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New players in the gene regulatory network controlling late B cell differentiation

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The differentiation of B cells into antibody-secreting plasma cells is associated with profound changes in morphology, lifespan, and cellular metabolism that are needed to support high rates of antibody production. These processes are driven by dramatic alterations to the transcriptional program and to the organization of the nucleus itself that in turn are regulated by the activity of a select group of transcription factors and epigenetic regulators. Although the core differentiation program is conserved in all mature B cells, subset-specific regulators, such as those found in B1 or memory B cells, provide additional complexity. Here, we review the key components of the gene regulatory network controlling B-cell terminal differentiation, with an emphasis on the new players and processes that have emerged in recent years.

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Introduction

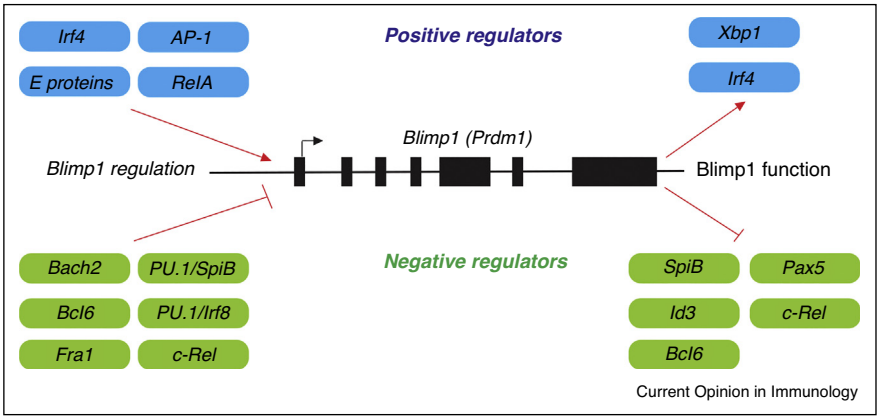
Humoral immunity represents an essential arm of the adaptive immune response that functions to target and eliminate foreign antigens while sparing self-tissue. Following the detection of foreign antigen via the B cell receptor (BCR), follicular B cells proliferate and undergo class switch recombination (CSR) before differentiating into either short-lived blasts or long-lived plasma cells and memory B cells. Plasmablasts are generated through an extrafollicular B cell response and provide early protection through the production of low affinity antibody. In contrast, most long-lived plasma cells are generated in specialized structures called germinal centers (GC), where, with T cell help, they undergo somatic hypermutation (SHM), which leads to the production of high-affinity antibody. Memory B cells are also generated in the GC and provide long-term protection by rapidly differentiating into antibody secreting cells (ASC) upon re-exposure to the same antigen [1]. In addition to follicular B cells, mature B cell subsets also include marginal zone (MZ) B cells and B1 cells that both function to respond rapidly to T cell-independent antigens, such as bacterial components. MZ B cells are located in the marginal sinus of the spleen, whereas B1 cells are predominantly found in the peritoneal and pleural cavities [2,3].

The terminal differentiation of B cells into ASCs is associated with the major re-organization of many cellular processes, which is reflected in their mutually exclusive transcriptomes [4]. These core events occur similarly in all mature B cell subsets, although there are variations in the sensitivity to the initiating signals, rate of differentiation, and longevity of the response between the distinct starting populations. The similarity in the differentiation of all B cell subsets has enabled the definition of a small group of key transcription factors that control a finely balanced gene regulatory network (reviewed in Refs. [5–7]). These transcription factors can be grouped into those that maintain the B cell fate (PAX5, BACH2, and BCL6) and others such as IRF4, BLIMP1, and XBP1 that are required for ASC generation and antibody secretion. In this review, we will highlight research published in the past few years that has built on this network, providing new transcriptional regulators as a well as highlighting functions for DNA methylation, chromatin modifications, and genome organization in the process.

The usual suspects

PAX5 is expressed throughout B-cell development and is required to maintain cellular identity. PAX5 functions to repress the expression of non-lineage B cell genes as well as several regulators of ASC formation including BLIMP1 (also known as *Prdm1*, Figure 1). In addition, PAX5 drives the expression of transcription factors associated with maintaining the B-cell fate including SpiB, IRF8, BACH2, and Aiolos [7]. BACH2 is a transcriptional repressor that is expressed throughout B-cell differentiation before being extinguished in ASCs. Like PAX5, BACH2 inhibits ASC formation by inhibiting the expression of BLIMP1 ([8–10], Figure 1). In the absence of BACH2, BLIMP1 is prematurely expressed, resulting in spontaneous ASC formation. As BLIMP1 represses *Aicda*

Figure 1



A *Blimp1*-centric view of the gene regulatory network controlling late B cell differentiation. Antibody secreting cell (ASC) promoting factors (shown in blue) including Irf4 (low level expression), E proteins, AP-1 and RelA promote the expression of the critical plasma cell gene *Blimp1/Prdm1*. BLIMP1 protein in turn drives the expression of genes encoding critical regulators of ASC survival (high level *Irf4* expression) and function (*Xbp1*) and suppresses the expression of genes associated with maintaining B cell fate (shown in green; *SpiB*, *Id3*, *Bcl6*, and *Pax5*). Other negative regulators of ASC formation (shown in green) including *Bach2*, *Bcl6*, *PU.1/SpiB*, *PU.1/Irf8* and *Fra1* maintain B cell identity by inhibiting *Blimp1* expression.

(encoding AID), the loss of BACH2 indirectly inhibits CSR and SHM. Low BACH2 expression also allows memory B cells to very rapidly differentiate into ASCs upon re-exposure to their cognate antigen [11]. In human B cells, BACH2 is repressed by an IL2/ERK/ELK1 signaling pathway [12•]. With the exception of BLIMP1, the functionally relevant targets of BACH2 currently remain poorly understood. Similar to BACH2, BCL6 primarily functions as a transcriptional repressor. BCL6 expression is predominantly restricted to GC B cells where it is required not only for GC formation but also to inhibit the DNA damage response in GC B cells and allow SHM to take place [13]. BCL6 has also been proposed to inhibit BLIMP1 expression in order to maintain GC B cells [14,15].

ASC formation and function, in contrast, rely on the coordinated activity of IRF4, BLIMP1, and XBP1. IRF4 is essential for both GC responses and for the differentiation and survival of all ASCs [16–18,19••,20]. At low concentrations, IRF4 induces the expression of *Aicda*, *Pou2af1* (encoding OBF1), and *Bcl6* [17,18,21], which are required for CSR and the generation of GC B cells, whereas high levels of IRF4 repress *Bcl6* [22] and activate *Blimp1* expression [18]. Within the B-cell lineage, BLIMP1 is exclusively expressed in ASCs, where it is essential for their generation from conventional mature B cell subsets [23••,24,25]. In contrast a proportion of B-1 cell derived ASC in the BM are proposed to be BLIMP1 independent [26]. BLIMP1 is dispensable for the survival of pre-existing ASCs and instead regulates many components of the unfolded protein response, both by direct binding and through the activation of XBP1 and ATF6 [19••], which are required for high-level antibody secretion. BLIMP1 represses many components of the B-cell program including *Bcl6*, *Id3*, *Myc*, and *Pax5*, thus completing the terminal differentiation process [23••,27–29].

New players

Although the above-mentioned gene regulatory network broadly describes the key events in the generation of ASCs, a number of new players have been identified in recent years that collectively when integrated into the current models offer the potential for a more holistic understanding of B-cell differentiation.

Aryl hydrocarbon receptor

The Aryl hydrocarbon receptor (AHR) is a ligand-induced nuclear receptor transcription factor that is expressed following B-cell activation. Similar to IRF4, AHR appears to play a dual role in the generation of ASC. AHR inhibits CSR by directly repressing *Aicda* [30•]. Furthermore, it restricts ASC formation by directly activating *Bach2*, which in turn represses *Blimp1*. AHR functions to restrict the size of the B-cell response upon immunization, as loss of AHR results in enhanced GC formation and antigen-specific ASCs, suggesting that it is an important regulator of B-cell differentiation.

PU.1, SpiB, and Irf8

The related Ets family transcription factors PU.1 and SpiB are known to bind DNA alone, to canonical Ets sites, or cooperatively with either IRF4 or IRF8 on composite Ets-IRF (EICE) sites [31]. Previously, we established that PU.1 and IRF8 together negatively regulate both CSR and ASC formation *in vitro* by simultaneously promoting the expression of *Bcl6*, *Pax5* and *Mef2c*

and repressing *Aicda* and *Blimp1* [32], functions that as noted above, are the opposite as those ascribed to IRF4 (Figure 1). SpiB in isolation plays a minimal role in the maintenance of GCs [33]; however SpiB/PU.1 double-deficient mice lack B cells due to a developmental block at the pre-B-cell stage and failed *Igl* recombination [34] and develop pre-B acute lymphoblastic leukemia at a high frequency [35]. When this developmental block was bypassed by conditional mutagenesis specifically in mature B cells, PU.1 and SpiB were found to be redundantly required for GC formation and the production of high-affinity antibody in response to immunization, despite paradoxically increased numbers of steady-state plasma cells *in vivo* [36]. *In vitro*, PU.1/SpiB negatively regulate ASC formation, with the loss of these factors resulting in reduced *Bcl6* and *Bach2* and a reciprocal increase in *Irf4*, *Blimp1* and *Xbp1* mRNA. PU.1 and SpiB control the expression of many components of the BCR signaling pathway and the receptors for CD40L, BAFF and TLR ligands, which enable B cells to appropriately respond to environmental cues. Thus the Ets and IRF families are key players in B cell differentiation, acting to link the extracellular signals with the intrinsic regulation of gene expression. Incorporating such extrinsic components is essential for a full understanding of the gene regulatory network to emerge.

Id3, E2A and E2-2

The Inhibitors of DNA-binding proteins (ID1-4) heterodimerize with basic helix-loop-helix proteins such as the E-proteins E2A and E2-2, preventing DNA-binding [37]. It was recently established that ID3 is both required for full GC formation [38•] and repression of ASC formation [39•], which led to an investigation as to which E proteins regulate this process. Although E2A alone is dispensable for B-cell differentiation, inactivation in mature B cells of E2A and E2-2 resulted in defective CSR, GC formation and ASC differentiation [39•,40•]. E2A and E2-2 drive the expression of many GC genes including *Bcl6*, *Bach2* and *Pou2af1*, as well as the genes required for ASC formation such as BLIMP1 (Figure 1).

AP-1

AP-1 is a heterodimeric transcription factor consisting of a Fos protein (including c-Fos, FosB, Fra1/2) and a Jun family member (c-Jun, JunB/D) that are often induced in response to extracellular cues. An AP-1 complex containing c-Fos is known to bind and activate the *Blimp1* promoter and thus promote ASC formation [41]. More recently, Fra1 has been shown to function to repress ASC differentiation, by competing with c-Fos for binding to the *Blimp1* promoter [42]. Thus the balance between the differentiation promoting and inhibiting AP-1 complexes influences the cellular decision to undergo ASC differentiation.

NF-κB

NF-κB is also a family of multi-subunit stimulus-induced transcription factors. In the context of late B-cell differentiation, the subunits, c-Rel and RelA, both activated by the canonical NF-κB signaling pathway, are differentially required for GC formation and ASC formation [43,44]. c-Rel is essential for the proliferation of activated B cells and both T-dependent GC formation and T-independent extrafollicular responses, resulting in impaired ASC differentiation. RelA, in contrast, is dispensable for GC, but plays a role in humoral immunity through the upregulation of *Blimp1* (Figure 1), although the extent of the deficiency in antibody production was much less pronounced with T-independent immunization.

Ying Yang 1

Ying Yang 1 (YY1) is a ubiquitously expressed transcription factor that is essential for the pro-B to pre-B transition [45]. Recently, it was observed that the promoters of GC signature genes were enriched for the YY1 consensus binding site [46], which led to studies that aimed to address whether YY1 was also involved in late B-cell development. Inactivation of YY1 after antigen stimulation revealed it was essential for GC formation and ASC differentiation [47,48]. Furthermore, using CD19-Cre it was shown that YY1 is required for the development of all peripheral B-cell subsets including B1 cells [48].

BHLHE41

B1 cells differ from conventional B cells in their BCR repertoire, location and differentiation kinetics, yet little is known about how their transcriptional control differs from other B cell subsets. BHLHE41 was recently identified a transcription factor that was dramatically upregulated on B1 cells relative follicular B cells, which prompted studies to address whether BHLHE41, or its close homolog BHLHE40, were involved in their development [49••]. Loss of BHLHE41 resulted in a dramatic reduction in B1a cells, beginning at the transitional stage, which was enhanced by the additional loss of BHLHE40, while B1b cells remained intact. BHLHE41 deficiency was also associated with an altered BCR repertoire. At the molecular level, BHLHE41 functions as a transcriptional repressor to control cell cycle and inhibit BCR signaling while allowing the pro-survival functions of cytokine signaling.

ARID3A

Another emerging regulator of the fate choice between B1 and conventional (or B2) B cells is the transcription factor ARID3A [50,51]. Inactivation of ARID3A from the pro-B cell stage results in a twofold expansion of splenic B2 cells, while splenic B1 cells were unaltered. However, the numbers of peritoneal B1a cells were dramatically reduced, although the mechanism for this remains uncertain.

DNA methylation

Although major advances have been made with regard to our understanding of how transcription factors regulate humoral immunity, it is clear that changes in epigenetic marks such as DNA methylation and histone modifications also impact on the process. The methylation of cytosine residues of CpG dinucleotides by DNA methyltransferases drives gene repression [52]. Repressed genes are characterized by methylation throughout their promoter and enhancer elements, while actively transcribed genes are characterized by methylation throughout the gene body (to prevent aberrant expression) and an absence of promoter and enhancer methylation [53].

Recently, two studies have characterized the methylation state of B cells as they transition to ASCs [54••,55••]. In human and mouse B cells, differentiation is associated with the hypomethylation of CpG loci, with almost no new methylation added during this process. Upon closer inspection, it was evident that demethylation occurred around key ASC genes including *Blimp1* and *Xbp1*, and was enriched for enhancer regions [54••,55••]. Demethylated regions in ASCs contained transcription factor binding motifs for NF-κB, IRF, POU homeobox and bHLH proteins (Figure 2, [54••]), matching well with the key regulators of the differentiation process discussed above. Finally chemical inhibition of DNA methylation resulted in increased ASC differentiation by cell cycle [54••], a finding that agrees with the coupling of *Blimp1* demethylation, and hence differentiation rate, to cell cycle progression [55••].

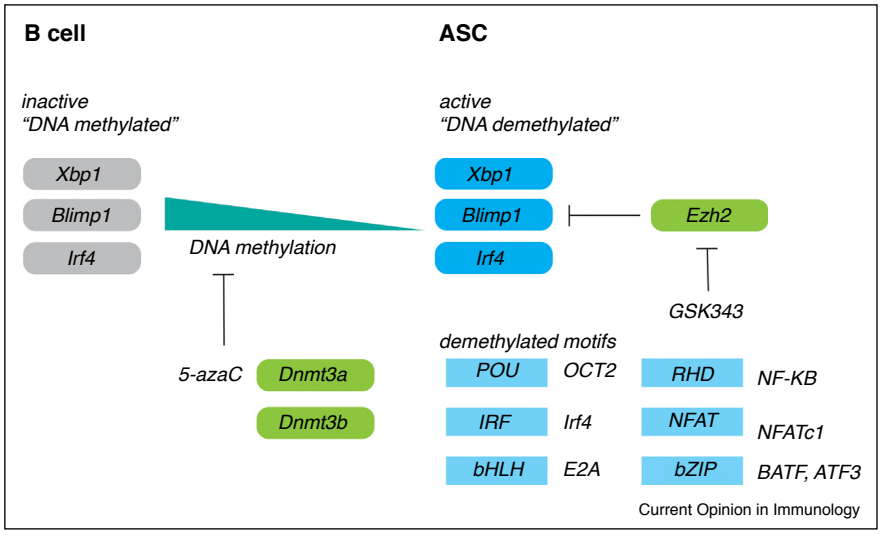
De novo methylation

During mitosis DNA methyltransferase 1 (Dnmt1) maintains the methylation state [56], while new methylation is incorporated by Dnmt3a and Dnmt3b [21]. Analysis of mice lacking Dnmt3a/b revealed that *de novo* methylation normally restrains the generation of GCs and antigen-specific immunoglobulin upon immunization [57••]. Loss Dnmt3a/3b had little effect on the gene expression programs of follicular B cells or GC B cells yet had a dramatic impact on genes that are normally downregulated in ASCs. The de-repression of ASC genes in the absence of Dnmt3a/3b was associated with decreased methylation and increased regions of chromatin accessibility, which were enriched for transcription factor binding motifs for bHLH, ETS, IRF, and ETS-IRF composite (EICE) sites [57••]. Thus the dynamic regulation of DNA methylation is required for appropriate gene expression during B-cell differentiation.

EZH2 and repressive histone marks

There has also been recent interest in the regulation of B-cell differentiation by chromatin modification. EZH2, the enzymatic component of the polycomb repressor 2 complex functions to silence gene expression through the trimethylation of histone H3 at lysine 27 (H3K27me3) and is essential for both B cell development [58] and the GC response [59–61]. Furthermore, EZH2 directly interacts with transcription factors such as BCL6 [62] and BLIMP1 [23••] in GC B cells and ASC respectively. In a recent study the importance of EZH2 for ASC formation was examined *in vitro* using the EZH2

Figure 2



Epigenetic regulators controlling late B cell differentiation. Antibody secreting cell (ASC) formation is associated with the demethylation of DNA (and hence increased transcriptional activity) around key transcription factor binding motifs and genes required for the process, including *Irf4*, *Blimp1*/*Prdm1* and *Xbp1*. Chemical inhibition of DNA methylation with 5-azaC accelerates ASC formation, which is opposed by Dnmt3a/b, which promote *de novo* methylation. Chemical inhibition of the epigenetic regulator Ezh2 (with GSK343), leads to a loss of the repressive histone mark (H3K27me3) in the promoter of a number of genes and an early induction of *Blimp1* and ASC formation.

inhibitor GSK343 [63]. EZH2 inhibition promoted differentiation in response to multiple stimuli. This effect was associated with the loss of H3K27me3 at the promoter of a number of genes and an early induction of *Blimp1* mRNA (Figure 2). T-independent immunization experiments revealed that EZH2 was required for ASC function, as in its absence, B cells proliferated poorly and maintained the expression of BLIMP1 repressed genes, including *Spib*, resulting in reduced ASC formation and Ig secretion [64•]. Thus, EZH2 functions at multiple points in the terminal differentiation process.

Genome organization

Although the regulators of distinct transcriptional and epigenetic landscapes of B cells and ASCs discussed above can act at a myriad of individual loci, it is also becoming apparent that these cell types have distinct three-dimensional organization of their genomes within the nucleus. Recent studies using the HiC technique to map DNA–DNA interactions genome-wide have revealed that PAX5 is essential for many of these long-range interactions within B cells [65••]. PAX5 binds in a large proportion of the anchors to intrachromosomal loops, most of which are lost upon PAX5 downregulation during ASC differentiation. ASCs in contrast, are proposed to be depleted of long-range intrachromosomal interactions and instead congregate *Blimp1*, *Xbp1* and unfolded protein response genes into spatially associated transcriptional hubs [66••]. The factors that control the distinct organization of the ASC genome remain uncertain.

Concluding remarks

Over the past few years, several new players have emerged that contribute to a greater understanding of how B cells differentiate into ASCs. This includes DNA sequence-specific transcription factors that impact on multiple aspects of this process as well as regulators of genome organization and the epigenetic landscape. However, many questions remain. Specifically, while current models propose that antagonistic interactions between key transcription factors regulate the B cell to ASC transition, there is still little understanding as to the order of events initiating this program. Moreover, any complete model of B cell terminal differentiation will need to incorporate the extrinsic signals that impact on cell proliferation and the type of responses generated, as well as the poorly characterized events that lead to the decision to become an ASC versus a memory B cell.

The function of NF-κB in B cell differentiation outlined in this review has been extended with the finding that the mutual antagonism between c-Rel and *Blimp1*, of each other's gene expression, controls the fate of activated B cells [67••]. This antagonism provides a bistable switch to cease the proliferative phase and initiate the ASC differentiation program. These findings were incorporated into an improved model of the ASC differentiation process.

Recently, the function of the PU.1 and IRF8 in late B cell differentiation has been extended to the *in vivo* situation with the finding that these factors act together to control follicular B cell homeostasis and GC formation [68].

Conflict of interest statement

Nothing declared.

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