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Current Opinion in Immunology

# Regulation of germinal center responses, memory B cells and plasma cell formation — an update

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Progress in understanding humoral immunity has been accelerated by the powerful experimental approaches of genetics, genomics and imaging. Excellent reviews of these advances appeared in 2015 in celebration of the 50th anniversary of the discovery of B cell and T cell lineages in the chicken. Here we provide a contemporary model of B cell differentiation, highlighting recent publications illuminating germinal center (GC), memory B cell and antibody-secreting plasma cell biology. The important contributions of CD4T cells to antibody responses have been thoroughly reviewed elsewhere.

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**Current Opinion in Immunology** 2016, **39**:59–67

This review comes from a themed issue on **Lymphocyte development and activation**

Edited by **James Hagman**

For a complete overview see the Issue and the Editorial

Available online 20th January 2016

http://dx.doi.org/10.1016/j.coi.2015.12.008



## Introduction

The antibody response employs intercellular communication, through direct contact and soluble mediators, in the exquisitely organised structure of secondary lymphoid organs [1–3]. These signals induce or silence genetic programs of activation, migration, survival and differentiation, all potentially modulated by epigenetic modifiers. Figure 1 illustrates the process and its participants as it occurs in the spleen; similar processes can be observed in lymph nodes (LN), or ectopically in inflamed tissue.

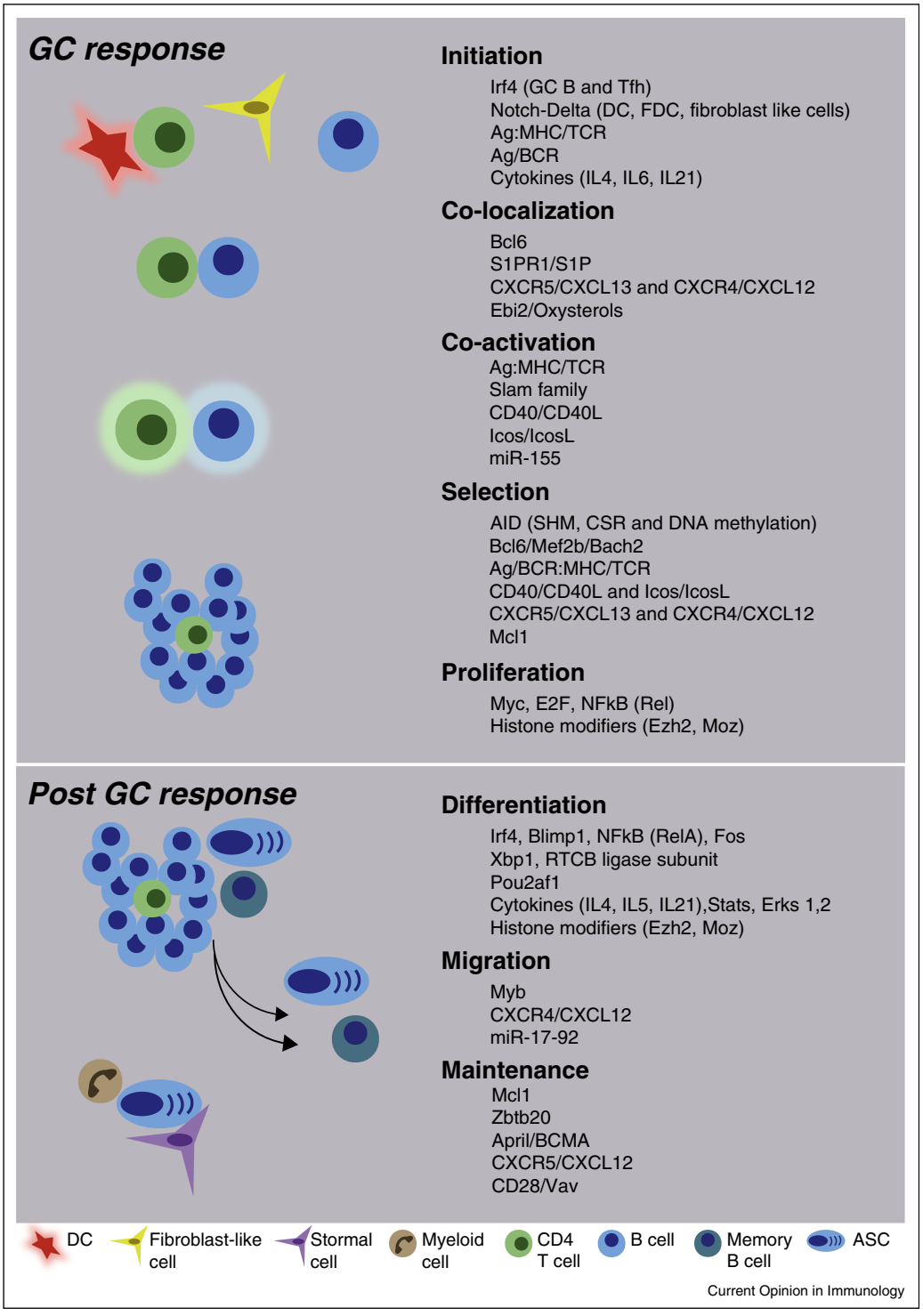
## Overview of the B cell response to antigen *in vivo*

In the resting state, polyclonal B cells and T cells are compartmentalized and non-dividing. Once antigen enters, it is captured by professional antigen presenting cells, including B cells, macrophages and dendritic cells (DC). Within 1 day in the mouse, a small number of B cells specific for the antigen (~1–3 cells initially; [4]) will migrate under the influence of changing chemokine receptor expression [5,6], drawn towards the T cell zone. Simultaneously, CD4+ T cells that have detected their cognate antigen presented on DC, will begin to express the transcriptional repressor Bcl6 [6], a master regulator of the T follicular helper cell (Tfh) lineage [7]. Bcl6 influences chemokine receptor expression, such that early Tfh cells migrate towards the B cell follicle under the influence of CXCR5 expression [8]. At the T:B interface, a cognate interaction occurs in which activated B cells provide further processed antigen to the T cell receptor (TCR), soliciting the secretion of cytokines (IL6 and IL21) that reinforce Bcl6 expression and the Tfh phenotype [8–11]. Subsequently, important receptor:ligand interactions are formed, including CD40:CD40L, ICOSL:ICOS, and homotypic interactions between SLAM proteins [12,13]. These interactions ensure that the cognate B and T cell remain in contact to initiate the next phase of the response.

A day or two later, the cognate B and T cells are moving again. The mature, antigen-specific Tfh move to the center of the B cell follicle. The activated B cells have multiple fates available to them [6]. First, some B cells will move to the interfollicular zone and differentiate into short-lived antibody secreting cells (ASC), providing a rapid, albeit low affinity, antibody response to the infection. Second, some B cells move into the pre-GC, likely driven by Bcl6 up-regulation and changes to chemokine receptor expression. There they will undergo affinity maturation in the developing GC. Lastly, some B cells will differentiate into early memory B cells, isotype switched but showing no evidence of affinity maturation. The distribution of any clone among these outcomes appears to be an intrinsic property of the clone, related to division potential and influenced by affinity but not by isotype [14••].

The GC reaction serves a number of critical roles. It is where somatic hypermutation (SHM) of immunoglobulin (Ig) variable region genes occurs, catalysed by activation-induced cytidine deaminase (AID; [15]). B cells cycle through the light and dark zones (LZ and DZ) of the GC undergoing iterative cycles of selection and rapid proliferation and mutation, respectively, timed intrinsically and facilitated by fluctuating CXCR4 and CXCR5 expression

**Figure 1**




The figure indicates the various cell types and molecules that come into play as B cells are activated and enter into a T cell dependent antibody response. The process follows a time frame (proceeding vertically down the page) that initiates in spleen and lymph nodes, and finishes in the bone marrow.

[16,17]. Affinity maturation occurs through competition by mutated B cell receptors (BCRs) of the GC B cells for the antigen decorating the follicular dendritic cells (FDC) and the limited number of Tfh available to provide help. Tfh are motile, interacting with many B cells and making frequent and durable contacts with cells expressing the most Ag/MHC on their surfaces. ICOS/ICOSL plays a central role in this process [18•]. The role of BCR signal strength is unclear; rather, a greater capacity to capture antigen and present it to Tfh cells appears to drive the process [19].

The GC is also the site of differentiation of long-lived memory B cells and plasma cells. The ASC exported from the GC ultimately persist in bone marrow niches that support their longevity [20,21], while memory B cells, although showing some predilection to remain in the organ of their formation [22,23], recirculate through the lymphoid system. Regulating the output of the GC is critical, as SHM potentially generates auto-reactive or otherwise mutated B cells that may lead to autoantibody production or to GC-derived B cell lymphomas [24,25].

## Regulators of GC B cells

A number of signalling molecules and transcriptional regulators have been implicated in GC biology [16,17]. While GC B cells require BCR signalling capacity, this is tempered by cell cycle-dependent fluctuations of Shp1 phosphatase levels [26•,27•]. Instead, the ability to capture and present antigen to Tfh cells, and to receive and respond to T cell signals is critical to affinity maturation in GC B cells [18•,28]. It was recently shown that by engineering a subset of B cells to express high Ag:MHCII levels, Tfh cell help was enhanced, and this increased expression of cell cycle and metabolism gene programs, including Myc, E2F and their target genes [29••]. This significantly shortened S phase in the selected GC B cells. In turn, affinity increased as cell cycle time decreased in the cells receiving strong T cell help.

Other recent work shows that CD40 and Icos ligands (CD40L, IcosL) cooperate through a feed-forward mechanism to ensure GC B cells with the highest affinities successfully compete for limited Tfh help [18•]. A B cell-intrinsic capacity to respond to IL21 and IL4 is also essential for optimal GC responses [30–32]. Impacting indirectly on GC B cell responses and antibody affinity are fibroblast-like cells (including FDC) in secondary lymphoid organs that express Notch ligands essential for development of Tfh, certain DC and marginal zone B cells [33].

A ''GC gene signature'' [34] highlighted the major signalling pathways (BCR, NFκB, CD40 and Myc) and signature transcriptional regulators of the GC (Figure 1). Bcl6, the master regulator of the GC, facilitates SHM by inhibiting the DNA damage response, and differentiation [35]. Bcl6 acts cooperatively with Bach2, another essential GC gene repressor [36], through co-regulation of several target genes [37].

The NFκB pathway is widely implicated in signalling in lymphocytes for activation, growth and survival. However, a surprisingly small proportion of GC B cells display the active nuclear form of NFκB. The use of conditional mutants of NFκB subunit genes has shown that specific members of the canonical NFκB family are differentially required, both temporally and mechanistically, for GC B cell maintenance and for plasma cell differentiation [38•,39•]. Processing NFkB1 is absolutely required for B cell differentiation and survival following antigen engagement, through the activation of IRF4 and Bcl2, respectively [39•]. c-Rel maintains the GC after the DZ and LZ are established (beyond day 7, to day 14 after immunization), not through supporting survival, but by up-regulating metabolic pathways supporting cell growth. Plasma cell differentiation is c-Rel independent. In contrast, RelA is dispensable for GC formation, expansion, class switch recombination (CSR) and affinity maturation, but its loss diminishes ASC differentiation *in vivo* and *in vitro*. Interestingly, RelA deficiency reduces Blimp1, but not IRF4 levels, *in vitro*.

Myc, another factor enabling proliferation and contributing to B cell transformation, is required for GC formation [40,41]. DZ B cells, some of the most rapidly dividing mammalian cells known, should universally express Myc, but surprisingly, it is expressed by only a minority of GC B cells, and these are dispersed through the LZ and DZ [17]. However, Myc+ GC B cells display the highest antigen affinity, reflecting a history of SHM and cell division. We have recently found that lymphocyte division is strictly dependent on Myc protein levels achieved upon initial activation (S Heinzel, PD Hodgkin and LMC, in preparation), which may help to explain how the successfully selected (higher affinity) GC B cell clones are able to spend longer in the proliferative phase in the DZ [42••].

An updated ''GC gene signature'' arose from our recent transcriptional profiling of peripheral B lineage cells in the mouse [43••]. Interestingly, some transcription factors not yet implicated in GC biology showed identical patterns of expression to known, essential GC regulators like Bach2, Pou2af1, Mef2b and its direct target, Bcl6 [44]. They include Mybl1 (also recognized by Ding *et al.* [45] as a Bcl6-regulated gene in a GC diffuse large B cell lymphomas), Phf19 (an epigenetic regulator; [46]) and Apitd1 (the DNA-binding component of the Fanconi anemia (FA) core complex that mediates genome maintenance [47]). These factors, among others [43••], deserve attention as potential new players in GC B cell biology.

## Regulators of B cell memory

Signals dictating GC B cell differentiation along the memory pathway remain undetermined. While the existence,

persistence and functionality of early memory B cells, arising prior to GC initiation, are now confirmed [48], the relevant molecular and cellular processes remain unclear. Despite this, much has recently been learned of memory B cell biology. Considerable importance was given to the existence of IgM memory, particular for its perceived unique capacity to differentiate into GC following reactivation, which was distinct from IgG memory, that showed a predisposition to differentiate into ASC [49,50]. Thus the IgM memory compartment is designated as the repository of very long-lived immune memory, only utilized when alternatives had failed and then used to restore the status quo in regenerating both PC and IgG memory B cells through re-running the GC reaction. Mechanistic support comes from the observation that IgG BCR are biochemically distinct from IgM BCR through the antigen-induced recruitment of GRB2 to a unique, conserved tyrosine motif in their cytoplasmic tails and their subsequently enhanced differentiation into plasma cells [51]. Conversely, a study of memory B cell subsets in mice, defined by expression of PD-L2, CD80 and CD73, concluded that the bias towards PC or GC differentiation could reflect the maturity of the memory compartment, and to isotype. That is, more recent or immature memory B cells, defined as negative for CD80 and PD-L2, preferentially reformed GC upon transfer and restimulation, while more mature memory B cells, (PD-L2- and CD80-positive), preferentially formed PC [52•]. These outcomes, however, may reflect the experimental system as much as intrinsic properties of the memory B cells themselves, as they used purified populations and were done in the absence of competing immunoglobulin.

A striking feature of T cell memory has been the discovery of resident memory cells, which show tissue tropism and are functionally specialised. Recent careful analysis of memory B cells formed either at the site of influenza infection, the lung in this case, or more distant, in the spleen, suggests that tissue tropism may also be a feature of B cell memory. Adachi *et al.* reported persistent GC in the lung with a higher incidence of V gene SHM and, most interestingly, significantly higher incidence of cross-reactive B cells able to neutralize flu escape variants [22]. While the basis of this difference is unknown, it might indicate that there is a degree of specialisation in aspects of the GC reaction occurring at the site of infection, especially if that site is not a specialised, secondary lymphoid organ. The generality of this finding, however, is yet to be determined as the distribution of memory B cells in Rhesus monkeys following influenza A infection showed a predilection for mediastinal lymph nodes rather than lungs [53].

The relationship between human memory B cell subsets has become somewhat clearer through use of RNA sequencing and through examination of clonality in the various subsets defined by isotype and CD27 expression. Budeus *et al.* [54] for example, found that the vast majority of memory B cells were GC-derived, were members of very large clones and that the same clone could be identified in multiple memory subsets, suggesting a stochastic distribution rather than one pre-determined by the location of the B cell, age of the human or the nature of the antigen. Interestingly, however, the nature of B cell memory is affected by the nature of the antigen when the antigen is persistent. At least this appears to be the explanation for the appearance of atypical memory B cells in responses to persistent parasites and viruses [55,56]. These memory B cells, with their unique phenotype of excessive inhibitory receptor expression, show significantly diminished signalling from the BCR such that proliferation and differentiation are effectively blocked [57].

The persistence of memory B cells remains an enigma. While some studies have indicated differences in persistence based on isotype, with IgM lasting longer than IgG [49], others indicate both IgM and IgG are equally longer-lived quiescent cells than naïve B cells [58]. Yet another study reported the surprising result of continuous sculpting of the IgA memory compartment through an ongoing response driven by the microbiota in the Peyer's patches, in both mice and humans [59••], suggesting continuous replenishment of memory. Autophagy has been reported as a unique requirement for memory B cell persistence, although the rationale remains unclear [60]. While memory B cells form independently of autophagy, transcriptional regulators of autophagy increase over time. The capacity to recycle cellular components likely contributes to the memory B cell's capacity for long-term survival.

## Regulators of ASC differentiation and maintenance

The majority of long-lived plasma cells arise from B cells selected in the GC. Signals that initiate their differentiation must extinguish the B and GC cell transcriptional programs (dominated by Pax5, Bcl6 and Bach2) to enable the opposing program of terminal differentiation (led by Irf4, Blimp1 and Xbp1) to act [61]. Plasmablasts then exit the GC and move through the blood to specialist niches in the bone marrow [20,62]. There, reticular cells and myeloid cells, principally eosinophils, interact with plasma cells to create a supportive niche in which they survive for long periods. Survival is mediated by signals from CXCL12, April and CD80 on the niche cells, and receptors CXCR4, BCMA and CD28, respectively, on the plasma cells, all ultimately coalescing on Mcl1 as the dominant pro-survival protein for ASC [63]. Plasma cells may actively orchestrate their life in the BM niche, as they are metabolically active, and secrete a number of inhibitory and stimulatory cytokines [43••,62].

Among the extrinsic signals that drive ASC differentiation are CD40L and cytokines from Tfh, signalling through

Erk1 and 2 and NFκB, where the role of RelA may be critical [38•]. They induce Blimp1, and reduce Irf8, Pax5, Bcl6 and Bach2 expression [62]. A signal from CD28 was recently shown to require Vav, but not PI3K, and to act in BM, but not splenic plasma cells, to positively influence Blimp1 expression and plasma cell maintenance [64].

Important intrinsic regulators of haematopoietic cells and B cells have recently been newly implicated in plasma cell biology, or their influences have been revealed through the generation of compound mutant mice. The importance of Ets family proteins Irf8 and PU.1 in inhibiting plasma cell differentiation, through competition with Irf4, was emphasized by accelerated ASC differentiation of double knockout (DKO) B cells *in vivo* and *in vitro* [65]. Similarly, the modest repression by Bcl6 and Bach2 on ASC differentiation was strongly increased in DKO mice, confirming their cooperation in maintaining the GC phenotype of B cells, but also highlighting many non-overlapping roles [37]. Antagonism between c-Fos and Fra1 for binding to AP-1 sites in the Blimp1 gene was recently shown to activate or silence its expression, respectively, and to consequently inhibit or abnormally enhance plasma cell differentiation [66].

Myb, long known as a mediator of differentiation in haematopoietic cells [67] is required for GC-derived plasmablasts to exit their organ of formation and migrate to the bone marrow for long-term survival [68•]. In the absence of Myb, Ig class switched plasmablasts, generated after immunization or infection, failed to enter the blood, but accumulated in the spleen, where they were mis-localized. The effect of Myb loss was B cell intrinsic, but did not impact GC formation. The primary defect detected was an inability for GC-derived plasmablasts to migrate in response to CXCL12 and thus contribute to the long-lived BM plasma cell pool.

A ''Plasma cell signature'' was identified through a comparison of the transcriptomes of B cell and plasma cells populations [43••]. It comprises ~300 genes that were >3-fold more highly expressed in the ASC populations compared to the B cell populations compared. Known regulators of plasma cell differentiation (e.g. Irf4, Blimp1/Prdm1, Xbp1) were confirmed, and gene expression differences between plasma cells residing in different organs or generated under different conditions were noted. This analysis will likely reveal new regulators of plasma cell behaviour. One example is Zbtb20, a BTB-POZ domain protein recently found to be required for the long-term survival of plasma cells in the BM [69•,70•]. Zbtb20 is a direct target of Irf4 activation that, when overexpressed *in vitro*, accelerates ASC differentiation. It may act from the GC stage to antagonise its close relative, Bcl6. Wang and Deepta *et al.* [69•] showed that the effect of Zbtb20-deficiency on ASC maintenance, which was adjuvant-dependent in their system, could be rescued by the pro-survival protein Bcl2 [69•].

Other putative novel regulators of plasma cell biology were suggested by patterns of expression that closely mimicked those of the 'master' ASC regulators Irf4 and Blimp1 [43••]. These include Cited2 (Cbp/p300-interacting transactivator), recently implicated in improved haematopoietic stem cell maintenance [71], Creb3l2 (CAMP Responsive Element Binding Protein 3-Like 2), a transactivator induced by ER stress [72], and Trib1 (Tribbles Pseudokinase 1) a regulator of MAPK kinases [73].

## Post-transcriptional and epigenetic regulation

Xbp1, a major facilitator of high-level Ig secretion by plasma cells [74], is regulated post-transcriptionally. Its mRNA is processed in response to the unfolded protein response (UPR) of the endoplasmic reticulum of highly secretory cells [75]. The stress sensing kinase IRE1α catalyzes the endonucleolytic cleavage of Xbp1 mRNA. Recently RTCB, the catalytic subunit of the tRNA ligase complex, was identified as the enzyme that ligates the processed mRNA to generate a transcript encoding mature, functional Xbp1s [76•].

Micro RNAs (miRNAs), which regulate mRNA stability and translation, impose a further level of regulation on many biological processes, including the humoral immune response. A recent example is miR-155, a known inhibitor of PU.1 expression [77]. Disabling the PU.1-miR-155 interaction sequence separately on both PU.1 mRNA and miR-155 revealed the PU.1-specific and broader effects of this regulatory axis, with only a partial overlap of gene expression changes between the mutants. Both mutations modestly elevated PU.1 expression and consequently increased PU.1 target gene expression. Many aspects of B cell-T cell interaction were affected, as was ASC differentiation.

MiR-148a is the most abundant miRNA in mouse and human plasma cells, and its levels are also strongly induced during ASC differentiation *in vitro* [78]. miR-148a targets the mRNAs for Bach2 and Mitf, known repressors of Blimp1 and Irf4, reducing their expression and thereby enhancing ASC differentiation. In addition, miR-148a was able to reduce the expression of cell death mediators PTEN and Bim.

Mir-17-92, which also influences early B cell development, has been shown, via conditional deletion, to facilitate homing of plasma cells generated during a T cell dependent response to the BM [79]. Selective depression of the IgG2c response was also seen. These effects were partly mediated by miR-17-92 directly targeting the chemokine receptor SIPR1 (which mediates cell egress from lymphoid organs) and Ikaros (which regulates Cγ2c germline transcription), respectively.

The role of epigenetic modification in GC B, memory B and plasma cells is now being appreciated [80]. For instance, the transcriptional profiles of naïve and memory B cells are similar, despite the cells responding with quite different behaviour upon exposure to antigen. Some of the difference has now been linked to different patterns of histone modification (histone 3 acetylation and methylation) between the two cell types. One model posits that co-existence of bivalent marks (both activating and repressive) on the same gene create a cell poised for rapid responses, typical of those occurring in a GC response. Acquisition or loss of such histone ''marks'' accompanies B cell activation, and mutation of the histone modifying enzymes changes B cell behaviour, sometimes subtly, *in vivo*. For example, Ezh2, a histone methyltransferase of the polycomb group, is highly expressed and required during division of GC B cells and plasmablasts, but is switched off in non-dividing memory and plasma cells. Interestingly, Ezh2 and Bcl6 share some target genes [81]. The histone acetyltransferase, Moz, also regulates GC B cell division, and contributes to Bcl6 expression [82]. Both regulate affinity maturation and memory B cell and ASC differentiation. Interestingly treatment of mice with histone deacetylase inhibitors (HDACi) revealed existing plasma cells and GC formation to be sensitive, but existing memory B cells remained largely unaffected [83].

Histones can also be modified by ubiquitination, and loss of MYSM1, a histone 2A deubiquitinase, was found to accelerate ASC differentiation [84]. MYSM1 represses differentiation mechanistically by coordinating histone modifications and transcription factor recruitment at the Pax5 gene, activating it. Both T cell dependent and independent antibody responses were heightened in the MYSM1 mutants.

DNA methylation patterns change significantly during the B cell response. Epigenetic gene regulation by DNA methylation is mediated by a family of DNA methyl transferases [85]. Very recent work has revealed that AID, the enzyme mediating SHM and CSR, catalyses the demethylation of many CpG sites in genes in B cells [86••]. In both mice and humans, AID was found to catalyse the vast majority of DNA methylome changes during the GC response, and these frequently co-localised with AID's deamination sites and double strand breaks. The role of AID in DNA demethylation, independent of its known role in affinity maturation and class switching, is a new area for investigation.

## Concluding remarks

While work over the past decade has provided great insight into the B cell response to antigen and the consequent differentiation, more recent work is defining its regulation in greater molecular detail. The mechanics of B cell selection and affinity maturation in the GC are more clearly understood, with the importance of Tfh-mediated help, and cell division regulation highlighted. There is scope for new insights into GC responses and humoral memory, through the identification of new regulators, while new roles for well-known proteins such as NFκB, Myb and AID are being discovered. Finally, the exquisite control and almost infinite versatility of the antibody response is likely the consequence of the fine-tuning of regulatory hierarchies by epigenetic or post-transcriptional means.

## Acknowledgements

LMC and DMT are funded by the Australian National Health and Medical Research Council, through a Program Grant (1054925) and Fellowships (637306 and 1060675), and by Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIIS.

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