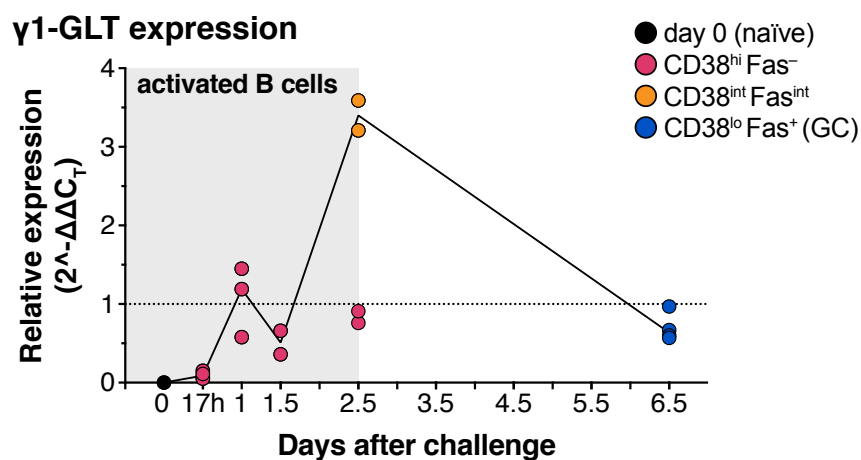
Figure 3.13. GLT expression is downregulated in GC B cells after NP-CGG immunisation. qPCR gene expression profile of donor-derived B1-8^{hi} tdT$^+$ B cells purified as shown in Fig. 3.12. Duplex qPCR analyses were conducted for γ1-GLT using *Actb* as a reference gene. Data is presented as a fold-change compared to day 1.0 values using the $\Delta\Delta C_T$ method. Dots represent individual mice. The black line connects the group medians for naïve (day 0), CD38^{hi} Fas$^-$ (17h – day 1.5), CD38^{int} Fas^{int} (day 2.5), and CD38^{lo} Fas^{hi} GC B cells (day 6.5). Data is representative of two independent experiments
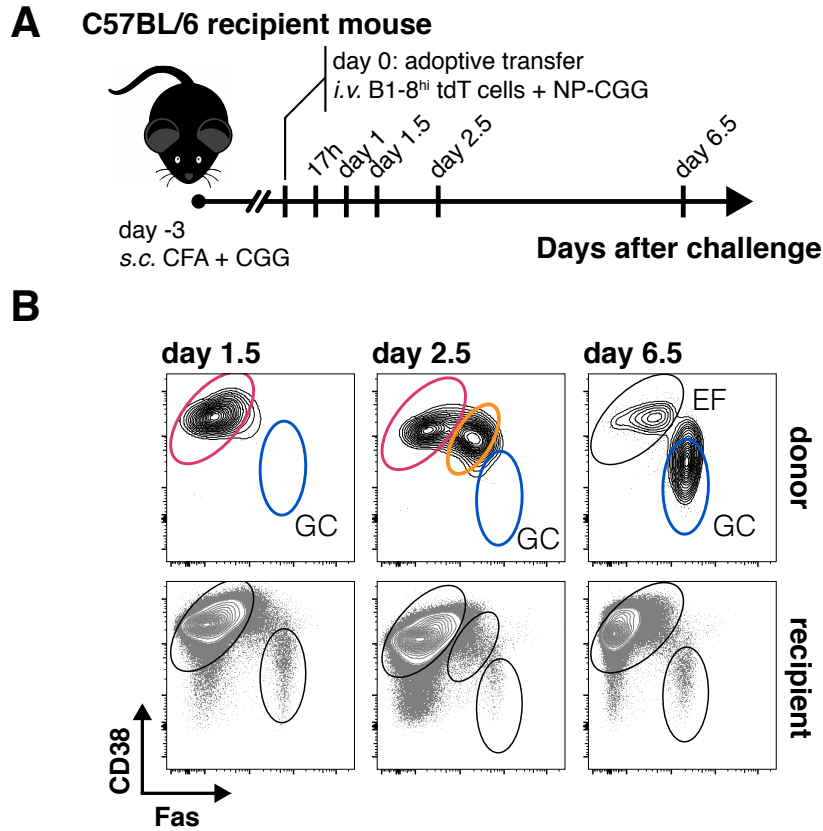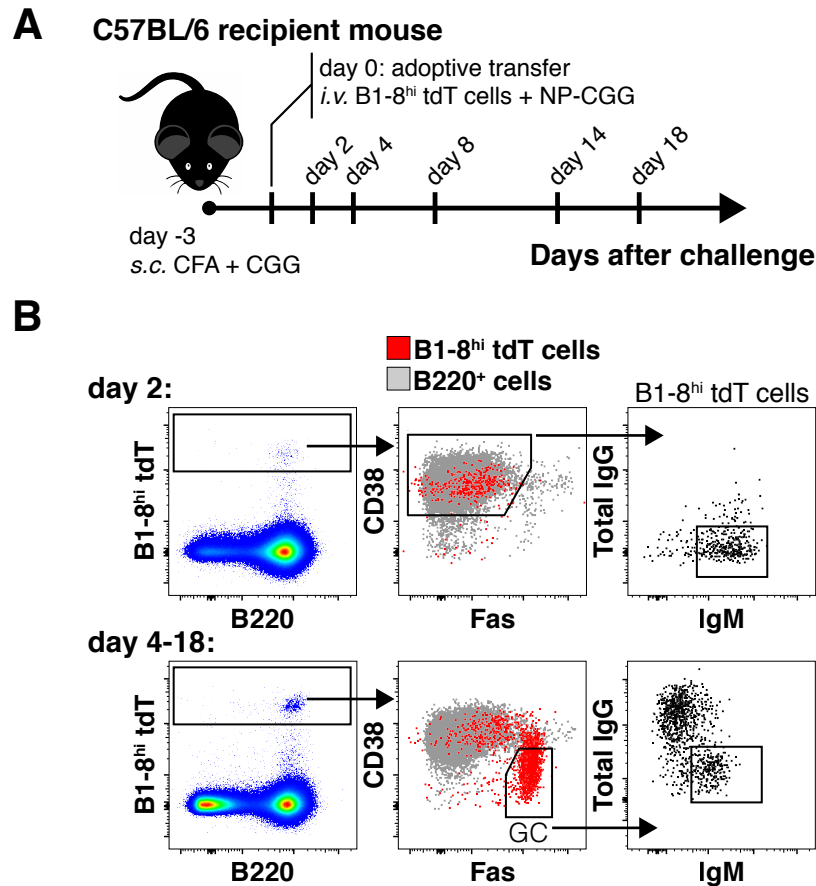
Figure 3.14. Adoptive transfer protocol and gating strategy used to identify B1-8$^{\text{hi}}$ tdT$^+$ B cells at late stages of the immune response against NP-CGG. A) Adoptive transfer protocol of B1-8$^{\text{hi}}$ tdT$^+$ B cells to investigate the late phases of the immune response to NP-CGG. C57BL/6 mice were immunised as described in Fig. 3.12. **B)** Flow cytometric plots showing gating strategy to analyse surface expression of total IgG at day 2 (top panel) and days 4 – 18 (bottom panel) in splenocytes harvested from mice immunised as in **(A)**. Donor-derived B1-8$^{\text{hi}}$ B cells were identified as tdT$^+$ B220$^{\text{hi/lo}}$ live cells (7AAD$^-$). Data is representative of two independent experiments.
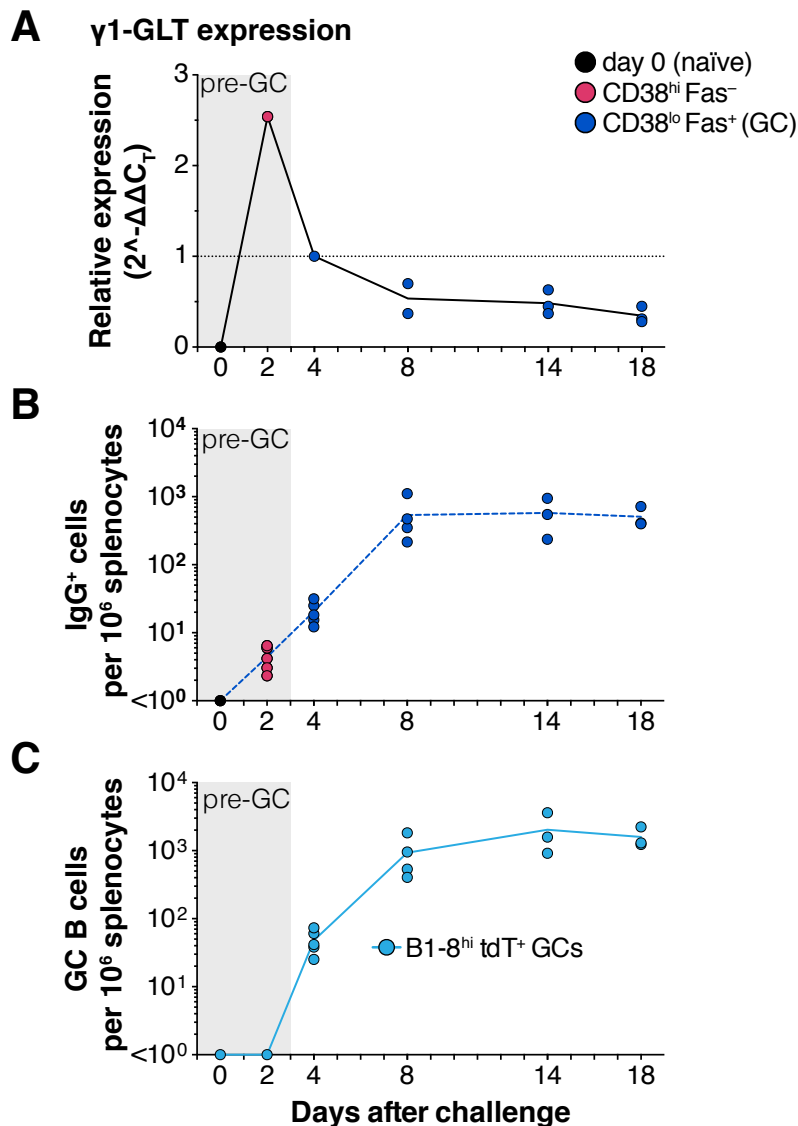
Figure 3.15. Expression of GLTs remains low in late GC responses. A) qPCR gene expression profile in purified donor-derived B1-8^{hi} tdT$^+$ B cells as shown in Fig. 3.14. Duplex qPCR analyses were conducted for γ1-GLT using *Actb* as a reference gene in the indicated subsets. Data is expressed as a fold-change compared to day 4 GC B cell values using the $\Delta\Delta C_T$ method. Dots represent individual animals and the black line connects the group medians for naïve B cells (day 0), CD38^{hi} Fas$^-$ activated B cells (day 2) and GC B cells (day 4 – day 18). **B-C)** Flow cytometric quantification of total IgG **(B)** in donor-derived B1-8^{hi} tdT$^+$ cells and total numbers of B1-8^{hi} tdT$^+$ GC B cells **(C)** in the spleens of C57BL/6 transferred mice identified as shown in Fig. 3.14. Total cell numbers were normalised to 1x10^6 splenocytes. Lines connect the group medians. Number of mice used in each time point: day 2 (n=5), day 4 (n=5), day 8 (n=4), day 14 (n=5) and day 18 (n=5). Data is representative of three independent experiments.

3.2.2 Switch circle transcripts: detection in primary immune responses

CSR is an irreversible DNA recombination event involving the genes encoding the C_H region of the antibody molecule: Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε and Cα (Chaudhuri and Alt 2004; Stavnezer et al. 2004; Cerutti 2008). As reviewed in Chapter 1, recombination of C_H leads to deletion of the intervening DNA between the two participating S regions, which forms an episome (see Fig. 1.2). The released episomal DNA, known as switch circle, contains the I_H promoter of the new isotype expressed by the switched B cell. Interestingly, the transcriptional machinery can be recruited to this promoter, which remains active for a brief window of time. The transcripts arising from the looped-out episomes, namely switch circle transcripts (SWCTs), have been used in different studies as a marker of successful isotype-recombination (Kinoshita et al. 2001; Litinskiy et al. 2002).

Formation of the non-coding SWCTs was assessed in all the adoptive transfer experiments performed in this thesis (SW_{HEL} and B1-8^{hi}; data not shown). Despite readily detection of SWCTs in B cells activated *in vitro* (Fig. 3.16) these by-products of CSR could not be detected at any time point during the *in vivo* primary responses, either in pooled or single antigen-specific B cells. This is likely to be due to the transient nature of these molecules and the low number of copies generated by a fraction of antigen-specific B cells. Of note, published studies measuring switch circle transcripts have been conducted using *in vitro* culture systems or during secondary (memory) immune responses that are typically characterised by a higher precursor frequency of responding B cells (Fagarasan et al. 2001; Kinoshita et al. 2001; Litinskiy et al. 2002; McHeyzer-Williams et al. 2015).

A

B
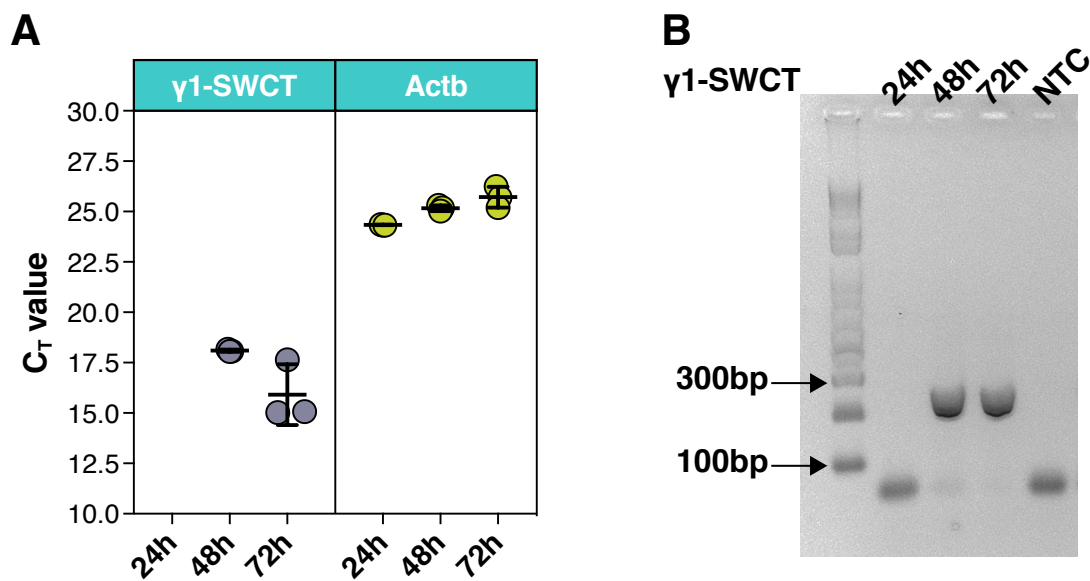
Figure 3.16. Detection of switch circle transcripts in cultured splenocytes.
A) Dot plot showing raw C_T values for γ1-switch-circle transcript (γ1-SWCT) and
the reference gene *Actb*. Purified naïve B cells from C57BL/6 wild type mice were
stimulated *in vitro* for 24h, 48h and 72h with IL-4 and LPS. Samples were pre-amplified
for γ1-SWCT for 22 cycles, and then subjected to qPCR analysis. **B)** Analysis of γ1-
SWCT expression as shown in **(A)**. cDNA fragments amplified by qPCR were resolved
by electrophoresis in a 1.5% agarose gel. NTC = no template control.

3.3 CSR in the context of polyclonal B cell responses

So far, we have characterised the induction of CSR using adoptive B cell transfers. These approaches have allowed us to study antigen-specific responses in a very controlled manner, in which differentiation into GC B cells or EFPBs, and maturation in GCs could be precisely timed. For instance, we can control exactly when B cells are first exposed to antigen, making the elicited immune responses exceptionally synchronised. Early stages of B cell activation are easily assessed, as increased numbers of antigen-specific cells are present before clonal expansion, and because the germline sequence of the BCR is known, any modification in antigen-affinity can be characterised over time (Brink et al. 2008; Zuccarino-Catania and Shlomchik 2015).

The SW$_{\text{HEL}}$ and B1-8$^{\text{hi}}$ mouse models have been carefully validated in the past by different groups, with no indication of altered immune responses due to the increased frequency of antigen-specific B cells (Paus et al. 2006; Chan et al. 2009; Shih et al. 2002; Schwickert et al. 2007). Nevertheless, we decided to test the modulation of CSR in a polyclonal repertoire without the need of Ig-transgenic mice. In the next sections we present the results obtained using the Cγ1-Cre:mT/mG mouse model and photoactivatable (PA)-GFP mice. Each of these models provide additional advantages when trying to answer the question of where and when CSR occurs.

3.3.1 Visualising the onset of GLT expression using Cγ1-Cre mice

To visualise the precise location in which isotype-switching is initiated in mice with a polyclonal BCR repertoire we used Cγ1-Cre:mT/mG mice (Casola et al. 2006; Muzumdar et al. 2007). This mouse model is a conditional Cre/loxP system, in which production of the γ1-GLT can be tracked by Cre-mediated GFP formation (Casola et al. 2006; Muzumdar et al. 2007). The Cre (cyclisation recombination) enzyme catalyses site-specific recombination by recognising a pair of 34-bp DNA target sequences named loxP (locus of crossover in bacteriophage P1), flanking a gene of interest (Feil et al. 2009).

Rajewsky and colleagues developed the Cγ1-Cre mouse line (in a C57BL/6 background) by inserting the coding sequence of the Cre recombinase in the Cγ1 locus – at the 3'-end of the last membrane coding exon (Casola et al. 2006). Transcription across the Cγ1 region results in the expression of a bicistronic mRNA containing the sequence of the γ1-GLT and the Cre enzyme (Casola et al. 2006). On the other hand, the mT/mG mouse is a two-colour Cre-reporter strain that shifts from red to green in cells where Cre is formed (Muzumdar et al. 2007). The mT/mG construct consists of two membrane-targeted fluorescent proteins, tdT (mT) and GFP (mG), under the control of the Rosa26 promoter. The tdT coding sequence is flanked by loxP-sites and it is constitutively expressed by default. When Cre is produced, the tdT sequence is excised allowing GFP formation (Muzumdar et al. 2007).

Therefore, when B cells from Cγ1-Cre:mT/mG mice are primed to switch to IgG1, expression of γ1-GLT is accompanied by formation of the Cre recombinase. In turn, Cre can target the mT/mG locus to enable production of GFP after excision of the tdT sequence. Given that this event is irreversible, cells and their progeny which induce Cγ1 transcription, are permanently tagged with GFP,

making this system a convenient tool to visualise *in situ* the expression of γ1-GLT in tissue sections by IF-microscopy.

Cγ1-Cre:mT/mG mice were *i.v.* immunised with SRBCs by Dr Yang Zhang and Dr Kai-Michael Toellner at the Institute of Immunology and Immunotherapy, University of Birmingham (Birmingham, UK). As shown in Figure 3.17, after immunisation the first GFP$^+$ cells were detected on day 2 and were found predominantly at the T:B border. By day 3, GFP$^+$ cells had expanded and could be found within primary follicles that had yet not formed GCs. Within the following 48 hours GFP$^+$ cells were seen filling both GCs and EF foci. Thus, similar to the SW$_{HEL}$ B cell response, class-switching in a polyclonal repertoire after SRBC immunisation is induced outside the follicles, 24h prior to GC formation.
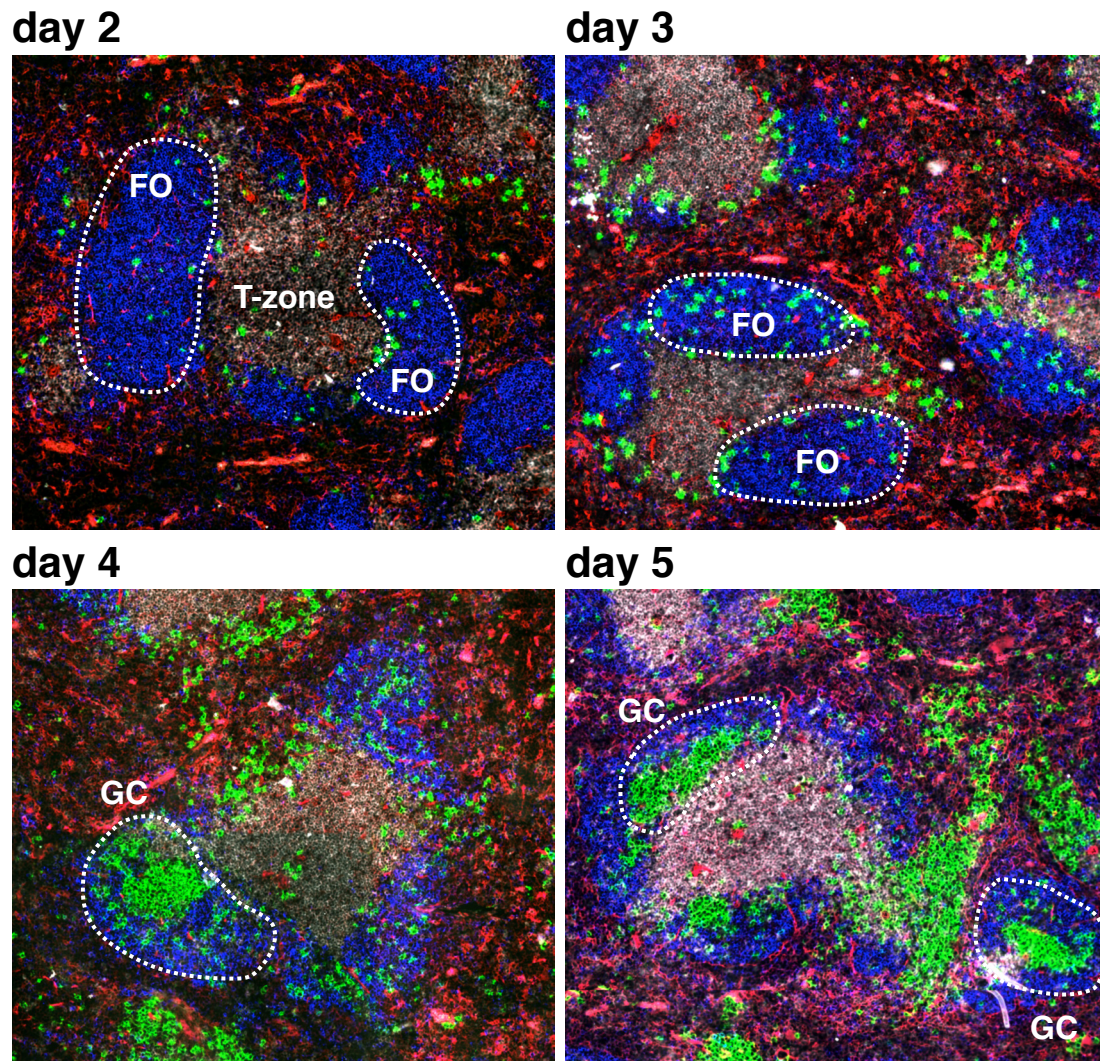
Figure 3.17. γ1-GLT expression is induced as early as day 2 in a polyclonal response after SRBC immunisation. Immunofluorescence (IF) images of frozen spleen sections from Cγ1-Cre:mT/mG mice after SRBC immunisation at the indicated time points: CD3 (grey), IgD (blue), Cγ1-Cre (green) and non-activated B cells (red). Data is representative of three independent experiments. Performed by Dr Yang Zhang and Dr Kai-Michael Toellner.

3.3.2 Studying CSR through phylogenic analysis of pho-toactivated GC B cells

In order to assess CSR in mice with a polyclonal repertoire without the need of adoptive transfers and exclude the possibility that increased exit of IgG$^+$ B cells from GCs might lead to underestimation of ongoing switching, we determined the timing of CSR in polyclonal GCs through phylogenetic analysis using PA-GFP mice (Victora et al. 2010). The hematopoietic lineage in these mice expresses a photoactivatable variant of GFP (PA-GFP), that shifts its excitation spectrum after one- or two-photon irradiation (Patterson and Lippincott-Schwartz 2002; Schneider et al. 2005). For instance, after two-photon irradiation at 830 nm, cells can be identified by two-photon excitation at 940 nm or by FACS using a standard 488 nm laser (Victora et al. 2010). This technique can be use to photolabel specific anatomical regions, such as the GC, within intact lymph node structures allowing the discrimination of individual GCs.

To examine CSR in GCs, *in situ* photoactivation of single GCs within lymph nodes from PA-GFP mice was performed by Dr Luka Mesin and Dr Gabriel Victora at the Rockefeller University (New York, USA) (Victora et al. 2010; Tas et al. 2016). This allows cells within the same GC to be fluorescently tagged, and then FACS-sorted as single GC B cells (Fig. 3.19A-C). GFP-PA mice were immunised with CGG in alum, and photoactivation followed by FACS-purification were performed 15 or 20 days later, to allow multiple rounds of division and SHM within GCs. The SHM burden in the V-region and the induction of recombination in the C$_H$ region was assessed by Igh mRNA sequencing in each cell. With this approach, clonal trees containing both switched and unswitched B cells can be used to establish the timing of CSR, where the CSR point can be inferred as the last common ancestor of the switched and unswitched cells. The number of somatic mutations at the inferred CSR point serves as a "molecular time stamp",

which can be compared to the total SHM burden of cells present in the GC at the time of analysis. Thus, CSR points occurring in cells with zero mutations would indicate CSR precedes SHM, and therefore occurs prior to GC onset; whereas CSR points occurring in cells that have accumulated mutations would suggest CSR is an ongoing process in GCs.

IgG1 is the most common isotype found in the CGG-alum response. To maximise the possibility of identifying IgM to IgG1 CSR events, we screened GCs for expanded IgM$^+$ and IgG$^+$ B cell clones, including in the analysis clones containing ≥ 4 IgM$^+$ cells (Fig. 3.18). This approach led to sequencing 13 clones, including all 8 clones that contained both IgM and IgG cells (Fig. 3.19A-B). Phylogenetic trees were displayed against the number of somatic mutations in each cell (x-axis in Fig. 3.19B), where the inferred CSR points are depicted as red filled triangles (Fig. 3.19B).

We found that several trees remained IgM, indicative of not having switched after entering the GC; these were attributed a CSR point of zero (red open triangles, Fig. 3.19B). Whereas the overall mutation burden in GC B cells was substantial (Fig. 3.19C, mean of 5 mutations per cell), most clones had switch points at zero mutations, with a few at one mutation, and only a single clone underwent switching at an inferred branch point bearing four mutations. Importantly, we found a number of highly expanded and diversified clones (*e.g.*, the top two clones at days 15 and 20 in Fig. 3.19B) for which CSR was either not detected or occurred in a common precursor with 0 or 1 mutations (Fig. 3.19B-C).

Thus, substantial SHM can occur in the absence of detectable CSR. Of note, no sequential switching events were detected in GCs, with IgG1, IgG2b and IgG3 always arising directly from IgM$^+$ cells (Fig. 3.19B). Mutational analysis of polyclonal GCs supports that CSR is restricted to the pre-GC or early GC periods, and is uncommon after cells have accumulated several mutations in mature GCs.
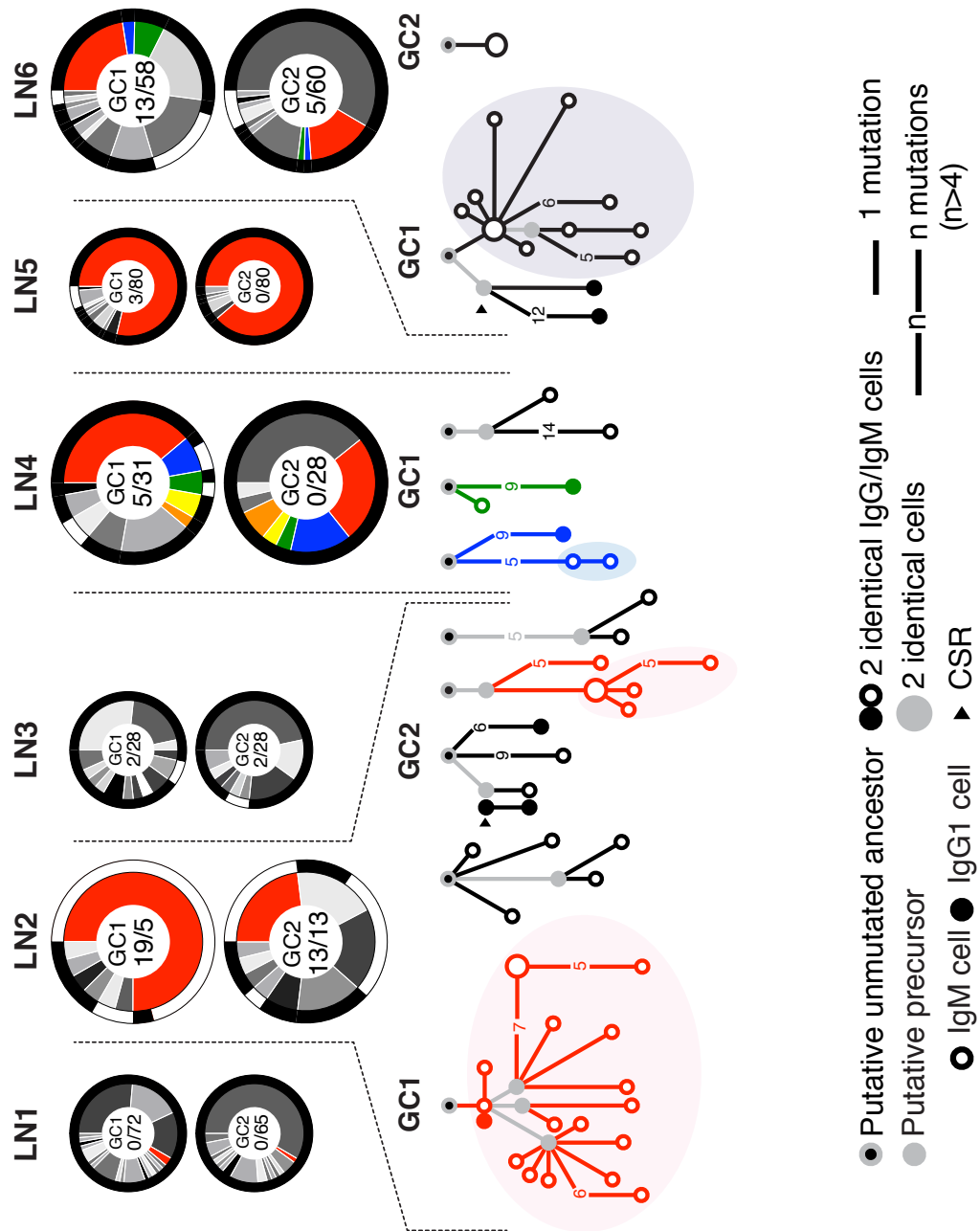
Figure 3.18. Clonal diversity of photoactivated GC B cells after CGG-alum immunisation. Charts showing clonal diversity of GCs isolated from popliteal lymph nodes (pLNs) after photoactivation in mice immunised 15 days before with CGG-Alum. Two individual GCs per pLN were photoactivated and separately FACS-sorted, as described (Tas et al. 2016). Pie charts show clonal distribution of sequenced Igh genes in each GC. In the inner ring, each slice represents one distinct clone represented in greyscale, coloured slices indicate clones that were found in both GCs (top and bottom pie charts) from the same pLN. In the outer ring the isotype IgG (black) or IgM (white) of each clone is indicated. Numbers in the centre of each chart are the total number of IgM/IgG cells sequenced. Clonal trees represent the phylogeny of IgM V_H sequences within each clone containing more than 2 cells per clone from pLNs that have more than 5 IgM cells (symbols according to the legend in the bottom panel). Pairs are from 5 different mice in 3 independent experiments. Performed by Dr Luka Mesin and Dr Gabriel Victora.

Figure 3.19. Lack of ongoing switching in IgM$^+$ B cells from established germinal centres.

3.4 Mathematical modelling of CSR dynamics.

The detection of IgM-dominated GCs by phylogenetic analysis at late time points in the response against CGG (Fig. 3.19), argues against the idea of ongoing switching within GCs. Hence, we next asked whether a dynamic downregulation of CSR early in GCs would be compatible with *in silico* models of GC reactions.

Starting from a state-of-the-art GC simulation, Dr Michael Meyer-Hermann and colleagues, (Meyer-Hermann et al. 2012; Meyer-Hermann 2014; Binder and Meyer-Hermann 2016), investigated the mean fraction of IgM^+ and IgG^+ B cells in GCs with the assumptions of constant versus decaying switching probability. Constant switching induces virtual GCs with a fraction of IgG^+ B cells significantly larger than 50% (Fig. 3.20A), which contradicts the presence of IgM-dominated GCs (Fig. 3.19). Thus, a model of constant switching *in silico* might require the additional assumption of a biased output of IgG^+ B cells. When we tested this idea, the empirical data obtained with the SW_{HEL} system (Fig. 3.5) could not be recapitulated either (Fig. 3.20B). In contrast, GC simulations with decaying switching probability, integrating the kinetics observed for $\gamma1$-GLT expression
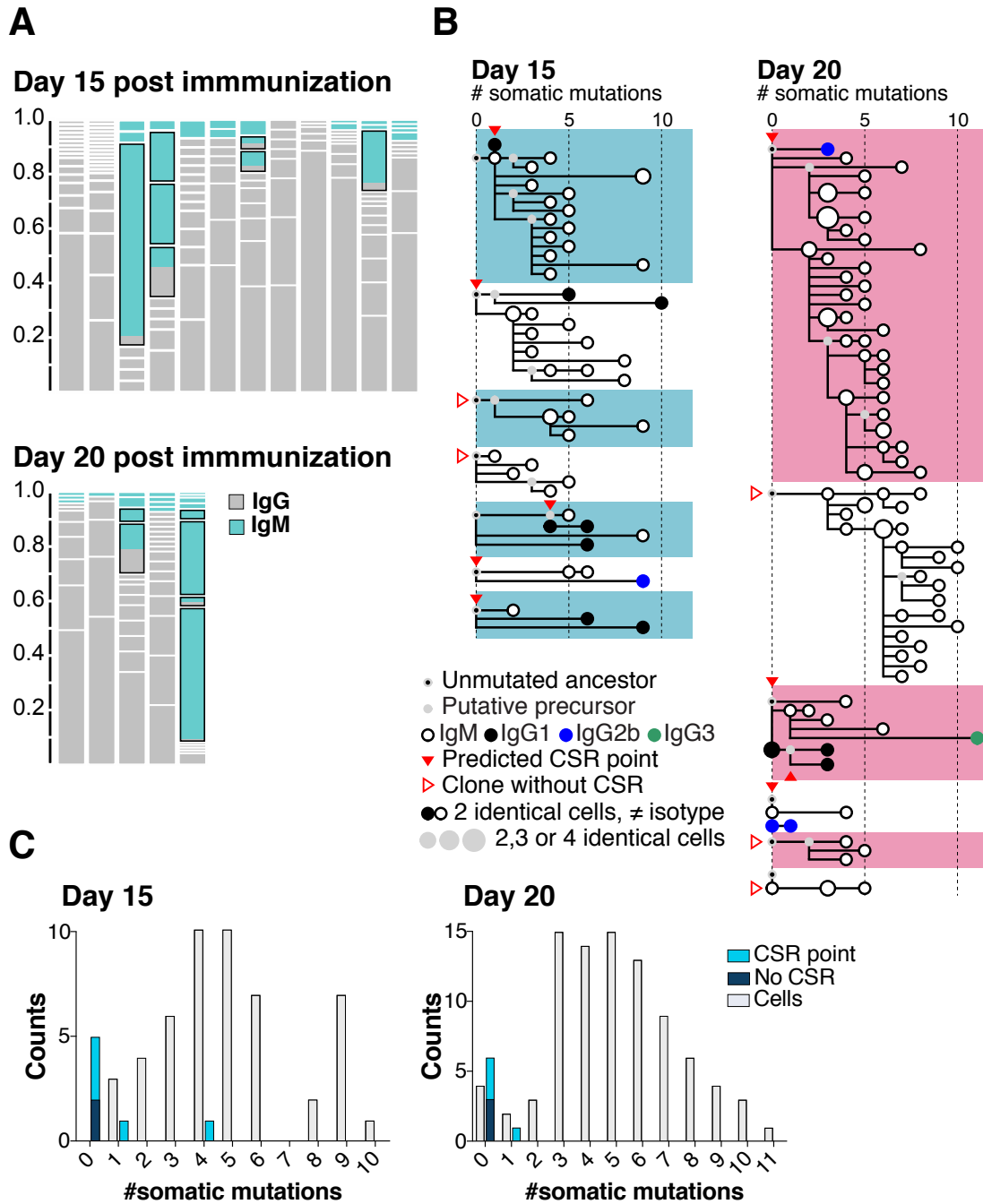
Figure 3.19. Lack of ongoing switching in IgM^+ B cells from established germinal centres. A) Schematic representation of the clonal and isotype composition of the GCs obtained from pLNs of PA-GFP mice immunised 15 or 20 days earlier with CGG in alum. Each column represents a single GC and the boxes in each column represent individual clones determined by phylogenetic analysis of single cell mRNA V_H sequences. The size of each box has been scaled to reflect the number of cells in each clone. Grey represents IgG^+ B cells and green represents IgM^+ B cells, as determined by Igh mRNA sequences. The boxes outlined in black indicate those clones selected for the somatic mutation analysis depicted in **(B)**, based on mixed composition by both IgG^+ and IgM^+ cells, and the presence of 4 or more IgM^+ cells. **B)** Charts showing clonal trees representing the phylogeny of V_H sequences within B cell clones (symbols according to the legend in the bottom panel). **C)** Summary of the data in **(A-B)** showing the SHM content of individual B cells at the time of the inferred switch event (filled red arrowheads). Clones containing only IgM^+ cells (empty red arrowheads) were pooled with those in which switching occurred at the level of the unmutated precursor (zero mutations). For each time point 5 different mice in 3 independent experiments were included. Performed by Dr Luka Mesin and Dr Gabriel Victora.

(Fig. 3.5) without any further assumption, was the only model consistent with the observed data (Fig. 3.20C). Thus, the diversity of isotypes found *in silico* with decaying switching probability supports a model with CSR limited to the first days of the GC reaction (Fig. 3.20C).

Next, we asked whether there is a critical time point to stop switching in GCs after which the diversity of isotype dominance would be lost. Starting from the model with decaying switching probability we shifted the time at which the decay starts from day 3.5 post-immunisation (GC onset) to day 19.5 (a late time point), keeping the integrated switching probability unchanged. The resulting isotype diversity of GC simulations dropped when CSR occurred later than day 6-7 post-immunisation (Fig. 3.20D). This suggests that isotype determination before the GC phase of intense B cell selection promotes diversification of the GC isotype dominance at later times. We conclude that an ongoing switching probability would homogenise the isotype distribution of GC output, while clonal selection occurring after a decayed switching probability favours a diversification of GC isotypes. This result strongly supports the model of a switching probability that decays over the first three days of the GC reaction.
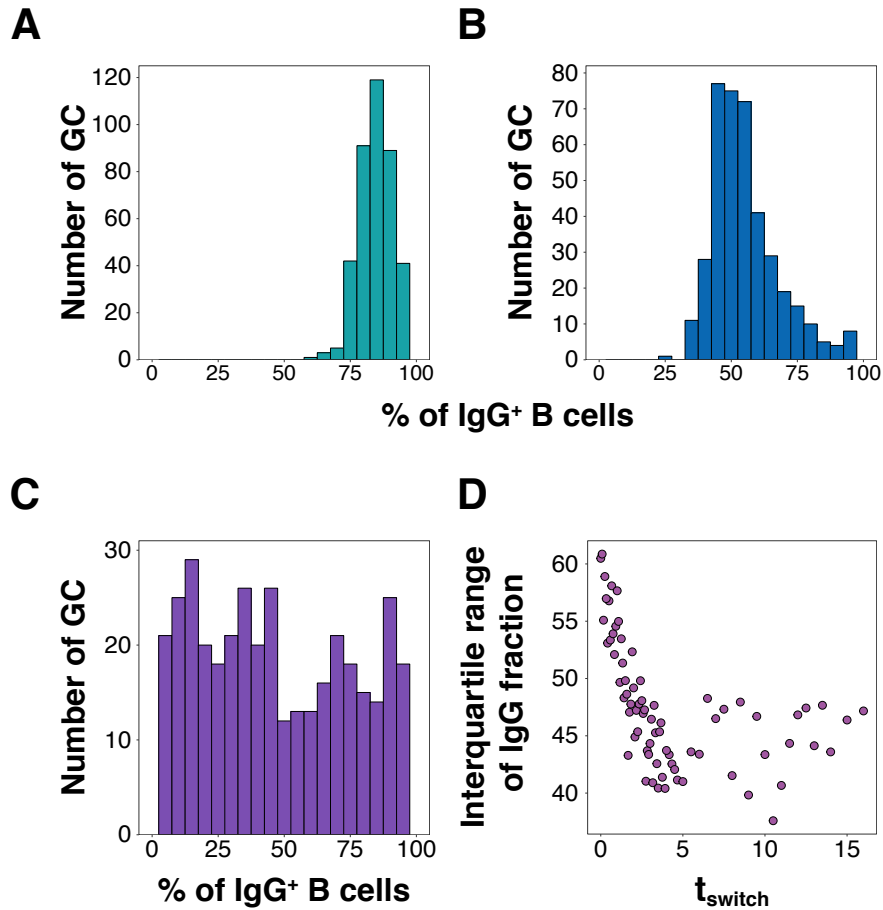
Figure 3.20. *In silico* **simulations of GCs with a decaying switching probability recapitulates the formation of IgM-dominated GCs. A-C)** Histograms showing distribution of IgG fractions at the end of affinity maturation *in silico.* **(A)** Model with constant switching probability of $p = 0.03$; **(B)** constant switching probability combined with an increased probability of IgG$^+$ cells leaving the GC; and **(C)** dynamic switching with an initial switching probability of $p = 0.15$ and a decaying switching probability of $\gamma = 0.035$ h^{-1} (see Fig. 3.5). Each distribution shows the fraction of IgG$^+$ B cells at day 21 after the onset of the GC reaction. **D)** Effect of class switch timing on the diversity of Ig-isotypes in simulated GCs. t$_{switch}$ (horizontal axis) denotes the time post GC onset (time post-immunisation minus 3.5 days) at which CSR has started with a decreasing probability. Each point corresponds to the interquartile range of the IgG fraction among B cells at the end of *in silico* GC reactions in 400 simulations. Modelling by Dr Sebastian Binder, Dr Philippe Robert and Dr Michael Meyer-Hermann.

3.5   Regulation of APE1 in GC responses

We next looked for mechanisms that might contribute to limit CSR within GCs. CSR relies primarily on activation of AID, UNG and APE1 to target S regions (Muramatsu et al. 2000; Rada et al. 2002; Guikema et al. 2007). As already discussed, the sequential activity of these enzymes results in the generation of DNA breaks needed for correct formation of class-switched antibodies (Chaudhuri and Alt 2004; Stavnezer et al. 2008).

The role of APE1 in the immune system has been difficult to study due to the lethality of APE1-deficient mice. Still there is accumulating evidence that APE1 is required in a dose-dependent manner during CSR (Masani et al. 2013; Xu et al. 2014), whereas it appears dispensable for SHM (Stavnezer et al. 2014). By contrast, SHM does not need APE1 but instead requires APE2 (Sabouri et al. 2009; Masani et al. 2013; Stavnezer et al. 2014).

We first interrogated an RNA-seq dataset of human naïve and GC B cells our team had generated in collaboration with Dr Lynn Corcoran (unpublished) to examine the expression of proteins associated with CSR and SHM. Expression of *AICDA*, *UNG* and *APEX2* were all increased in GC B cells, whereas *APEX1* appeared downregulated (Fig. 3.21), consistent with a previous report in bulk mouse GC B cells (Stavnezer et al. 2014).

Having identified a critical component of CSR downregulated in GCs, we next asked whether the downregulation of APE1 was also a feature of both DZ and LZ B cells (Fig. 3.22). In tonsil sections subjected to histologic analysis, APE1 was barely detectable across well-established GCs (Fig. 3.22A). We then confirmed this observation by western blot using FACS-purified tonsillar DZ, LZ, unstimulated naïve B cells (Fig. 3.22B), and naïve B cells stimulated for 72h with IL-21 and CD40L (referred to as activated B cells). Our data revealed a significant

decline in APE1 protein in both DZ and LZ areas of human GCs compared to naïve or activated B cells (Fig. 3.22C-D). Thus, in addition to greatly diminished GLT induction in GC B cells, APE1 downregulation emerges as a novel mechanism to prevent CSR in GCs.

Finally, we considered whether BCL6, the master transcription factor of the GC-cell fate, could be actively repressing APE1 formation in GCs. BCL6 is a transcriptional repressor that was first characterised in 1993 (Baron et al. 1993; Ye et al. 1993). Soon after its discovery, it was proven that BCL6 is essential for B cells to become GC B cells (Dent et al. 1997; Ye et al. 1997). A key role of BCL6 in the commitment to the follicular fate was extended later to T_{FH} cells, revealing that BCL6 is also necessary in T cells following a GC pathway (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009). Today, a great deal of knowledge has been generated regarding the structure of BCL6 and its capacity to represses different genes and pathways. ChIP-on-chip experiments have predicted that BCL6 can specifically modulate over 400 genes in GC B cells including: anti-apoptotic genes, cell cycle modulators and genes involved in the DNA-damage response, among others (Ci et al. 2009; Basso et al. 2010). Based on this theoretical framework, BCL6 appeared as an ideal candidate to modulate APE1 expression in GC B cells.

We reanalysed a ChIP-on-chip datasets (Ci et al. 2009) published by our collaborator Dr Jose Polo. This revealed that BCL6 binds the promoter region of *APEX1*, but not *APEX2* in human primary GC B cells (Fig. 3.23), as well as the promoters of the well-known BCL6 targets, TLR1 and BCL6 (Ci et al. 2009). Therefore, we propose that BCL6 is likely to modulate the expression of APE1, and thus prevent induction of CSR in GC B cells.
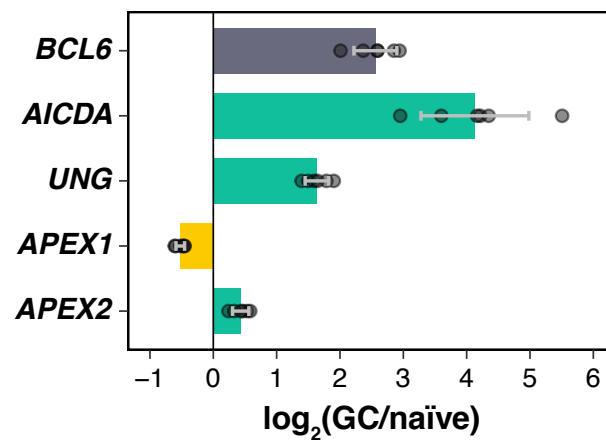
Figure 3.21. RNA-seq analysis of selected target genes expressed by human GC B cells. Plot showing relative mRNA expression of human *BCL6*, *AICDA*, *UNG*, *APEX1* and *APEX2* genes. Samples were analysed by RNA-seq purified from tonsillar naïve B cells and GC B cells. Data is presented as the $\log_2$ fold-change between reads per kilobase per million reads (RPKMs) of GC B cells relative to those on naïve B cells. The bars represent means and error bars indicates $\pm$ standard deviations. Dots represent individual donors (n=5). Samples were processed by Dr Ilenia Papa (the Vinuesa group) and data was generated by Dr Lynn Corcoran.
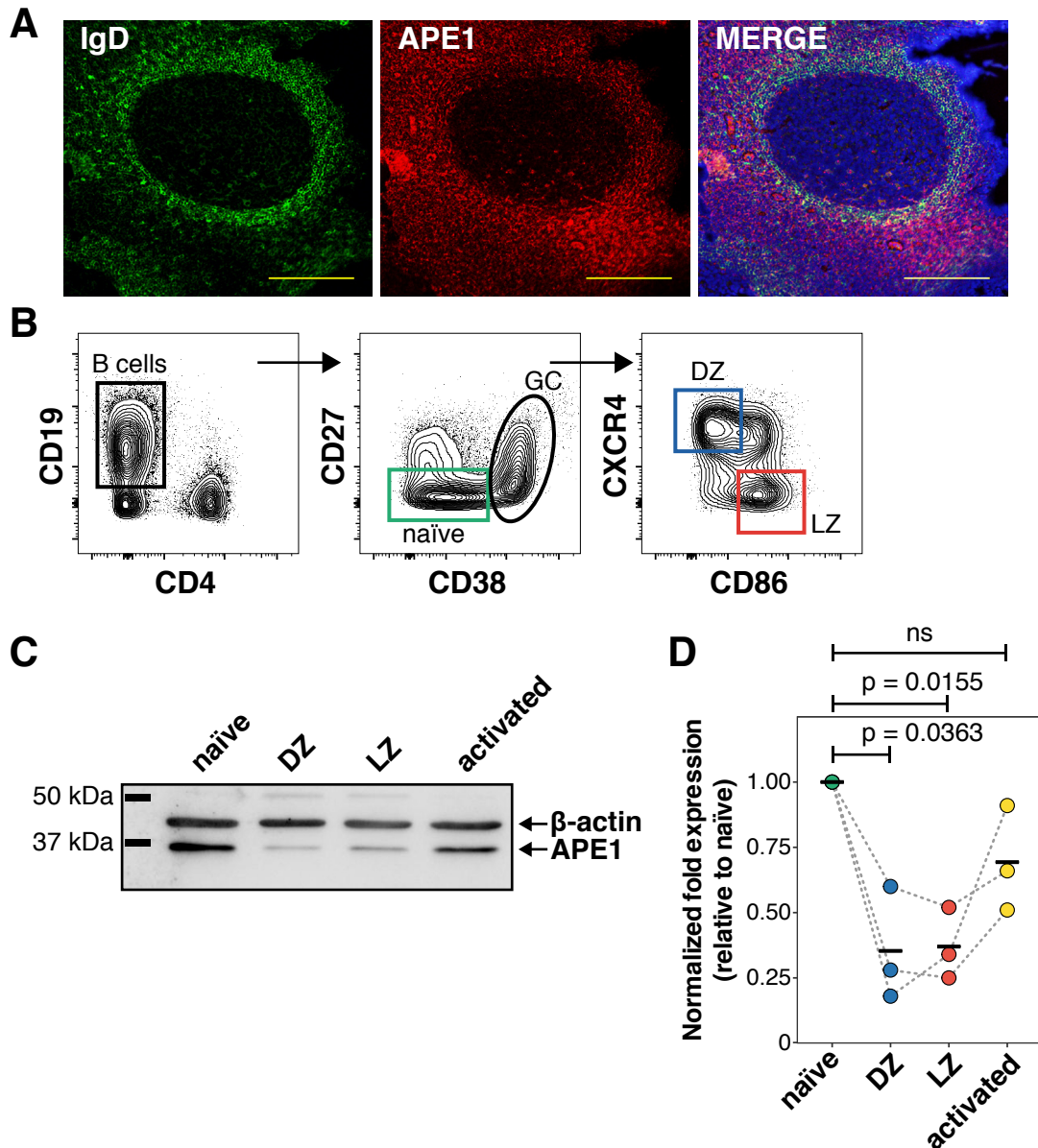
Figure 3.22. APE1 is downregulated in human GC B cells. A) Immunofluorescence (IF) images of frozen human tonsil samples showing APE1 (red), IgD (green), and DAPI (blue). Scale bars = 200 µm, 20x magnification. **B)** Flow cytometric plots showing the gating strategy to purify naïve, DZ and LZ B cells from human tonsils. Activated B cells correspond to naïve B cells stimulated *in vitro* for 72h with IL-21 and CD40L. **C)** Western Blot of human APE1 protein in naïve, DZ, LZ and activated B cells. β-actin was used as a loading control. **D)** Quantification of APE1 protein levels by densitometry as for blot in **(C)**. APE1 expression was normalised using β-actin levels. Expression levels were normalised to those of naïve B cells by densitometric analysis. Horizontal black bars represent means and dotted grey lines connect samples derived from the same tonsil donor. Numbers on top indicate the respective p-value from two-tailed paired t-test analysis, n = 3.
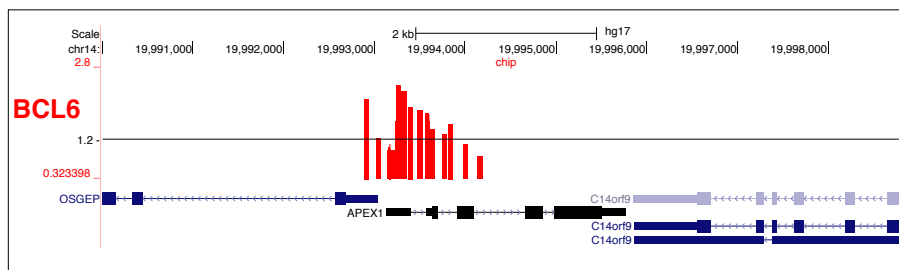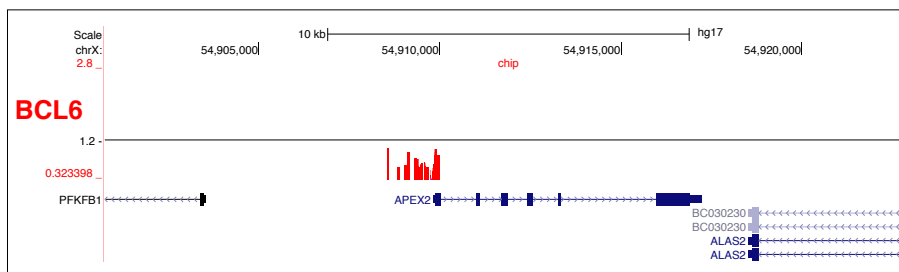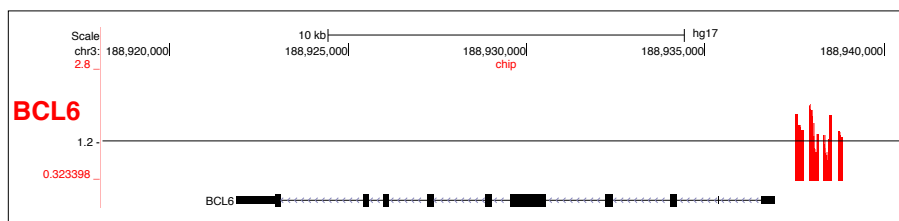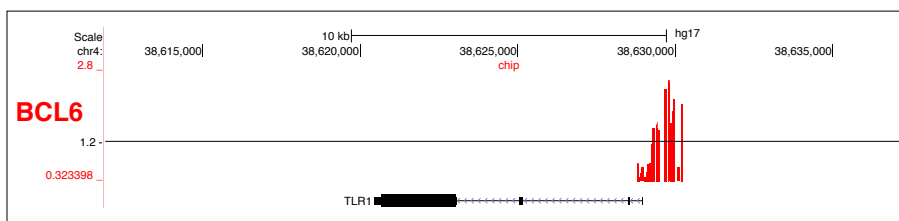
Figure 3.23. APE1 expression in human GC B cells is modulated by BCL6.
Regions where BCL6 binds to the promoter region of the genes encoding for APE1
(APEX1), APE2 *(APEX2)*, BCL6 and TLR1, as determined by ChIP-on-chip. Data
was reanalysed from Ci et al. 2009 by Dr Jose Polo.

Chapter 4

Discussion

4.1 Summary

The precise location where CSR takes place still remains a subject of intense debate and controversy. Nonetheless, the prevalent view assumes that GCs are the privileged sites where CSR occurs. The present study provides evidence that CSR is initiated prior to GC formation during a primary immune response against TD-antigens. We used transgenic mouse models that allow us to unequivocally identify the phenotypic changes that B cells undergo as they become early GC B cells or EFPBs during the course of an immune response against model antigens. We observed that a comparable proportion of class-switched B cells were found in the EF and follicular areas of the spleen, with no further enrichment within GCs. Analysis of GLT expression at the single cell level in purified IgM$^+$ activated B cells and IgM$^+$ GC B cells provided evidence that isotype switching was largely not triggered nor executed in mature GCs: neither GLTs were produced at significant amounts, nor unswitched clones became switched. This effect was independent of the immunising antigen, the longevity of the GC response or the adjuvant used. Similar results were observed when GLT formation was

assessed in a polyclonal B cell response using Cγ1-Cre:mT/mG mice. Likewise, phylogenetic trees constructed from *Igh*-mRNA sequencing of photoactivated GC B cells demonstrated that CSR ceased soon after the onset of SHM, and revealed the existence of IgM-dominated GCs, which by mathematical modelling were unlikely to occur under the assumption of ongoing-switching.

We conclude that isotype switching is a process that is activated before GC formation and proceeds normally regardless of whether B cells differentiate in GCs or EFPBs. Thus, our work challenges the general belief that CSR is a GC process.

4.2 APE1 vs APE2 in CSR

Here we have shown that CSR is initiated at early stages of B cell activation, with no evidence of ongoing switching in established GCs. It is likely that low levels of AID, which was first detected on day 2.5, in conjunction with APE1 are sufficient for CSR to occur before GC formation; whereas high expression of AID and APE2 in GCs might be required for SHM.

When we tested the expression pattern of APE1 by RNA-seq, IF-microscopy and western blot (WB), all methods showed a significant reduction of this protein in GC B cells. Moreover, by WB we observed that both DZ and LZ B cells had diminished APE1 levels. This data was also confirmed in single cell studies, where fewer GC B cells expressed *Apex1* mRNA compared to early activated B cells. In regard to the low levels of AID, CSR is considered very efficient in nature, as it can occur even in the presence of a single double-strand break (DSB) (Stavnezer et al. 2008). Besides, AID can attack anywhere within the S region, which can extend as long as 10-12kb, increasing considerably the likelihood of creating sufficient DNA breaks to successfully induce CSR. Based on this evidence, we propose a mechanism whereby CSR is induced early during B cell

activation, and then downregulated in GCs due to the lack of APE1 protein in these micro-environments. We further hypothesise that this reduction is mediated by the transcriptional repressor BCL6, as shown by ChIP-on-chip analysis (Ci et al. 2009).

The downregulation of APE1 in GC B cells is surprising, considering that APE1 is essential for cell survival and ubiquitously expressed in all cells, with an estimate of 0.35 - 7x10^6 molecules/cell (Xanthoudakis et al. 1996; Fung and Demple 2005; Al-Safi et al. 2012). Besides being the core apurinic/apyrimidinic (AP) endonuclease for CSR, APE1 is a multifunctional enzyme that orchestrates many cellular processes (Tell et al. 2009). APE1 is probably best known for its vital role in the BER pathway, an important arm of the DNA damage response that repairs damaged bases arising spontaneously in the genome or mediated by physical or chemical agents (Fortini and Dogliotti 2007; Krokan and Bjørås 2013). The most common single base lesions are induced through hydrolysis of dC residues to dU, the attack of reactive oxygen species (ROS), UV-light, or alkylating agents (Demple and Harrison 1994; Thakur et al. 2014). In all of these cases, the main intermediate to repair the damaged base is the generation of AP sites that can be mended by APE1 (Thakur et al. 2014). If not properly repaired, these lesions could lead to cell cycle arrest (by stalling DNA replication), DNA cytotoxicity, apoptosis, or more severe defects, such as deleterious mutations, neurodegenerative disorders or cancer (Fung and Demple 2005; Krokan and Bjørås 2013).

In order to restore genomic integrity, the most basic BER components needed are: i) a DNA-glycosylase, *e.g.*, UNG, that recognises and removes the modified base leaving an abasic site; ii) an AP-endonuclease that cleaves the phosphodiester bond of the abasic site creating a DNA single-strand break (SSB) with a 3'-OH terminus; and iii) downstream BER proteins, such as DNA polymerase β (Polβ), scaffold protein X-ray cross-species complementing 1 (XRCC1), DNA ligase I

(LIG1) and ligase III (LIG3), that replace the missing nucleotide and seal the DNA backbone (Krokan and Bjørås 2013). The process involving the replacement of a single nucleotide is part of the short-patch BER pathway, as opposed to the long-patch BER pathway where nucleotides around the damaged base are also displaced (Christmann et al. 2003; Sancar et al. 2004).

APE1 is indispensable to maintaining the integrity of the DNA and safeguard the cell survival. The DNA-repair function of APE1 is encoded in the C-terminal domain, which is conserved across many species (Tell et al. 2005). This domain harbours several catalytic activities: AP-endonuclease, 3'-5' exonuclease, 3'-phosphatase, and 3'-phosphodiesterase that suit different types of AP-sites (Thakur et al. 2014). Through its N-terminal domain APE1 can engage in protein-protein interactions, endowing APE1 with the capacity to coordinate the correct recruitment of other BER components (Tell et al. 2005). Under normal circumstances APE1 recruits XRCC1, which acts as a scaffold protein that binds DNA Polβ, and the ligases LIG1 and LIG3. DNA Polβ is a high-fidelity polymerase specific of the BER pathway with no reported roles in DNA replication or other processes. This polymerase can fill single DNA gaps with a low error frequency of 10^{-3}–10^{-4} per nucleotide incorporated (Sweasy 2003).

APE2 is the second of three enzymes found to possess AP-endonuclease activity in mammals (Hadi et al. 2002). The cellular function of APE2 is just beginning to be elucidated. Biochemical studies have shown that APE2 has weaker endonuclease activity but stronger 3'-5' exonuclease and 3'phosphodiesterase activities compared to APE1. It is believed that APE2 does not play a significant role in the short-patch BER pathway (Al-Safi et al. 2012); however, its exonuclease activity might be important to repair mismatched nucleotides originated from oxidative DNA damage (Burkovics et al. 2006b). A unique feature of APE2 is the capacity to interact directly with proliferating cell nuclear antigen (PCNA) through a PCNA-interacting protein (PIP) motif (Burkovics et al. 2009). PCNA

is a DNA sliding clamp that increases the processivity of DNA Polδ and Polε during DNA replication (Moldovan et al. 2007). PCNA also serves as a scaffold protein for translesional polymerases (error-prone), such as Polη, Polκ, and Polι (Haracska et al. 2001).

Regarding the role of AP-endonucleases in CSR, initial results using siRNA inhibition suggested that APE1 was not necessary for CSR. Nevertheless, it was later shown that APE1 haploinsufficient B cells had reduced class-switching to all isotypes after *in vitro* stimulation and possessed lower number of DSBs in the Sμ region (Guikema et al. 2007; Schrader et al. 2009). Furthermore, complete deletion of the APE1-coding gene in the cell line CH12F3 provided definitive evidence of the requirement of APE1 for CSR (Masani et al. 2013). In contrast, the role of APE2 in CSR is more controversial. APE2 has a weaker endonuclease activity *in vitro* that is usually masked by APE1. Nonetheless, primary B cells deficient in APE2 were shown to possess reduced CSR (Guikema et al. 2007; Schrader et al. 2009). However, independent studies using an APE2-knockdown cell line gave opposite results, as CSR was not affected by the lack of APE2 (Sabouri et al. 2009; Masani et al. 2013). The implications of these differences still remain a puzzle (Stavnezer and Schrader 2014).

Despite the conflicting results, the consensus is that APE1 is the main endonuclease in CSR, whilst APE2 is needed during SHM (Masani et al. 2013; Stavnezer et al. 2014). Our finding that APE1 is donwregulated in GC B cells, in addition to published data by Stavnezer and colleagues (Stavnezer et al. 2014), helps to clarify how DNA lesions introduced during SHM are spared from correct repair by the BER pathway. In this model, APE1 downregulation in GCs might be needed to avoid the recruitment of the error-free BER machinery, composed by DNA Polβ (Stavnezer et al. 2014). The accurate repair of dU residues generated by AID and UNG would play against the incorporation of somatic mutations in the IgV region needed for affinity maturation during the cyclic rounds of

mutation/selection undergone by GC B cells. This idea is also consistent with the finding that Polβ is not downregulated in GC B cells (Schrader et al. 2009), as complete abrogation might be risky in cases where off-target mutations arising outside the Ig locus demand faithful repair through the BER-pathway. Thus, the activity of DNA Polβ to properly correct base lesions in GC B cells is tuned by decreased levels of APE1 (Schrader et al. 2009).

GC B cells are highly proliferative cells and some of the deoxyuridine:deoxyguanosine (dU:dG) mismatches left by AID and UNG are not repaired before DNA replication. These dU residues are normally replaced by deoxythymidine (dT), resulting in dT:dG mismatches. It is in those cases where the 3'-5' exonuclease activity of APE2 is thought to play an important part (Stavnezer et al. 2014). APE2 would recognise these mismatches and incise the DNA, allowing access of the error-prone DNA Polη through its interaction with PCNA (Stavnezer et al. 2014). Interestingly, it has been shown that the exonuclease activity of APE2 is enhanced by binding to PCNA, increasing as well the frequency of miss-incorporated nucleotides (Burkovics et al. 2009).

In summary, the reduced expression of APE1 would limit the activity of the error-free DNA Polβ in GCs. This effect coupled to upregulation of APE2, which in turn can physically interact with PCNA and engage error-prone DNA polymerases, would explain the "correct" introduction of point mutation during SHM. This however opens the discussion as to how CSR can proceed in B cells that possess high levels of APE1 and the high-fidelity DNA Polβ, and also how B cells undergoing CSR avoid correct AP-site repair at the same time that lesions in other genomic regions are properly repaired. In this regard, it is likely that the error-free BER pathway lead by Polβ still can repair a proportion of the instigated lesions; however, Polβ would become overwhelmed by the high number of dU residues localised in specific areas of the IgV genes and S regions (Schrader et al. 2009). Another explanation might be the presences of yet-to-be discovered

accessory proteins guiding the CSR machinery exclusively to the Ig locus. If such components exist, a plausible mechanism could involve the recognition of AID-DNA complexes.

Another likely reason to downregulate APE1 in GC B cells comes from the observation that APE1 can negatively control the expression of c-MYC by cleaving its mRNA (Barnes et al. 2009). As reviewed in Chapter 1, c-MYC is upregulated in positively selected LZ B cells, allowing these cells to migrate to the DZ to undergo further rounds of proliferation and mutation. Therefore, high levels of APE1 might interfere with the correct transit of GC B cells across the DZ/LZ driven by c-MYC.

APE1 is also known as redox effector factor 1 (Ref1), due to its redox activity found in the N-terminal domain (Tell et al. 2009). APE1 works as a redox co-factor controlling the activation of several proteins and transcription factors, including early growth response protein-1 (Egr-1), NF-κB, p53, hypoxia inducible factor-1α (HIF-1α), cAMP response element binding protein (CREB) and activator protein-1 (AP-1), among others (Tell et al. 2009). APE1 can reduce cysteine (Cys) residues in these transcription factors controlling their binding capacity to target genes (Tell et al. 2005). The redox state of APE1 is also critical in modulating its own activity, which is controlled by reduction of the Cys 310 (Kelley and Parsons 2001). Thus, it will be interesting to determine whether the redox activity of APE1 and its own redox state may also be important to regulate CSR in GC B cells.

4.3 Pathogenic gene insertions and lymphomas

Programmed DNA damage during SHM and CSR is a tightly regulated event, yet off-target activity of AID outside the IgV and S regions has also been reported to contribute to genomic instability (Liu and Schatz 2009). In fact, $\sim$95%

of all lymphoid cancers are believed to have a B cell origin, produced through aberrant gene translocations and fusions, or mutations affecting *cis*-regulatory elements (Nussenzweig and Nussenzweig 2010). Generation of DSBs in non-Ig genes by AID has been implicated with certain types of cancers. For instance, in Burkitt's lymphoma translocations of *c-myc* into the 3' IgH enhancer or S regions are well known examples of oncogenic rearrangement mediated by the dysregulation of AID (Ramiro et al. 2004). Unlike other enzymes with the ability to target DNA, AID has no sequence specificity. In theory, it can deaminate any dC present in a single-stranded DNA (ssDNA) configuration. Nonetheless, AID mainly favours dC bases immerse in "RGYW" DNA motifs (R = purine base, Y = pyrimidine base, and W = dA or dT) (Vaidyanathan et al. 2014).

Despite extensive research, how AID is recruited specifically to the Ig gene loci is still unknown. In this line, the presence of cofactors guiding AID to the appropriate genomic regions, interaction with RNA pol II and the direct recognition of secondary DNA structures (*e.g.*, R-loops) by AID are among the possibilities (Gazumyan et al. 2012; Vaidyanathan et al. 2014). To date, no conclusive mechanism has been provided. AID can only target dC nucleotides exposed in transcribing DNA, and the rate at which mutations are incorporated is directly proportional to the rate of transcription (Di Noia and Neuberger 2007; Gazumyan et al. 2012). Assuming that no other forces than the transcriptional state of the chromatin participate in the recruitment of AID and considering that during SHM the rate of mutations is $\sim$10^6 times higher than background levels (Di Noia and Neuberger 2007), restricting CSR – which also contributes with a high frequency of DNA lesions – from taking place in GC B cells would help to reduce the likelihood of pathogenic translocations due to the numerous DSBs induced by AID. It is worth mentioning that, it is unclear how off-target DSBs are faithfully repaired during CSR.

Another potential advantage of limiting CSR in the GC comes from the

observations of oncogenic or autoimmunity-inducing gene translocations or insertions occurring in the IgH locus during CSR (Nussenzweig and Nussenzweig 2010; Tan et al. 2016): these occurrences would make this recombination process particularly risky in GCs, where not only cells are intensely proliferating, but also their output is destined to become long-lived memory B cells or plasma cells.

Lanzavecchia and colleagues have described a novel process, whereby B cells can produce broadly reactive antibodies (brAbs) against *Plasmodium falciparum*, the causing agent of the most severe cases of human malaria (Phillips et al. 2017), via the interchromosomal insertion of distant DNA regions into the IgH locus (Tan et al. 2016). These brAbs were isolated from human donors infected with malaria by their capacity to recognise and opsonise erythrocytes infected with *P. falciparum*. The target antigens were identified as members of the RIFIN-family, a diverse group of proteins expressed by the parasite that are associated with immune escape (Phillips et al. 2017).

A deeper characterisation of the antigen-binding domain of brAbs revealed the presence of a non-immunoglobulin DNA fragment interspacing the VDJ-segment. The gene insert was identified as the collagen-binding domain of the leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), which was necessary and sufficient to bind RIFIN-members (Lanzavecchia et al. 2016). The brAbs exhibited signs of SHM in the V_H region, where the point mutations Pro-106-Ser and Pro-107-Arg were associated with loss of collagen-reactivity; and Thr-67-Leu, Asn-69-Ser and Ala-77-Thr with increased binding to parasite-infected red blood cells (Tan et al. 2016).

Although no conclusive mechanism has been found, it is believed that the gene insertion would occur by DNA translocations promoted by RAG1/2 recombinases. However, only partial recombination signal sequences (RSSs) were found flanking the LAIR1-insertion, and both LAIR1 donor alleles were present in their respective locus (chromosome 19) (Tan et al. 2016). Moreover, expression

of RAG proteins in mature B cells is normally associated with pathogenic DNA insertions (Nussenzweig and Nussenzweig 2010). This raises some interesting questions as to how the collagen-binding domain of LAIR1 is translocated into the IgH locus. It has been proposed that this might involve another mechanism called "templated-sequence insertions (TSIs)" that was discovered as an alternative pathway to repair DSBs, where DNA sequences from distant genomic regions are inserted to patch these lesions (Onozawa et al. 2014; Tan et al. 2016). This process occurs through a reverse-transcribed mRNA intermediate that neither affects nor deletes the template DNA sequence (Onozawa et al. 2014). In this context, AID-induced DNA lesions could also work as the substrate for this pathway.

The interchromosomal insertion of specific gene portions scattered across the genome represent a powerful feature by which the antibody repertoire can be further diversified. However, if not carefully controlled it might lead to oncogenic insertions, aberrant gene fusions or the generation of auto-antibodies. In the particular case of LAIR1-containing brAbs, the antigen-specificity is given by a domain that binds a self-component (collagen) and its reactivity was only lost due to SHM. Considering that gene insertions might occur via a DNA-DSB intermediate, such as the ones created during CSR, we proposed that this might represent another reason to suppress CSR in GC B cells, as the mutational burden arising through SHM combined with CSR might be too high for the cells to cope with. Therefore, separating two processes that induce a high-rate of mutations in space and time would help to reduce the likelihood of deleterious gene insertions in GC B cells.

4.4 CSR and formation of IgM$^+$ memory B cells

The evolutionary advantage of preventing CSR in GCs is likely to come from the need to allow production of sufficient IgM$^+$ memory B cells (Reynaud

et al. 2012), and thus a diversified Ig-isotype pool upon IgM-memory reactivation during secondary infections with either evolving or antigenically-related pathogens (Bernasconi et al. 2002; Pape et al. 2011). While IgG$^+$ memory B cells are associated with effector functions as they tend to differentiate rapidly into plasmablasts upon antigen re-challenge, IgM$^+$ memory B cells are more likely to re-enter the GC reaction to further improve the BCR affinity (Inoue et al. 2018). This is consistent with the observation that primary IgM$^+$ memory B cells have fewer mutations in the CDR (Blink et al. 2005; Anderson et al. 2007), which translates in lower affinity for antigen and eventually better plasticity to adapt in recall responses.

Employing a single-cell analysis approach to track the fate of memory B cells, the McHeyzer-Williams group has shown that memory B cells can reinitiate GC reactions during a short-term secondary immune response (tested 70 days after the first challenge) (McHeyzer-Williams et al. 2015). However, the authors found that IgM$^+$ memory B cells had little impact in recall responses, being class-switched memory B cells the main precursor of secondary GCs. These GCs were fully functional, with cells able to remodel the BCR and presumably undergo sequential class-switching. Nonetheless, no formal kinetics were conducted to determine the exact location where CSR was triggered in memory B cells (McHeyzer-Williams et al. 2015).

This apparent discrepancy on the role of IgM$^+$ memory B cells after re-challenge may be explained by the titres of circulating antibodies, which are directly linked to the immunisation regime (*i.e.* the type of antigen/adjuvant), and the time at which the boost is performed. Jenkins and colleagues have shown that the capacity of IgM$^+$ memory B cells to rejoin GCs is regulated by the serum levels of antigen-specific antibodies elicited during the primary response (Pape et al. 2011). When primary antibodies were present (day 320 after the first challenge), IgM$^+$ memory B cells were neither activated, nor seeded GCs and the

formation of antibody-secreting cells depended primarily on the differentiation of class-switched memory B cells. In contrast, when IgM$^+$ memory B cells were transferred into unimmunised mice, they were strongly activated and capable of forming GCs. Interestingly, activation of naïve recipient B cells was inhibited by class-switched memory cells but not by IgM$^+$ memory cells (Pape et al. 2011). In conclusion, the half-life of the antibodies secreted during the primary response has a profound effect in the recruitment, activation and outcome of IgM$^+$ memory B cells.

It is well known that humoral memory in humans can last for many years, even decades. In this line, IgM$^+$ memory B cells are considered more stable over time compared to their class-switched counterpart, with steady levels in mice at day 450 after the first challenge (Pape et al. 2011). Thus, IgM$^+$ memory cells represent a reservoir of lymphoid-memory that can be induced at recall when the levels of primary protective antibodies have declined. Once activated, they can form new GCs where they become effector cells or new memory B cells that feed the memory pool with new members (Pape et al. 2011). This idea further supports the hypothesis that IgM$^+$ memory B cells are necessary to maintain a reservoir of antigen-experienced B cells that can be rapidly engaged upon infection but are not functionally committed to any particular process or pathway, preserving their capacity to switch to any isotype. This, in turn, results in a much broader spectrum of antigen-specific B cells, in terms of BCR affinities and functional properties, filling the memory B cell compartment after each immunisation.

Similar to the work we have done here in a primary TD-response, it will be interesting to study in the future how CSR is regulated in this pool of memory B cells after boost. We speculate that memory B cells should be able to recapitulate the very same processes observed during activation of naïve B cells.

4.5 Limitations of this study

In this manuscript we present evidence to support that CSR ceases within mature GCs. We provide data obtained from adoptive transfer experiments of B cells carrying a transgenic BCR (SW$_{\text{HEL}}$ and B1-8$^h i$) reacting against model antigens, and also in polyclonal systems where the endogenous repertoire of B cells was activated after CGG or SRBC immunisation. As already discussed, these models have proven to be extremely reliable and capable of recapitulating all the processes experienced by the immune system in physiological conditions. Still, it will be interesting to interrogate how CSR is modulated after infection with intact pathogens, such as bacteria, viruses and other microorganisms.

It is important to highlight that in the past, studies aiming to unravel the connection between CSR and GC B cells were based on models that affected the normal development of GCs. None of them were however, designed to specifically test the selective requirement of GCs for CSR. Hence, these findings relied heavily on secondary effects due to the lack of adequate T:B encounters, or cognate T:B interactions besides the absence of GCs. In this respect, the loss of GCs was originated by disorders affecting the normal organisation of lymphoid structures, proliferation and survival, lack of lymphokines needed to modulate the correct migration of B and T cells, and deletion of key molecules or activators, such as BCL6, NF-κB and CD40, among others. It is therefore likely that in those studies, the lack of CSR was a side effect of a more complex condition associated with impaired immune responses. Thus, the link between CSR and GCs remained a correlation rather than a causal effect. We hope the work presented here will open the debate for new and thorough characterisation of CSR and other GC processes.

Our single-cell qPCR studies were fundamental in demonstrating that CSR is downmodulated by GC B cells. Although, the frequency of GC B cells expressing

GLTs at day 6.5 by single-cell qPCR was almost negligible, it is difficult to know at this stage whether this small fraction of GC B cells are just late comers joining the GC reaction that have already class-switched at the T:B border, or they are actual GC B cells inducing CSR *in situ*. One way to address this problem could be through BCR-sequencing to compare the SHM content of these cells. In this line, the phylogenic analysis of IgV gene sequences from immunised PA-GFP mice suggests that CSR becomes a rare event after the onset of SHM in mature GCs. Thus, most of the switching has already occurred at the early phases of B cell activation before GC formation. A second interpretation is that these isolated events could be needed to promote sequential-switching to more distal isotypes, although we did not find a single sequential switching event in the GC phylogenic trees we examined. Analysis of a larger data set may be required to draw definitive conclusions on sequential switching in GCs. Mathematical models might also help to predict how much contribution, if any, these rare events found in GCs would have in the formation of class-switched B cells after each round of selection in the GC. Another possibility to discard induction of CSR in GC B cells may include the expression of switch-circle transcripts (SWCTs), which would give a better idea about ongoing CSR.

As mentioned in Chapter 1, very few studies have successfully assessed SWCTs on freshly isolated murine GC B cells. In our single-cell qPCR studies we employed published protocols, where target-specific pre-amplification of cDNA was used to assess formation of $\gamma1$ and $\gamma2$b-SWTCs. While these protocols worked quite nicely during the *in vitro* optimisation in stimulated naïve B cells, they could not be replicated in *ex vivo* isolated samples. This might be attributed to the transient nature of SWCTs, which tend to disappear more rapidly compared to GLTs and even switch-circles (Kinoshita et al. 2001). Correct detection of SWCTs might require a more exhaustive time-course study with intervals of 12-24h, similar to the experiments performed in bulk-cell populations, or using a more

sophisticated technique, such as single-cell RNA sequencing (scRNA-seq). This would solve many of the problems and challenges described by other groups.

While a substantial effort has been made to ascertain the precise location where CSR is induced after immunisation, the focus of this thesis has been placed on the changes that B cells undergo over time. CSR is guided by a combination of signals delivered by antigen, cytokines and direct help from T cells. In this regard, linking our observations with the activation of T_{FH} cells might be necessary. Of great interest in our lab is also the role of T follicular regulatory (T_{FR}) cells in controlling humoral responses, further exploration of T_{FR} cells and their contribution in modulating CSR might open new research avenues. A key component needed for correct CSR is the presences of cytokines. Profiling the composition of cytokines released to activate B cells, along with any local change in the GC milieu might help in the interpretation of our findings. Probably, the low levels of GLTs detected in GC B cells are the result of reduced or limiting concentration of cytokines.

4.6 Future Directions

- We have observed early induction of γ1- and γ2b-GLT expression by HEL-binding B cells, NP-reactive cells and in polyclonal responses where the endogenous repertoire of B cells have also been study. It will be important to see if these observations are also true in the context of natural infections using intact pathogens.

- Our data suggest that the cues needed to induce CSR *in vivo* are delivered at early stages, but not upon or during GC-formation in a primary TD-antibody response. Further investigation is needed to understand the regulation of CSR during secondary immune responses.

- Changes in the composition of cytokines and the frequency of cognate T_{FH} cell help are an important component to guide isotype-switching in B cells. Further exploration of these two arms would greatly enhance our understanding of CSR dynamics in TD-responses.

- Germline transcription is an essential for CSR, allowing the access of AID and formation of GLTs, which is an essential prerequisite for CSR. Although there is good correlation between GLT expression and successful isotype-switching, SWCTs are considered a more definitive marker of active CSR. Despite our efforts, SWCT-detection was not feasible in our kinetic studies. scRNA-seq could prove useful to overcome the technical difficulties associated with their detection in primary tissues.

- An important aspect of this work was to provide a mechanism that could helps us to explain how CSR is modulated during a primary immune response. Of particular interest to us is the role of APE1 in controlling CSR within GCs. Further characterisation of how APE1 interacts with other BER-pathway members and their precise roles during CSR is something that needs to be dissected. For instance, it has been reported that APE1 can increases the activity of UNG, leading to formation of more AP-sites. APE1 activity is also modulated post-transcriptionally through phosphorylation, alkylation and redox control. Such processes and mechanisms might hold great importance for the regulation of CSR in GCs, therefore the need of additional studies.

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