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Memory B cells: Effectors of long-lived immune responses

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Immunological memory is the phenomenon whereby B and T cells have the unique ability to respond with heightened kinetics and efficacy to subsequent encounter with Ag relative to the initial exposure. In this review, we examine recent developments in the phenotypic characterisation of memory B cells, with an emphasis on the definition and functional properties of memory B-cell subsets in humans. Gene expression differences are also considered in light of the unique functional and survival properties of memory B cells, and mutations that alter memory formation and function are also examined. Finally, we consider recent advances in the understanding of germinal center B-cell differentiation through analysis of transcription factor networks operating in these B cells.

Key words: B cells · Cell differentiation · Memory cells

Introduction

The ability of the immune system to "remember" previous encounters with pathogens, and to respond with heightened kinetics and efficacy compared with the initial exposure, is a defining feature of the adaptive immune response of higher vertebrates. The contribution of B cells to immunological memory encompasses two distinct populations of cells that are generated during primary immune responses, long-lived plasma cells (PC), which continue to secrete high levels of neutralising Ig for protracted periods of time well after Ag clearance, and memory B cells, which can rapidly proliferate and differentiate into PC following recurrent exposure to the initial immunising Ag, thereby simultaneously increasing the precursor frequency of Ag-specific memory B cells and enriching the pool of Ag-specific Ig [1–4]. The ability of naïve B cells to differentiate into memory and PC underlies the success of most – if not all – vaccines currently in use. Despite this, and the fact that we have known about the phenomenon of humoral immunity for centuries, we still do not have a thorough understanding of the biology of memory B cells. Here, we will highlight recent advances, and outstanding questions, in elucidating some of the critical aspects underlying the generation, function and maintenance of memory B cells.

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Development of serological memory is dependent on germinal centres

Following initial interactions between protein Ag, naïve B cells and other cells within the lymphoid tissue microenvironment such as $CD4^+$ T cells, macrophages and DC, the Ag-specific naïve B cells then undergo one of two fates: some of them rapidly differentiate into short-lived PC, which provide a first wave of defence against the invading pathogen, while others migrate to the B-cell follicle and seed a germinal centre (GC) [1–5]. Within GC several cardinal molecular processes necessary for the evolution of long-lived serological immunity take place: somatic hypermutation (SHM) and class switch recombination (CSR) of the Ig V and C region genes, respectively, expressed and secreted

by the responding B cells, selection of high-affinity variants, and subsequent differentiation of these cells into memory B cells and PC [1–5]. When GC formation is impaired, either by the targeted inactivation of specific genes (see section *Identification of genes required for memory B-cell generation lessons from mutant humans and mice*) or deletion of $CD4^+$ T cells [6], long-lived memory B cells fail to develop. Thus, the generation of sustained serological memory is critically dependent on the ability to establish GC reactions.

Definition of memory B cells

Historically, memory B cells have been defined as long-lived IgD^- B cells, indicating that they had undergone CSR [7–11]. Memory B cells have also been identified by their ability to bind specific Ag [3, 12–14]. Another defining feature of memory B cells is the accumulation of somatic mutations within their Ig V region genes [3]. Indeed, IgD^+ B cells expressed unmutated Ig V region genes, and thus corresponded to naïve B cells, while those expressed by IgD^- B cells were heavily mutated [9, 15]. Much of the initial phenotypic characterisation of memory B cells was performed using human B cells, since relatively large numbers of IgD^- and IgD^+ B cells could be recovered from peripheral blood (PB) and tonsils. These studies revealed that memory B cells were larger than naïve B cells and expressed higher levels of co-stimulatory and activation molecules such as CD80, CD86 and CD95 and had down-regulated expression of CD23 [10]. Later studies suggested that expression of CD27 identified human memory B cells because $CD27^+$ B cells exhibited the morphological (increased size), phenotypic ($CD23^-CD80^+CD86^+CD95^+$) and molecular (somatically mutated Ig V region genes) features of IgD^- B cells [16–18].

The delineation of human naïve and memory B cells according to differential expression of IgD or CD27 allowed the isolation and detailed analysis of the functional attributes of these cells following stimulation with a diverse array of mitogens, including mimics of T-cell help (*i.e.* CD40L), or exposure to Ag (*i.e.* anti-BCR reagents), TLR ligands or cytokines [5, 8, 10, 19–26]. Although these analyses are largely restricted to *in vitro* experiments, they yielded important findings regarding the biology and function of naïve and memory B-cell subsets. The rates of proliferation and differentiation of naïve and memory B cells *in vitro* mirrored those of primary and secondary immune responses *in vivo*, with a greater proportion of memory cells entering division earlier, undergoing more rounds of division and becoming plasmablasts than naïve B cells [8, 19–26]. The greater responses of memory compared with naïve B cells are unlikely to simply result from heightened expression of CD40, TLR or cytokine receptors on memory B cells – in fact, although resting memory B cells do not express IL-21R, *in vitro* (and presumably *in vivo*) activation induces its expression allowing activated memory B cells to respond to the stimulatory effects of IL-21 to a greater magnitude than naïve B cells, which constitutively express IL-21R [24]. Rather, memory B cells appear to have undergone a re-wiring process during their generation such that they have an intrinsic advantage over their

	Naïve ($IgM^+IgD^+CD27^-$)	GC ($CD38^{hi}CD20^{hi}$)	IgM Memory $IgM^{hi}IgD^{lo}CD27^+$	IgG,A Memory (IgM/D^-CD27^+)	FCRL4 Memory ($FCRL4^+$)	PC ($CD38^{hi}CD20^{lo}$)
Location	PB 2° lymphoid	2° lymphoid	PB, 2° lymphoid (MZ in spleen)	PB 2° lymphoid	MALT	BM 2° lymphoid
Proliferation	+	++	++	+++	+++	-
Differentiation	+	+++	++	+++	+++	-
TLR responsiveness	7, 9 co-activation		7, 9, 10	7, 9, 10		9, 10
Chemokine Receptors	CXCR4 CXCR5, CCR7	CXCR4, CXCR5	CXCR4 CXCR5, CCR7	CXCR4 CXCR5, CCR7	CCR1 CCR5	CXCR4 (CCR9, 10)
SHM	-	++/-	++/-	++/-	++/-	++/-
Survival Bcl2, A1 $Bclx_L$	+	- ++	++	++		
Phenotype CD80, CD86, CD95 ABCB1	- -	++ -	+ +	+ +		+ (CD86, CD95) -
Cell Cycle	p21	CyclinB1/2, E1/2	CyclinE1	CyclinE1		
Transcription factors	PAX5 Spi-B KLF4, KLF9 PLZF	PAX5 Spi-B Bcl6 MTA-3	PAX5 Spi-B Notch2	PAX5 Spi-B RUNX1	PAX5 Spi-B RUNX2	IRF4 Blimp1 XBP-1

Figure 1. Characteristics of the major human B-cell subsets. Each subset was defined according to the indicated phenotype. *Proliferation* and *Differentiation* refer to the potential of those B-cell populations to respond when exposed to the appropriate stimuli. *Chemokine Receptors* is defined by either chemotaxis or gene expression data. *SHM* indicates that there are B cells within these memory subsets expressing mutated Ig V region genes. The *Cell Cycle* proteins and *Transcription factors* listed are unique to or distinctive of those subsets and are not intended to be an exhaustive list. See text for relevant citations for the entries in this figure.

naïve precursors that allows them to respond with heightened kinetics and vigour to all types of stimuli [27, 28]. Together, memory B cells can be defined as long-lived quiescent B cells expressing somatically mutated Ig V genes that are capable of eliciting rapid and robust responses compared with corresponding Ag-inexperienced naïve B cells.

Memory B-cell subsets

Immunologists have a great fascination with partitioning cells of the same lineage into distinct populations, usually based on key attributes. Memory B cells have also been subjected to such compartmentalisation. For instance, analysis of Ig expression by Ag-binding murine or $CD27^+$ human B cells revealed that a large proportion of them in both species continues to express IgM, thus demonstrating the existence of IgM^+ memory B cells, in addition to the classic population of IgG^+ (and to a lesser extent IgA^+) memory B cells (Fig 1; [16, 17, 25, 29, 30]). The identification of IgM^+ memory B cells in humans based on expression of CD27 is consistent with previous findings of human splenic, tonsil, PB and BM B cells expressing somatically mutated Ig μ heavy chain genes [9, 31–33].

The origin and function of human IgM^+CD27^+ memory B cells has become a topic of debate [27]. Since these cells are present in patients with immunodeficiencies due to mutations in genes (*e.g. CD40L*, *CD40*, *ICOS*, *SH2D1A*) that cripple GC formation [34–37], one hypothesis is that these cells represent a distinct lineage of innate-type B cells that are generated independently of GC and actually undergo SHM during diversification of the pre-immune repertoire; thus, they should not be considered as *bona fide* memory cells [34, 35]. It has been proposed that human IgM^+CD27^+ B cells have exclusive roles in immunity against polysaccharide T-independent type pathogens such as encapsulated bacteria [35, 38]. Recent studies have indeed demonstrated that IgM^+CD27^+ B cells are distinct from classic isotype-switched memory B cells with respect to CDR3 spectratyping of their Ig variable genes [39] and their ability to be generated in humanised SCID mice in the absence of T cells [40]. Thus, at least during the neonatal period, IgM^+CD27^+ B cells may undergo a unique developmental program. However, there is considerable evidence to suggest that IgM^+CD27^+ B cells, as well as the analogous population in rodents, make significant contributions to secondary immune responses in adults [14, 27, 30, 41]. It is also worth emphasising that analysis of the Ag specificity of human IgM^+CD27^+ B cells demonstrated that these cells were not enriched for reactivity against polysaccharide/bacterial Ag [42], which is consistent with the abilities of both IgM^+ and IgM^- $CD27^+$ B cells to participate in immune responses against TD and T-independent Ag *in vivo* [41]. Lastly, while IgM^+CD27^+ B cells can be generated in patients who lack GC, there is a numerical deficiency in these cells compared with normal individuals [27, 34–37]; this actually demonstrates that most IgM^+CD27^+ B cells are generated in a GC-dependent manner. Taken together, it is highly likely that IgM^+CD27^+ B cells represent a heterogenous population of B cells that have specialised functions during distinct stages of development.

Further heterogeneity has been reported for human and murine memory B cells. Several groups have noted that some IgG^+ B cells in human spleen, tonsil and PB do not express CD27 [37, 43–45]. These $CD27^-IgG^+$ B cells appears to be a memory subset since they exhibited features of classic memory cells including mutated Ig V genes and expression of co-stimulatory molecules [44, 45]. Interestingly, the frequency of $CD27^-$ memory B cells is expanded in SLE patients compared with healthy controls [45]. $CD27^-IgG^+$ and $CD27^+IgG^+$ memory B cells differed from one another with respect to the distribution of IgG subclasses, with $CD27^+IgG^+$ B cells expressing IgG1, IgG2 and IgG3, while $CD27^-IgG^+$ cells preferentially expressed IgG1 and IgG3 [43, 44]. While these phenotypic differences imply functional specialisation of $CD27^-$ and $CD27^+$ B memory B cells in terms of the quality of the IgG that they might produce and the type of pathogen they may respond to, detailed functional analyses of $CD27^-IgG^+$ and $CD27^+IgG^+$ memory B cells have yet to be reported.

Examination of human tonsils also revealed the existence of another population of $CD27^-$ memory B cells that could be identified by expression of the inhibitory receptor FCRL4 (Fig. 1; [46–48]). Most of these cells (>60%) expressed IgG; however ~25% also expressed IgA [47]. This is distinct from PB $CD27^-$ memory B cells that lacked IgA^+ cells. In normal individuals, $FCRL4^+CD27^-$ B cells appear to be restricted to mucosal associated lymphoid tissue (MALT), because they were not detected in BM, spleen, lymph node or PB [47, 48]. This may reflect increased expression of specific chemokine and homing receptors. Alternatively, even though FCRL4 has been described to have inhibitory function *via* its association with SHP-1 [46], it may contribute to the positioning of $FCRL4^+$ memory B cells in mucosal sites. Functional assessment of $FCRL4^-$ and $FCRL4^+$ memory B cells demonstrated that $FCRL4^+$ B cells underwent less proliferation than $FCRL4^-$ cells; however, they secreted much higher levels of Ig *in vitro* [47]. The unique localisation of $FCRL4^+$ memory B cells in MALT and their rapid differentiation into PC suggests they may have important roles in mucosal humoral immune responses (Fig. 1).

Interestingly, and in contrast to healthy individuals, $FCRL4^+$ memory B cells have recently been found in the circulation of patients infected with HIV [49]. Moir *et al.* [49] observed that the reduced responses and unique expression of inhibitory receptors by $FCRL4^+$ memory B cells in HIV patients were features of "exhausted" effector/memory-type $CD8^+$ T cells that are generated in response to chronic viral infection. Accordingly, they proposed that HIV infection induces B-cell exhaustion and dysfunction in a subset of memory B cells that has the potential of eliciting protective Ab responses against HIV. This may explain the poor humoral immune responses that are evoked in individuals following exposure to HIV.

Studies of Ag-specific B cells in mice partitioned memory B cells into $CD80^-$, $CD80^+CD35^{lo}$ and $CD80^+CD35^{hi}$ subsets

[50]. Interestingly, these populations differed from one another with respect to the frequency of SHM, with many (>70%) of the $CD80^-$ and $CD80^+CD35^{hi}$ memory B cells expressing unmutated Ig V region genes, while >80% of $CD80^+CD35^{lo}$ memory B cells had undergone SHM [50]. This finding of memory B cells expressing unmutated Ig V region genes [50] confirms that made by Schittek and Rajewsky [15] that ~25% of IgM^-IgD^- B cells harbour germline Ig V region genes.

Thus, in both humans and mice, multiple subsets of memory B cells have been identified and, in some cases, further interrogated for biological function or perturbed development in cases of disease. The exact relationship between each subset (*i.e.* whether one subset is the progenitor of another) as well as the relative contribution of each to the establishment and maintenance of long-lived humoral immune responses should be the focus of future studies.

Mechanisms underlying the rapid kinetics of secondary humoral immune responses

As detailed above, a defining feature of memory B cells is the ability to respond rapidly following re-exposure to a pathogen or immunising Ag. There are several features of memory B cells that would allow them to achieve this. First, the precursor frequency of Ag-specific memory B cells is substantially greater than that of Ag-binding naïve B cells [3]. Thus, there are simply more memory B cells available to respond to pathogenic insults. Second, memory B cells are strategically located at sites of Ag drainage, such as the splenic marginal zone, the mucosal epithelium of the tonsil and the BM, while naïve B cells are positioned within lymphoid follicles [5, 10, 17, 31, 33]. Notably, the spleen has recently been found to be the predominant site for localisation of long-lived memory B cells, evidenced by the demonstration that the frequency of virus-specific memory B cells in the spleen exceeded that in the PB by three- to tenfold [51]. The frequency of B cells in the human spleen and PB is ~50 and ~10% of all lymphocytes, respectively. Furthermore, there is a significantly greater frequency of memory B cells in human spleens than in PB (*i.e.* ~50 *versus* ~25%) [17, 52]. These numerical differences would indicate that there are up to 100 times more Ag-specific memory B cells in the spleen than the circulation. Third, the process of SHM increases the affinity of the memory cell for specific Ag [1]. Fourth, these cells constitutively express the co-stimulatory molecules CD80 and CD86 [10, 17, 18, 50]. Fifth, the cytoplasmic domain of switched Ig isotypes provides memory B cells with biochemical properties distinct from naïve B cells. Thus, engagement of the BCR on murine B cells engineered to express either IgM, IgG or chimeric IgM/G molecules (where the cytoplasmic domain is derived from IgG) resulted in a greater and more sustained signalling cascade in IgG^+ or chimeric IgM/G^+ B cells compared with IgM^+ B cells [53]. This may underlie the ability of IgG^+ and chimeric IgM/G^+ B cells to produce >100-fold more Ig *in vivo* than IgM^+ B cells in response to specific Ag [53, 54].

There are several caveats to the interpretation of these results. First, these B cells expressed IgG or chimeric IgM/G throughout development. Thus, in contrast to *bona fide* memory B cells, they did not acquire the switched Ig isotype during the normal process of a GC or undergo Ag-induced affinity maturation and selection. Second, analysis of human B-cell subsets have demonstrated that both IgM-memory and isotype-switched memory cells enter division earlier and undergo greater numbers of division than naïve B cells, suggesting that determinants in addition to the cytoplasmic domain of switched Ig isotypes contribute to the heightened response of memory B cells [24, 25, 28, 55]. These limitations notwithstanding, memory B cells would clearly have a competitive advantage over naïve B cells inasmuch as they could capture and present more Ag to $CD4^+$ T cells *via* their BCR, stimulate these cognate $CD4^+$ T cells *via* CD80/CD86 and then receive appropriate help. This, together with prolonged signalling through surface IgG, would result in the clonal expansion of memory B cells and their differentiation into PC. These features of memory B cells, combined with their increased precursor frequency, would contribute to the rapidity and efficacy of secondary humoral immune responses.

In addition to these physical and phenotypic features, microarray analyses of human and murine naïve and memory B cells have identified specific genes and/or signalling pathways that may underlie the superior anamnestic immune response. In general, memory B cells express increased levels of key survival genes (*Bcl-2*, *A1*), which correlates with their improved survival *in vitro* and *in vivo*. Memory B cells also express reduced levels of negative regulators of the cell cycle (*e.g. p21*, *WAF-1*, *KLF4*, *KLF9*, *PLZF*) and increased levels of pro-proliferative genes (*cyclin E1*) compared with naive B cells, which suggests that the ability of naïve B cells to enter the cell cycle and thus undergo differentiation is more stringently regulated than that of memory B cells [28, 55–59]. Interestingly, global gene analysis of murine BCR transgenic IgM^+ (*i.e.* "naïve"), IgG^+ and chimeric IgM/G^+ (*i.e.* "memory"-like) B cells revealed that while Ag stimulation induced expression of a large suite of genes in naïve B cells, only a fraction of these were induced in "memory" B cells [53]. This was unexpected, since it was anticipated that, due to their greater response, memory B cells may up-regulate expression of a large number of genes following exposure to Ag. This led the authors to propose a "less-is-more" hypothesis whereby BCR stimulation on memory B cells results in diminished expression of genes that function to prevent survival of naïve B cells and/or their differentiation into PC [53]. This is supported by the findings for human B cells [28] in that it is the lack of expression of negative regulatory genes in memory B cells, rather than heightened expression of pro-responsive genes, that allows these cells to rapidly respond to repeated Ag stimulation.

Further studies in mice found that memory B cells expressed increased levels of components of specific intracellular signalling pathways – adenosine receptor 2A, isoforms of protein kinase C, bone morphogenic protein receptor 1a, leukemia inhibitory factor receptor – that are known to promote activation and

differentiation of numerous cell types [60]. While the function of several of these genes has been established in non-lymphoid cells, their specific function in memory B cells is incompletely defined. Irrespective of this, these findings have provided an impetus to elucidate the biology of these genes in B cells and determine whether they contribute to the characteristic differences between the responses of naïve and memory B cells.

Mechanisms underlying the longevity of memory B cells

The primary B-cell response in humans peaks approximately 4 wk after Ag exposure. Thereafter, the frequency of memory cells declines rapidly [61, 62]. Despite this, memory B cells can persist at low but detectable frequencies for essentially the life of the host (>50 years in humans) [11, 61, 62]. The processes that contribute to this phenomenon are unknown. While initial studies proposed that memory B cells required continual exposure to Ag for their persistence [63], an elegant *in vivo* BCR-specificity switching model demonstrated that Ag is not necessary for their prolonged survival [64]. Similarly, the survival of established memory B cells is independent of sustained T-cell help [6].

It was also proposed that polyclonal or bystander activation of memory B cells by innate stimuli such as TLR ligands contributed to the maintenance of humoral immunity by facilitating expansion of memory B cells and their differentiation into PC [22]. This hypothesis was supported by the finding that a greater frequency of memory B cells was actively turning over *in vivo* compared with naïve B cells (~2.5 *versus* <0.5%, respectively) [43, 65], and by the complementary proposition that Bcl-6 regulated the self-renewal/proliferative capacity of memory B cells [66]. However, it is unclear whether the increased basal proliferation *in vivo* of memory over naïve B cells is a direct result of polyclonal stimulation or simply reflects intrinsic differences between the cell cycle machinery that allows memory cells to respond more rapidly than naïve cells. Furthermore, since Bcl-6 is not expressed by resting memory B cells [57, 59], it is unlikely that this transcription factor contributes to memory cell longevity or turnover. Supporting this conclusion is the observation that ectopic expression of Bcl-6 in human GC B cells repressed their differentiation into "memory"-like cells *in vitro* [59].

Another possibility is that survival of memory B cells is supported by BAFF (B-cell activating factor belonging to the TNF family), since (i) BAFF is required for the generation of mature B cells in mice, (ii) memory B cells express BAFF-R and TACI, two receptors for BAFF [67] and (iii) the frequency of memory B cells is reduced in some individuals with mutations in *TACI* [68]. While this is an attractive possibility, it is also unlikely to be correct because studies in mice and non-human primates have demonstrated that inhibiting BAFF signalling has no effect on the memory B-cell compartment, even though naïve B cells were depleted or reduced in these systems [69–71]. Thus, while several scenarios have been proposed to explain the longevity of memory B cells, they either remain unproven or have been disproven. Consequently, the mechanisms responsible for this striking feature of memory B cells are enigmatic, and remain an area for investigation.

Memory B-cell generation and persistence – lessons from mutant humans and mice

The inability to form GC, either due to intrinsic B-cell defects or extrinsic defects in accessory cells, has highlighted the fundamental requirement for GC in the generation of long-lived serological memory responses. The ability to detect and enumerate human memory B cells based on CD27 expression has facilitated analysis of B-cell differentiation in genetic immunodeficiencies characterised by reduced levels of serum and Ag-specific antibodies. Patients with loss-of-function mutations in genes encoding CD40/CD40L [34], NEMO (NFκB essential modulator) [72], ICOS (inducible co-stimulator) [36], SAP (SLAM-associating protein) [37, 52] and CD19 [73, 74] have dramatic reductions in the frequencies and absolute numbers of memory B cells. These patients are essentially devoid of isotype-switched memory B cells and lack the vast majority (>60%) of IgM memory B cells. Memory B cells are also reduced, but to a lesser extent, in some patients with mutations in *TACI* [68] and rheumatoid arthritis patients undergoing anti-TNF therapy [75]. Analyses of genetically engineered mice have largely confirmed these findings from humans and have also identified additional molecules crucial for GC formation and serological memory [4]. While these studies of mutant humans and gene-targeted mice have clearly revealed a requirement for particular genes for memory B-cell development, they have struggled to distinguish between a gene product being required for GC formation or being required for the formation or maintenance of memory. This is due in large part to technical limitations in the ability to delete genes in a temporal or tissue-specific manner.

However, this may now be changing with the appearance of strains containing inducible forms of Cre recombinase or expressing Cre from promoters that either become active after B-cell activation or are expressed late in development. This includes Cγ1-Cre in which deletion of floxed genes is restricted to B cells initiating CSR to IgG1 [76] and an AID-Cre strain in which Cre is expressed from a bacterial artificial chromosome transgene under the control of the AID promoter and thus restricted to B cells undergoing CSR and/or SHM [77]. Additional strains that will be useful in resolving the role of particular genes in the formation and persistence of humoral memory include hormone-inducible forms of Cre, Cre expressed in mature, follicular B cells and Cre under the control of the *prdm1* promoter and thus expressed in Blimp1$^+$ PC. The Cγ1-Cre strain, for example, has been used to delete IRF4 or PLCγ2 from activated B cells, revealing the roles of the encoded proteins in the formation and maintenance of isotype-switched B-cell memory. IRF4 was not required for memory cell formation but was required for both induction of AID – and hence CSR – and the generation of PC,

from both naïve and memory B cells [78]. Stage-specific deletion of PLCγ2, which is downstream of the BCR and required for calcium flux following Ag binding, revealed it to be crucial for GC and memory B-cell formation and, in a particularly useful insight, memory cell survival [79]. This last fact was revealed by the inducible deletion of *plcγ2* in established memory B cells, one of the few ways available to address this question. Interestingly, deletion of PLCγ2 had no impact on affinity maturation, emphasising the independence of this process from BCR signal transduction. AID-Cre has been used to delete the transcription factor E2A from GC B cells, resulting in defective development despite normal induction of AID and CSR [77]. An example of late-stage gene deletion providing insight into the regulation of humoral immunity involves the Rev3 subunit of Polζ, an error-prone polymerase involved in DNA repair [80]. Deletion of Rev3, critical for Polζ function, in $CD21^+$ B cells revealed the polymerase to be redundant in SHM but essential for double strand break repair [80]. These experiments required tissue-specific deletion as germline Polζ deletion is lethal [80].

The use of Cγ1-Cre has yielded another chapter in the role of CD95 in regulating B-cell differentiation. CD95, the death receptor mutated in the *lpr* strain of mice, was shown to be highly expressed on GC B cells [10, 81]. While originally thought to have little or no role in GC B-cell activity [81], it was subsequently shown to be required to limit the GC reaction [82]. In the absence of CD95, GC B cells continued to proliferate, mutate and generate memory cells containing highly mutated Ig V genes. It now appears that CD95 expression in the GC is critical for maintaining B and T-cell homeostasis as mice in which CD95 was deleted using Cγ1-Cre developed a lethal lymphoproliferative disease that mimicked the disease that developed in mice that lacked CD95 in all B cells [83], suggesting the involvement of CD95 in processes in the GC even more fundamental than affinity maturation.

While analysis of mice harbouring germline deletions of candidate genes continues to provide insight into GC formation and memory B-cell generation, studies of transgenic mice have also been informative. For instance, mice transgenic for a dominant-negative version of the signalling component Ras were found to elicit normal primary humoral immune responses, as measured by the kinetics of GC and memory B-cell generation and secretion of Ag-specific IgG. However, there was impaired recruitment of high-affinity post-GC B cells into the memory compartment and, following re-exposure to the immunising Ag, these memory B cells failed to differentiate into PC, thus resulting in a very weak (and low affinity) secondary immune response. The impaired memory B-cell response was corrected by transgenic expression of Bcl-2, suggesting that Ras is required for survival of Ag-stimulated memory cells [84]. Finally, mice transgenic for the gene encoding GC-associated nuclear protein (GANP), which has DNA-primase activity and is thought to be involved in DNA replication, generated Ab of up to 100 times higher affinity compared with controls. Although the mechanism is not yet understood, it involves in part altered Ig V gene selection [85]. Somewhat surprisingly, the heightened affinity in GANP-transgenic mice complements the diminished affinity seen in GANP-deficient mice [86].

Similar over-expression studies have also been performed using human B cells infected *in vitro* with retroviruses encoding genes of interest. Spi-B, a transcription factor belonging to the Ets family, is expressed in naïve, GC and memory B cells but is down-regulated in PC [87]. Consistent with this, it is down-regulated during the differentiation memory B cells into PC *in vitro* [87]. Interestingly, enforced expression of Spi-B in human activated B cells prevented their differentiation into PC *in vitro*; this was achieved by repressing expression of the requisite PC transcription factors Blimp-1 and XBP-1 [87]. Thus, it is possible that sustained expression of Spi-B is necessary to preserve the memory B-cell pool and memory cell identity. The molecular mechanisms responsible for maintaining Spi-B expression in memory B cells are yet to be determined.

Another recent development in the analysis of memory formation is the role of micro-RNAs (miR) [88]. One in particular, miR-155, stands out. Deletion of miR-155 revealed it to have a role in GC formation and output by regulating the differentiation of DC and $CD4^+$ T cells [89, 90] and promote PC formation [91]. Also of relevance to memory formation is the finding that both miR-155 and miR181b are negative regulators of AID expression [92–94]. In a broader sense, multiple miR have been found to be differentially expressed in a stage-specific manner throughout B-cell differentiation, with unique expression patterns noted for naïve, GC, memory and PC [95, 96]. Furthermore, miR are aberrantly expressed in B-cell malignancies [97]. Thus, the restricted expression and function of particular species of miR in B-cell subsets are likely to reveal an important role in regulating the differentiation of activated B cells into GC, memory and PC lineages, and perturbations to this process may manifest as malignant transformation.

Transcription factor networks involved in memory B-cell formation

Genetic studies and other analyses examining the distribution of transcription factors within GC have led to the formulation of potential transcriptional networks regulating GC B-cell differentiation (Fig. 2). One such network has Bcl-6 at the centre due to its absolute necessity, its function in promoting GC B-cell proliferation, in protecting GC B cells from the consequences of DNA damage and potentially in suppressing differentiation along either the memory or PC pathways [59, 98]. In this scheme, Bcl-6 is maintained by external stimuli operating through IRF8 [99], STAT5 [66] and/or STAT3 [100]. Exactly what the external stimuli are remain to be determined but cytokines are likely candidates. Down-regulation of Bcl-6, which accompanies B-cell differentiation into both PC and memory cells (Fig. 2; [59]), can be achieved through direct repression by IRF4, itself induced by CD40-mediated activation of NFκB [101]. Thus T-cell–B-cell interactions in the GC may down-regulate Bcl6 expression, opening a window of opportunity

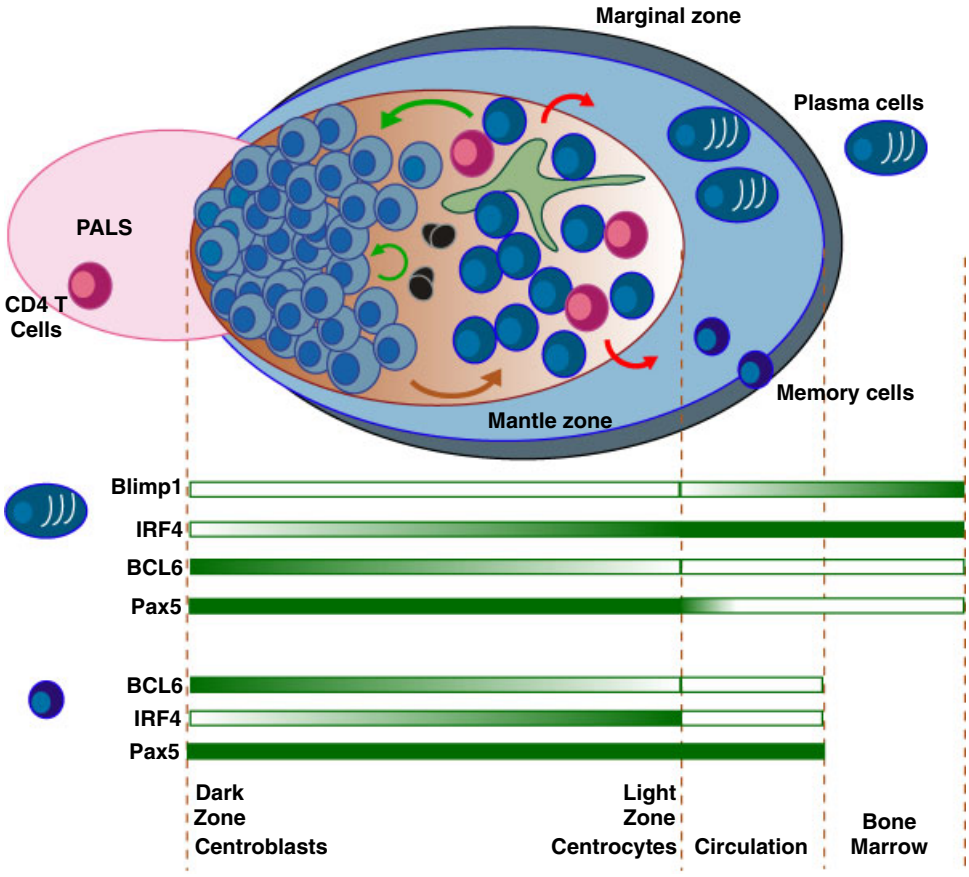

Figure 2. Schematic representation of the germinal centre reaction and the formation of memory B cells and PC. The dark and light zones of a prototypical GC, sitting within a follicle and adjacent to the T-cell-containing periarteriolar lymphoid sheath (PALS), are indicated. The mantle and marginal zones are indicated, separated by the marginal sinus (data not shown). Potential migration of centrocytes and centroblasts are indicated by arrows, as is proliferation of centroblasts. Apoptotic cells are shown in black and a single follicular DC (FDC) is shown in the light zone. Emigration of differentiated PC and memory B cells is also indicated from the light zone. Differences in expression of transcription factors relevant to the formation of memory B cells are shown. Changes associated with PC formation and with memory B-cell formation are indicated in a separate section. For example, expression of IRF4 expression increases in the light zone and further in PC but it is extinguished in memory B cells. Bcl6, in contrast, is highest in the dark zone and then extinguished in both PC and memory B cells. See text for relevant citations and a more detailed explanation of GC B-cell differentiation.

for differentiation. While this scheme explains the formation of PC and memory B cells (Fig. 2), it does not explain recirculation of B cells within the GC. Recent intravital imaging (reviewed in [102]) and extrapolation from experimental data [5], have posited that GC B cells migrate within GC, circulating between the dark zone – a region of proliferation and SHM – and the light zone where selection and differentiation occur. Indeed, expression of the chemokine receptors CXCR4 and CXCR5 varies on GC B cells in a manner consistent with inter-zonal migration being driven by sensitivity to CXCL12 and CXCL13, a conclusion supported by analysis of gene-targeted mice [102]. There may therefore be conditions in the GC light zone that influence the fate of GC B cells, directing them towards differentiation and emigration on the one hand, or recycling within the GC on the other, presumably coincident with altered expression of Bcl6. The combination of factors that influence this decision are still to be defined but presumably involve CD40 and T-cell derived cytokines.

Concluding remarks

Despite knowing of immunological memory as a phenomenon for centuries, the cellular and molecular processes involved in its generation and maintenance are only now being revealed. Significant progress has been achieved on several fronts, defining important molecular and cellular interactions. This has been achieved through the analysis of abnormal situations, either engineered in mice or spontaneously arising in human immunodeficiencies and through the use of screening systems such as microarrays. Collectively this has identified subsets of memory B cells, molecular configurations associated with heightened responsiveness and longevity, and transcriptional networks that predict the alternate differentiation pathways open to Ag-activated B cells. It remains to integrate the cellular and molecular advances more fully, providing a map of memory formation and possibly the means to guide B cells to particular, desirable outcomes.

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Abbreviations: **BAFF:** B-cell activating factor belonging to the TNF family · **CSR:** class switch recombination · **GANP:** GC-associated nuclear protein · **GC:** germinal centre · **miR:** micro-RNAs · **PB:** peripheral blood · **PC:** plasma cell · **SHM:** somatic hypermutation

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