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Epigenetic gene regulation in plasma cells

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SUMMARY

Humoral immunity provides protection from pathogenic infection and is mediated by antibodies following the differentiation of naïve B cells (**nB**) to antibody-secreting cells (**ASC**). This process requires substantial epigenetic and transcriptional rewiring to ultimately repress the nB program and replace it with one conducive to ASC physiology and function. Notably, these reprogramming events occur within the framework of cell division. Efforts to understand the relationship of cell division with reprogramming and ASC differentiation in vivo have uncovered the timing and scope of reprogramming, as well as key factors that influence these events. Herein, we discuss the unique physiology of ASC and how nB undergo epigenetic and genome architectural reorganization to acquire the necessary functions to support antibody production. We also discuss the stage-wise manner in which reprogramming occurs across cell divisions and how key molecular determinants can influence B cell fate outcomes.

Keywords

Plasma cells; B cell differentiation; epigenetics; transcriptomics; cell division; heme

1. INTRODUCTION

The humoral arm of the adaptive immune system relies on robust differentiation of naïve B cells (**nB**) into antibody-secreting cells (**ASC**) or plasma cells. Upon antigen encounter, nB become activated, rapidly proliferate, and undergo substantial reprogramming events, with a subset differentiating to ASC (Figure 1). Once B cells are activated, interactions with CD4 T cells play a major role in determining the cell fate choices, antibody specificity and isotype class, and ultimately longevity of the response. Stimulation with T cell-independent (**TI**) antigens, such as lipids and polysaccharides, leads to the formation of short-lived plasma cells (**SLPC**). TI antigens can be subdivided into two types[1,2]. TI type I antigens

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CONFLICT OF INTEREST

The authors have no conflicts to report.

include pathogen-associated molecular patterns that bind pattern recognition receptors, such as toll-like receptor recognition of bacterial cell wall components or DNA. TI type II antigens have highly repetitive, multivalent structures that activate B cells by crosslinking of their B cell receptors (**BCR**). TI type I antigens elicit large polyclonal responses, whereas TI type II antigens engage the BCR and thus induce antigen-specific B cell response. Stimulation with a protein antigen induces a T cell-dependent (**TD**) response in which B cells migrate to germinal centers (**GC**) and undergo somatic hypermutation and affinity maturation, ultimately resulting in the generation of BCR with higher antigen affinity. The final output of the GC reaction is long-term immunological protection provided by the generation of long-lived plasma cells (**LLPC**) and memory B cells[3]. Whether derived from TI or TD responses, antibodies secreted by ASC provide protection from pathogens[4]. Thus, the formation of ASC and antibodies is the cornerstone of adaptive immunity.

2. UNIQUE BIOLOGY OF A PLASMA CELL

2.1 Metabolic adaptations to manufacture and secrete antibodies

The primary function of an ASC is the manufacturing and secretion of antibody molecules. Remarkably, ASC are capable of secreting up to 10,000 antibodies per second[5]. To support the high rate of antibody secretion, nB undergo significant morphological and bioenergetic changes as they differentiate from a quiescent B cell that does not secrete antibody to an ASC[6]. This change in metabolism is essential to meet the energy demands required for rapid proliferation of responding B cells and the translational requirements of ASC[7,8]. Responding activated B cells (**actB**) utilize both glycolysis and oxidative phosphorylation (**OXPHOS**), whereas GC B cells also utilize fatty acid oxidation[9,10]. As actB divide and differentiate towards ASC, they gradually increase their capacity to perform OXPHOS[9]. This shift towards OXPHOS is in part due to an increase in transcription of more than 100 components of the electron transport chain tricholaric acid cycles[9], as well as activity mediated by Protein Kinase C β (**PKC-β**), which is induced in actB following antigen stimulation[11,12]. PKC-β was shown to promote mTORC1 signaling to facilitate mitochondrial remodeling and heme biosynthesis, which is necessary for ASC formation. While PKC-β-deficient cells accumulated reactive oxygen species and failed to differentiate, these phenotypic changes were reversed by treatment with hemin suggesting a mechanistic link between PKC-β and heme biosynthesis[13]. ASC primarily rely on OXPHOS to support antibody secretion, and this metabolic switch is dependent on BLIMP1, one of the master transcriptional regulators of ASC fate. Experimentally, promoting OXPHOS metabolism in responding B cells using dichloroacetate[14–16] results in increased ASC formation, suggesting a role for OXPHOS in promoting differentiation[9]. However, within the ASC lineage there appears to be differences in oxygen consumption. While SLPC consume oxygen nearly at their maximal possible levels, bone marrow LLPC exhibit a high spare respiratory capacity[17].

Another metabolic difference specific to LLPC is the high surface level expression of the glucose transporter GLUT1[17]. Interestingly, despite ASC reliance on glucose to support their metabolic demands, genetic deletion of GLUT-1 does not completely block ASC formation, indicating that glucose uptake depends on additional transporters or pathways[18,19]. Another glucose transporter, GLUT-6, is likely to play a similar role in ASC[20,21]. Additionally,

a large fraction of sugars are shunted to hexosamine biosynthetic pathways in ASC for antibody glycosylation[17], which modulates antibody effector functions[22]. Inhibiting glycosylation using tunicamycin resulted in significant ASC death due to an increase in misfolded proteins and chronic induction of the unfolded protein response (**UPR**)[23]. Furthermore, differences in glucose uptake and subsequent pyruvate import into the mitochondria have been attributed to differences in the longevity of SLPC versus LLPC, thus pointing to cross-talk between metabolism and cell survival[17]. Therefore, while LLPC use glucose for various processes, OXPHOS is still an important source of energy. Additionally, the unique metabolic features of LLPC might allow survival in the hypoxic BM environment[24]. These studies highlight the unique bioenergetic adaptations of ASC metabolism and how distinct modes may be utilized to promote differentiation and support the demands of antibody generation.

The reliance on OXPHOS is a unique feature of ASC. One of the key drivers of mitochondrial metabolism is the molecule heme. Heme is an iron containing porphyrin ring and is a key part of the cytochrome proteins that function in the electron transport chain and facilitate OXPHOS metabolism[25]. In addition to its role in the mitochondria, heme can influence B cell fate outcomes through additional mechanisms (Figure 2). One mechanism by which heme promotes ASC formation involves direct binding to the transcription factor BACH2 resulting in its degradation[26]. BACH2 maintains B cell fate by repressing BLIMP1, thus heme directly promotes ASC formation by degrading BACH2 and allowing for the induction of the ASC transcriptional program. In addition, heme can be degraded through the action of heme oxygenase-1 (HO-1) into carbon monoxide, biliverdin, and iron. Iron is an essential cofactor for many epigenetic erasers, such as histone[27] and DNA demethylases[28] as well as certain histone deacetylase 8 (HDAC8)[29,30]. Mice fed an iron-deficient diet exhibited diminished GC B cells and ASC in response to antigen. These findings were further corroborated by ex vivo studies that showed that treatment with an iron chelator impaired B cell proliferation and differentiation[31]. The impaired ASC response observed following iron depletion was attributed to the failure of H3K9 demethylases to promote the expression of cyclin E1, which regulates entry into S phase of the cell cycle, and resulted in G1/S arrest of iron-deficient B cells[31]. These data point to heme as a central metabolite with the ability to influence B cell fate through multiple mechanisms.

Memory B cells are able to rapidly differentiate into ASC compared to antigen-inexperienced naïve B cells; however, the mechanisms that facilitate these enhanced secondary responses are not fully understood. Memory B cells were found to harbor unique epigenetic and transcriptional features with primed accessible chromatin surrounding genes important in ASC differentiation, including BLIMP1[32]. Compared to naïve B cells, memory B cells upregulated their iron homeostasis metabolic pathway and possessed greater basal levels of heme, as determined using the expression of HO-1[32]. Treatment of mouse and human naïve and memory B cells with hemin promoted ASC formation and led to an increase in both basal and maximal respiratory capacity[32]. Although, heme can act through multiple pathways to promote ASC formation (Figure 2), the results mirrored the increase in ASC observed following dichloroacetate treatment to enhance OXPHOS metabolism. Thus, recent studies have demonstrated the critical role of iron and heme in regulating

cell fate decisions. More work is necessary to elucidate the precise mechanism(s) and factors regulated by heme/iron (BACH2, OXPHOS, demethylases) and how they impact ASC formation. Additionally, these data highlight the need to understand the interplay and crosstalk between metabolic byproducts and their impact on reprogramming through chromatin modifying enzymes that are dependent on these metabolites[33].

In addition to upregulating metabolism to support antibody secretion, ASC also support their energy needs by autophagy, a process that allows cells to degrade proteins and reuse the resulting metabolic intermediates[34]. This process involves engulfing cytoplasmic contents in a double-membraned vesicle that is delivered to the lysosome for degradation and recycling. When evaluating autophagy in ASC differentiation from murine B cells, significant autophagic induction was observed in both in vitro and in vivo generated ASC, independent of the initial stimulation[35]. The importance of this pathway is illustrated by the fact that deletion of *Atg5*, an essential component of the autophagy pathway, resulted in increased ER stress, reduced cellular ATP, and a decrease in ASC survival[36]. In contrast, an increase in antibody secretion on a per cell basis was observed, suggesting that autophagy may be necessary to limit antibody secretion, thus reducing the ATP expenditure and promoting cell survival.

The high rate of antibody production also requires substantial adaptations of the secretory apparatus and UPR, which is normally induced during stress and initiated by the accumulation of misfolded proteins in the endoplasmic reticulum (**ER**)[37]. The UPR consists of three highly conserved signal transductions pathways triggered by three main UPR-inducing ER stress sensors: 1) PKR-like endoplasmic reticulum kinase (**PERK**), 2) activating transcription factor 6 (**ATF6**), and 3) inositol-requiring enzyme 1α (**IRE1α**)[38]. The PERK pathway leads to an overall suppression of protein translation[39,40]; however, very limited PERK activation in ASC is observed in vitro[41]. In fact, genetic ablation of PERK or CHOP (C/EBP homologous protein), a downstream component of the PERK pathway, has no impact on plasma cell survival in vitro[42,43]. ER stress that exceeds the capacity of adaptive UPR leads to activation of the apoptotic program partially induced by CHOP[44]. However, it has been reported that LLPC are less sensitive to ER stress associated apoptosis[45], although the underlying mechanism remains to be elucidated. The ATF6α pathway can augment ER quality control processes and drive ER expansion, but deletion of ATF6α did not impact antibody secretion or survival in vitro and in vivo[46].

The response initiated by IRE1α is best understood in ASC. IRE1α is a ribonuclease and splicing factor that promotes the splicing of X-box protein 1 (**XBP1**) mRNA to a more stable *Xbp1* isoform[47]. Once generated, XBP1 promotes the expression of genes necessary for the expansion of the secretory apparatus[48,49]. The importance of XBP1 is highlighted by in vivo studies, where deletion of *Xbp1* impeded the ability of ASC to secrete antibodies. XBP1-deficient ASC exhibited normal protein folding but altered glycosylation and lipid synthesis, leading to the failure to mount a proper UPR[50]. These findings indicate that XBP1 is not required for ASC formation but rather for antibody secretion[49,51]. While XBP1 is typically thought of as the master transcription factor regulating the UPR response initiated due to the high antibody secretion, a recent study demonstrated that actB upregulate UPR-related genes prior to becoming an ASC[52]. Upregulation of the actB UPR program was

regulated by mTORC1 signaling and the adaptor protein Raptor and occurred prior to XBP1 activity[52]. This important finding indicates that part of the actB program is to prepare for subsequent antibody synthesis by initiating and building the transcriptional networks to deal with the stress of protein production and secretion.

2.2 Antibody-secreting cell survival and homing

ASC are able to survive in multiple niches in mammals. Both SLPC and LLPC can reside in secondary lymphoid organs, such as the spleen, bone marrow (**BM**)[53,54], and in tissues such as the gut[54–56]. Since ASC are generated in secondary lymphoid organs, migration of ASC to their survival niche is facilitated by the expression of chemokine receptors. This process is initiated by upregulation of S1PR1, which allows for entry into the bloodstream[57], followed by expression of specific chemokine receptors that direct ASC to their niche. Expression of CXCR4 promotes homing to the BM[58]. Blocking the CXCR4-CXCL12 axis diminished ASC homing to and retention in the BM[59], resulting in obvious accumulation of ASCs in the spleen and a concordant decrease in BM ASC[60]. CXCR3 promotes homing to inflamed tissues[61], while CCR10 and CCR9 promote the migration of IgA+ ASC to the gut, with CCR9 being restricted to the small intestine[62]. Other factors that have been implicated in ASC maturation or rention in the BM include: very late antigen 4 (VLA4)[63], CD44[64], CD28[65], and CD93[66] on PCs, as well as ZBTB20[67,68] and Aiolos[69].

Lasting humoral immunity relies on the long-term survival of LLPC. However, not all ASC are created equally. TI responses predominantly generate SLPC that die within a few days. On the other hand, ASC formed following stimulation with TD antigens are able to home to the BM and persist for decades[57,70,71]. Thus, understanding the factors regulating ASC survival is crucial to the improvement and development of successful vaccines. The BM is the primary niche of LLPC owning in part to the presence bone marrow stromal cells and cytokines, such as APRIL and BAFF, which promote the expression of the anti-apoptotic gene *Mcl1* and thus promoting ASC survival[72,73]. A recent study identified two novel factors, fibronectin and YWHAZ, that were secreted by stromal cells that promote survival of human LLPC in vitro[74]. Treatment with antibodies targeting these proteins reduced PC survival in the culture. Furthermore, the combination of mesenchymal stromal cells secretome (including fibronectin and YWHAZ), APRIL, and hypoxic conditions improved the long-term survival of LLPC in vitro and likely in vivo[74]. However, even within the BM, there is heterogeneity in the lifespan of ASC suggesting that other factors, including cell intrinsic differences are at play.

3. B CELL DIFFERENTIATION IS A COORDINATED MULTISTEP PROCESS

3.1 Initiation of the antibody-secreting cell transcriptional program

B cells and ASC express mutually exclusive gene expression programs[75]. As a result, B cell differentiation into ASC requires significant transcriptional rewiring that is coordinated by transcription factors (**TF**)[75]. For example, *Pax5*[76,77], *Bach2*[78–80], and *Ebf1*[81] are important for establishing or maintaining the nB program, while *Bcl6*[82–86] and *Irf8*[87,88] regulate actB fate. B cell activation leads to a progressive upregulation of IRF4, which in turn promotes the expression of the master ASC regulator, BLIMP1[89,90]. BLIMP1[91] (encoded

by *Prdm1*) extinguishes the B cell program and orchestrates the ASC program. BLIMP1 alone coordinates many distinct phenotypes associated with ASC, including: 1) division cessation through repression of *C-myc*[92]; 2) loss of MHC class II antigen presentation by repressing expression of the class II transactivator (CIITA)[93]; and 3) establishment of the ASC program via repression of PAX5[94,95], a critical TF that maintains B cell fate programs and represses *Prdm1*. While recent work has demonstrated that repression of *Pax5* is not essential for formation of *bona fide* ASC, normal ASC gene expression programs are significantly dysregulated when PAX5 remains expressed in ASC[96]. The activity of these factors coordinates programming essential for each cellular stage and have been reviewed extensively[70,75]

Although there are differences in the cell types that emerge and timing of TI and TD B cell responses, few differences in SLPC or LLPC have been identified, suggesting that the processes that lead to ASC formation are likely similar[7,97,98]. Indeed, SLPC contribute to early protective antibodies to influenza[99], and more recent work demonstrated that extrafollicular responses correlated with early neutralizing antibodies in critically ill COVID-19 patients[100]. Thus, SLPC contribute to early protection from infection and can be induced during both TD and TI antigen responses.

3.2 Cell division is an essential process during B cell differentiation

Inhibition of proliferation after stimulation of peripheral blood mononuclear cells with pokeweed mitogen prevented the generation of ASC, implicating cell division as essential for B cell differentiation to ASC[101]. The development of carboxyfluorescein succinimidyl ester (**CFSE**)[102] or CellTrace (**CT**) dyes allowed for the relationship of cell division and ASC formation to be assessed. These dyes covalently bind free amines on the surface and inside of cells and become diluted through cell proliferation. Application of these dyes has led to the appreciation that cell division is intimately linked to reprogramming events leading to ASC formation[103–107].

To investigate the relationships between cellular division and differentiation in vivo, a model system was employed where CT-labeled splenic nB cells were transferred into a B cell-deficient μMT[108] hosts followed by inoculation with LPS to stimulate differentiation[105]. Fine temporal mapping of ASC differentiation revealed that B cells began dividing rapidly 24–36 hr after LPS inoculation and differentiation required a minimum of 8 cell divisions[105] that first appeared at 60 hr[107]. Challenging host mice with a 10,000 fold range of LPS only impacted the frequency of cells that entered the response but did not change the 8 division requirement for ASC formation[107]. These results are consistent with previous reports indicating ex vivo LPS inoculation results in a quantal all-or-none stimulation paradigm[109]. Intriguingly, when CT-labeled nB were transferred to wild-type (**WT**) hosts, the majority of ASC were observed in division 8 but could also be detected as early as division 5 (Figure 3). However, when MYD88-deficient[110] mice are used to prevent host cells from responding to LPS, the requirement of 8 cell divisions before ASC formation was restored[107]. These data indicated that currently unknown cell extrinsic effects in WT mice can impact the cell division kinetics of B cell differentiation. We speculate that other LPS-responding cells present in WT hosts, such as macrophages and dendritic cells[111], can

influence B cell differentiation. Further supporting this hypothesis comes from experiments in which the TI II antigen 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll was used to stimulate B cells via BCR. Here, a similar requirement for 8 divisions was observed irrespective of whether the hosts were WT or μMT[107]. Collectively, these data support the concept that there is a conserved differentiation path to an ASC and that a minimum number of cellular divisions are required before differentiation can occur. This raises the question as to what happens during each of those divisions. As described below, chromatin, epigenetic, and transcription changes occur to allow for the engagement of metabolic pathways necessary for high levels of antibody secretion.

3.3 Chromatin accessibility changes indicate epigenetic control of B cell differentiation

Epigenetic mechanisms, such as DNA methylation and histone modifications, function to prevent or promote accessibility of DNA to TF[112]. Therefore, identifying the regions that change accessibility during B cell differentiation can reveal critical regulatory elements and factors that control B cell reprogramming to ASC. The development of the assay for transposase accessible chromatin-sequencing (**ATAC-seq**)[113,114] has revolutionized the study of accessible chromatin landscapes. Using the in vivo model system described above, ATAC-seq was applied to discrete divisions during B cell differentiation to better define the cis-regulatory changes that occur during ASC formation[106]. These included actB cells in divisions 0, 1, 3, 5, and $CD138^{+}$ASC[115,116] in division 8. Differentially accessible regions (**DAR**) were determined for all samples compared back to division 0 and revealed that progressive changes in chromatin accessibility occurred as the cells divided, with the majority of chromatin accessibility changes occurring in ASC. TF essential for ASC formation were enriched in ASC DAR including NF-κB, AP1:IRF heterodimers (AICE; high affinity IRF site)[117], and IRF:IRF homodimers (ISRE; low affinity IRF site)[118,119]. Accessibility surrounding NF-κB and AICE sites increased progressively as cells divided, whereas accessibility surrounding ISRE was unique to ASC. These data are consistent with the concentration-dependent activity of IRF4 and high levels of IRF4 expression promoting ISRE binding in ASC[90,118–120]. Furthermore, these data described a hierarchy of TF activity that is linked to cell division during B cell differentiation. Strikingly, comparing undivided cells to ASC revealed a subset of promoters that was accessible in division 0, but not expressed until ASC formation in division 8, suggesting that epigenetic mechanisms play a role in regulating this “primed” set of genes. These primed genes showed strong enrichment for the gene ontology terms “cell cycle” and “DNA replication” indicating that they might allow the cells to rapidly respond after stimulation[106]. Indeed, a subset of these primed promoters were enriched for the repressive histone modification H3K27me3, and pharmacological inhibition of the enzyme responsible for H3K27me3 deposition (EZH2) resulted in increased expression of primed genes, such as *Prdm1*. These data indicate that proper control of H3K27me3 is critical for appropriate expression of ASC-inducing genes and point towards the role of the epigenome to control the timing and magnitude of gene expression during B cell differentiation.

4. B CELL FATE PROGRAMMING AND HETEROGENEITY

B cells encountering an immune challenge respond asynchronously, with cells distributed in all cell divisions and a predictable fraction of ASC forming[104,107]. Ex vivo stimulation of B cells has led to a stochastic model of differentiation to describe the population-level immune response while accounting for heterogeneity in cell fates[121–124]. Tracking individual cell fates (whether the cells will divide, die, or differentiate) over time revealed that unrelated cells in the same division have variable outcomes[122,123]. In contrast, sibling cells in the same division often have the same fate[122,123]. These data suggest that cell division alone does not determine the fate of a cell and that each division is actually a heterogeneous mix of cells that have adopted distinct fate outcomes.

Recent advances in single cell (**sc**) RNA-seq now allow for a molecular characterization of heterogeneity within immune populations during an immune response[125,126]. This can be leveraged to gain a better understanding of cellular transitions, or “trajectories”, that may provide insight into the processes that govern B cell fate decisions. scRNA-seq was applied to the in vivo adoptive transfer system described above to better understand the transcriptional programming that drives B cell heterogeneity[107]. Trajectory analysis ordered cells by cell division leading to ASC formation and revealed a bifurcation event in actB that occurred around divisions 3–5 during B cell differentiation, with one branch leading to ASC. These data indicate that B cell fates were instructed during the earliest stages of B cell activation and that a subset of B cells are destined to become ASC. ActB that followed this branch were designated “ASC-destined”, while cells that followed the alternative branch were denoted “non-ASC”. ASC-destined actB upregulated critical gene sets for differentiation including MYC-target genes[127,128] and OXPHOS[9] compared to non-ASC actB in the same division. IRF4, in tandem with BATF at AICE motifs, enforced early reprogramming events that drove cells down the ASC-destined branch. Importantly, ASC-destined cells could be distinguished by loss of CD62L (L-selectin) and may be leveraged in future work to identify cells along each branch. In contrast, non-ASC actB exhibited transcriptional signatures indicating they were responding to inflammatory stimuli and may have a role in shaping the overall immune environment. In this branch, MHC-II expression was maintained. There are likely additional molecular determinants that precede or follow the IRF4-dependent bifurcation event. Future work is also needed to better understand if the programming of cells along each path is fixed or plastic. Ultimately, understanding the full scope of factors that control the path to an ASC has significant therapeutic potential because it may aid in our ability to control desired immune outcomes.

5. EPIGENETIC CONTROL OF ANTIBODY-SECRETING CELL DIFFERENTIATION

As described above, the transcription factor networks regulating plasma cell fate have been well established. In addition, there is also a growing body of evidence suggesting the crucial role of epigenetics in fine-tuning the magnitude of the immune response[129,130]. Epigenetics is the study of heritable mechanisms that alter gene expression without changes in the DNA sequence. Although multiple epigenetic modifications exist[112,131], here we will discuss the

consequences of deletion of some epigenetic modifiers that control DNA methylation and histone modification.

5.1 DNA methylation

The methylation of cytosine at CpG dinucleotides is a central epigenetic modification that controls gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of TF to DNA[132]. DNA methylation localized to promoters or enhancers results in gene repression. Alternatively, methylation across the gene body coupled with loss of methylation at promoters or enhancers supports gene expression[133]. DNA methylation is mediated by one of three DNA methyltransferases (**DNMT**). DNMT3A and DNMT3B promote de novo DNA methylation[134], while DNMT1 maintains DNA methylation during cell division[135]. Loss of DNA methylation can occur either via passive loss of methylation during cell division or an active process mediated by Ten-eleven translocation methylcytosine dioxygenases (TET1, TET2, TET3). TET enzymes promote DNA demethylation in a step wise manner by conversion of mCpG into 5-hydroxymethylcytosine (**5hmC**), 5-formylcytosine (**5fC**) and 5-carboxylcytosine (**5caC**), which leads to unmethylated DNA[136].

Using the same in vivo model system described above, DNA methylation and transcription were examined in discrete divisions leading to ASC formation[105]. These included cells in division 0, 1, 3, 5, and 8, with cells in division 8 further delineated based on CD138 status to separate ASC ($CD138^+$) from non-ASC ($CD138^-$)[115,116]. This study revealed that B cell differentiation is coupled to cell division and is associated with a progressive loss of DNA methylation, with only a small number of loci gaining de novo DNA methylation during ASC formation[105]. Differentiation of human B cells observed similar trends and indicated the reconfiguration of the DNA methylome was connected to the cell cycle[137]. Importantly, loss of DNA methylation occurred at many key genes essential for ASC formation, including *Prdm1*, *Irf4*, *Xbp1*, and was enriched at B cell specific enhancer regions[105,137]. TFs critical for the differentiation of ASC were enriched within demethylated enhancer regions and included IRF family members, BATF (AP-1 family member), NF-κB, and E2A (reviewed in[130]). Selective demethylation at regulatory regions critical for ASC differentiation suggests that these regions are targeted. Deletion of the DNA demethylase genes encoding TET2 or TET2 and TET3 in hematopoietic stem cells or class-switched B cells led to a reduction in ASC and antibody titers with a corresponding expansion of GC B cells due to a failure to induce the transcriptional program necessary for GC exit[138,139]. While deletion of *Tet2* and *Tet3* using inducible Cre-ER^{T2} followed by ex vivo stimulation did not alter the frequency of ASC generated, there was a significant defect in class switch recombination due to a failure to demethylate the *Aicda* (AID) locus[140]. Furthermore, inhibition of DNA methylation[105] or enhancement of TET enzymes via ascorbic acid (vitamin C)[141] led to an increase in ASC, thus providing functional evidence for the essential role of DNA demethylation in ASC formation.

Despite the fact that de novo DNA methylation occurs only at a small number of loci, those changes are necessary for restraining the commitment to the ASC fate. B cell conditional deletion of *Dnmt3a* and *Dnmt3b* led to an increase in GC B cells, as well as ASC with

an aberrant transcriptional profile. DNMT3A/B-deficient ASC upregulated genes associated with lysosome function, transcription, as well as various metabolic pathways[142]. However, despite the well-established role of DNA methylation in ASC formation, it is yet to be determined how these enzymes are recruited to the specific loci. Additionally, more work is needed to uncouple passive and active demethylation events and determine the timing in which these processes occur.

5.2 Enhancer of zeste homolog 2 (EZH2)

Enhancer of zeste homolog 2 (**EZH2**) is the catalytic component of the Polycomb Complex 2 that mediates histone 3 lysine 27 trimethylation (**H3K27me3**), which is associated with gene silencing[143–145]. The active loss of H3K27me3 is mediated by two demethylases: ubiquitously transcribed tetratricopeptide repeat, X chromosome (**UTX**) and JmjC Domain-Containing Protein 3 (**JMJD3**)[146,147]. Mutations in EZH2 are frequently found in Diffuse Large B-cell Lymphoma and Follicular lymphoma, which led to great interest in understanding its role in B cells. In fact, EZH2 was shown to function at multiple stages of B cell development and differentiation[148–150]. EZH2 is necessary for GC formation and was shown to cooperate with BCL6 and the CBX8-BCOR complex to repress genes associated with ASC fate, as well as cell cycle inhibitors, such as CDKN1A, to allow for the rapid proliferation of GC B cells[148]. Furthermore, gain of function EZH2 mutant proteins contribute to follicular lymphoma by allowing the mutant B cell to persist and expand in the light zone of the GC in a manner independent of T cell help[151]. In addition, EZH2 has been shown to directly interact with BLIMP1 to mediate gene repression[95]. Consistent with these findings, deletion of EZH2 led to an upregulation of Blimp1 target genes and failure to repress the B cell transcriptome. Phenotypically, EZH2-deficiency led to a decrease in ASC formation in vivo, and a profound proliferation defect, with EZH2-deficient B cells accumulating in cell divisions 3–5[152] (Figure 3). Furthermore, deletion of *Ezh2* led to a failure to upregulate genes associated with the UPR, glycolysis, and OXPHOS, which correlated with reduced ability of the cells to secrete antibodies and perform glycolysis, as well as lower basal respiration rate. Thus, EZH2-dependent gene repression is necessary to repress the B cell program and initiate metabolic and secretory reprogramming during ASC formation.

5.3 Lysine-specific histone demethylase 1A (LSD1)

Histone H3K4 methylation is associated with gene expression, with monomethylation (H3K4me1) associated with enhancers, dimethylation (H3K4me2) with enhancers and gene bodies, and trimethylation (H3K4me3) at RNA polymerase engaged promoters[153]. Demethylation of H3K4me1/2 (as well as H3K9me1/2) is mediated by the monoamine oxidase Lysine-specific histone demethylase 1A (**LSD1**)[154,155], which has been shown to physically interact with BLIMP1[156] and regulate ASC formation[157]. Deletion of LSD1 led to a failure to decommission nB enhancers, resulting in reduced differentiation of LSD1-deficient B cells into ASC. Furthermore, LSD1-deficiency led to downregulation of genes associated with cell cycle and proliferation, including E2F- and MYC target genes, which corresponded with impaired proliferation[157] (Figure 3). In the context of the GC, LSD1 was shown to physically interact with BCL6 to repress enhancers of BCL6 target genes, including nB enhancers that are either lost or poised in GC B cells[154]. Interestingly,

the function of LSD1 in the GC is independent of its catalytic activity, suggesting that it is part of a larger complex with other actions. Pharmacological inhibition of the catalytic domain did not impede GC formation seen in the genetic knockout[154]. In fact, the Tower domain of LSD1, which interacts with the corepressor complex CoREST, was necessary for survival of lymphoma cell lines[154]. While deletion of LSD1 leads to a reduction in GC B cells, the opposite is true for the H3K4 methyltransferase (**KMT2D**)[158]. The increase in GC B cells was attributed to increased/enhanced proliferation of KMT2D-deficient follicular B cells, as the phenotype was not observed when KMT2D was conditionally deleted in GC B cells[158]. The role of H3K4 methyltransferases in ASC formation remains to be elucidated. However, evaluating the role of the specific H3K4 methyltransferases is complicated by the existence of four partially redundant H3K4 methyltransferases[153].

Conditional deletion of *Lsd1* during B cell development resulted in a significant decrease in marginal zone B cells (**MZB**) but not on follicular B cells (**FoB**)[159]. MZB represent a small subset of splenic B cells that predominately respond to TI antigens[160,161]. Loss of MZB was in fact due to the reprogramming of cells towards the FoB compartment, as the top 200 FoB genes were upregulated in LSD1-deficient MZB. Moreover, chromatin accessibility data indicated that NF-κB like motifs were affected. Further analyses showed that the non-canonical NF-κB family member p52 interacted with LSD1, and that LSD1's activity through the BAFF signaling pathways during transitional B cell development to MZB, was diminished in LSD1-deficient cells[159].

5.4 Disruptor of telomeric silencing 1-like (DOT1L)

Disruptor of telomeric silencing 1-like (**DOT1L**) facilitates methylation of H3K79, which is associated with gene expression and has been of interest in B cells due to its role in leukemias characterized by translocation of the mixed lineage leukemia (**MLL**) gene. In the tumor setting, MLL frequently forms fusion complexes with DOT1L-interacting proteins such as AF10, AF4, and ENL. As a result, DOT1L is recruited by MLL fusion complexes to promote malignant gene expression and therefore is a target for drug development[162]. In the context of B cell differentiation, DOT1L deficiency impaired GC and ASC formation in response to TI and TD antigens in vivo[162–164]. The failure to mount a robust response following stimulation in vivo was attributed to a failure of DOT1L-deficient B cells to upregulate genes associated with cell movement and migration, resulting in aberrant cell localization within secondary lymphoid organs[164]. Interestingly, differentiation of DOT1L-deficient B cells or inhibition of DOT1L ex vivo resulted in an increase in ASC. However, while DOT1L-deficient B cells expressed key ASC genes (CD138 and BLIMP1), these ex vivo generated ASC failed to fully downregulated B220 or CD19 suggesting incomplete differentiation. Additionally, ex vivo cultured DOT1L-deficient B cells failed to upregulate the expression of BACH2, MYC, MYC-target genes, and EZH2. Consistent with downregulation of EZH2, DOT1L-deficient B cell upregulated EZH2-target genes, including *Cdkn1a*, suggesting a mechanistic link between the two epigenetic enzymes[163].

6. 3D ARCHITECTURE DURING B CELL DIFFERENTIATION

In addition to changes in transcription factor networks, DNA methylation, and histone modifications, B cell differentiation is also associated with substantial 3D reorganization of the genome[165]. The advent of chromatin confirmation capture techniques allowed for a closer examination of DNA architecture, which coupled with whole genome sequencing (Hi-C), revealed the presence of topologically associated domains (**TAD**). TAD are regions of the genome that tend to frequently interact compared with regions outside of TAD[166,167]. Although this field is young and rapidly progressing, there have been important observations regarding the stage-specific reorganization of the 3D genome and the key enhancer-promoter interactions that mediate B cell differentiation and function.

A comparison of the genomic profiles of nB, actB, or ASC revealed substantial differences in their genomic architecture. B cell differentiation leads to a significant increase in the number of DNA loops and a shift from long-range to mostly short-range interactions[168,169]. These changes were also associated with alterations in the epigenetic landscape, such as gains in the active histone modification H3K27ac at regions surrounding key genes critical for ASC, including *Prdm1*, *Atf4*, *Ell2*[169]. Fine scale mapping of chromatin architecture changes by time and cell division ex vivo revealed that the first wave of chromatin reorganization occurred just prior to the first cell division[165], a process that is likely driven by MYC[168]. Genome organization remained largely unchanged during dividing actB until ASC formation, at which a second wave of changes were observed[165]. The essential role of rewiring the 3D architecture and DNA loop formation is further exemplified by studies investigating the role of *Smc3*, the catalytic component of the cohesion complex, in GC formation and B cell differentiation[170]. Genetic deletion of *Smc3* impeded GC formation; however, haploinsufficiency of *Smc3* resulted in expanded GC and a block in ASC formation. This indicates that the final step of ASC differentiation requires major reorganization of the genome architecture compared to other differentiation stage and is sensitive to changes in SMC3 levels.

Furthermore, the study of 3D architecture revealed the presence of multi-enhancer genes and multi-gene enhancers in actB. The multi-enhancer genes were enriched for gene ontology pathways, such as MHC-II antigen processing and ER associated degradation, while the latter group included genes associated with metabolism and DNA replication[171]. The important role of such multi-gene enhancers in regulating cell fate decisions was illustrated by another study which evaluated the 3D architecture in GC B cells. This work revealed the presence of an enhancer that functioned as a locus of region control (LRC; a GC-specific enhancer cluster) by interacting with many GC signature genes. Deletion of this region using CRISPR led to abolished GC formation[172].

In addition to the global genome architecture studies mentioned above, local enhancer-promoter interactions are also important and dynamic during B cell differentiation. Analysis of the MHC-II in mice and humans has revealed the complex interplay between TF, cis-regulatory elements, and genetic variation in the regulation of antigen presentation and potentially adaptive immunity[173–175]. The entire MHC-II locus contains a set of loops that are orchestrated by the CCCTC-binding factor (**CTCF**), which is known to regulate

3D interactions[176] and the cohesion complex[177–179]. CTCF was required for maximal MHC-II gene expression[174], and in murine cells, the MHC-II locus is reorganized into distinct compartments when MHC-II gene expression is repressed in ASC[180]. In the era of genomics, a new set of super enhancers enriched for multiple transcription factor binding sites, active histone modifications, the Mediator complex, and cover large domains greater than 10kb in size have been described[181]. A super enhancer located between the *HLA-DRB1* and *HLA-DQA1* genes (termed *DR/DQ-SE*) is one of the most acetylated regions of the B cell genome and contains the highest density of genetic polymorphisms in the human genome[175]. Deletion of the *DR/DQ-SE* led to significant changes in 3D loops, enrichment of histone modifications and ultimately lower levels of MHC-II gene expression that impaired the ability of B cells to stimulate allogenic T cell proliferation in a mixed lymphocyte reaction assay[175]. These studies highlight how genetic variation within a super enhancer can impact the 3D architecture of a single locus and ultimately result in variation in MHCII gene expression across a population. This indicates that non-coding genetic variation may be as important as HLA allelic variation within a population. Further work will help delineate the factors controlling MHCII enhancer-promoter interactions and how variation impacts the adaptive immune response.

7. CONCLUDING REMARKS AND OUTLOOK

B cell differentiation to ASC is the cornerstone of humoral immunity. The transition from a quiescent nB to an ASC is a remarkable biological feat that requires substantial physiological alterations, including a shift in metabolism, ER stress pathways, and autophagy to sustain immunoglobulin production. Significant advances have been made in our understanding of the transcriptional and epigenetic events that coordinate these changes, and it has become clear that cell division and epigenetic reprogramming are intimately intertwined, with discrete divisions representing distinct stages of B differentiation[105–107].

Following initial activation in vivo to a TI antigen, nB cells begin to proliferate between 24 and 36 hr post-stimulation[107] In cell divisions 1–2, accessibility changes at promoters and distal elements are observed and transcriptional amplification is initiated for essential ASC gene sets, including Myc target genes and OXPHOS (Figure 4).[9,105]. By 48 hr post-stimulation, cells that are able to continue proliferating have progressed as far as division 5[107]. In divisions 3–5, DNA hypomethylation occurs around enhancer regions containing NF-κB and AP-1 motifs, and cells continue to upregulate / repress pathways initiated in the initial divisions[105,106]. Additionally, a critical decision point facilitated by IRF4 designates a portion of actB to follow a reprogramming path to an ASC during these cell divisions. Cells that do not follow this path contain transcriptional signatures indicating they are responding to inflammatory stimuli and may ultimately contribute to the immune response. By 60 hr, cells that continue to divide are observed in division 8, with a subset differentiating to ASC (Div 8+)[107]. During these final divisions before ASC formation is observed, massive epigenetic and transcriptional changes occur. Cell division defects following the deletion of many factors, such as LSD1 and EZH2, that contribute to actB reprogramming are often observed in these divisions[152,157] (Figure 3). We speculate that the coupling of actB reprogramming with cell division and cell-cycle promoting genes may have evolved to provide a fail-safe when reprogramming goes awry. Remarkably, substantial differences

in epigenetic and transcription reprogramming exist in actB and ASC in division 8. This includes >50,000 demethylated loci and >1,500 differentially expressed genes[105]. BLIMP1 is one factor essential for the transcriptional changes observed in division 8 ASC, as deletion of BLIMP1 had minimal impact on gene expression in earlier divisions. Additionally, low affinity IRF:IRF (ISRE) and E2A motifs are uniquely enriched in accessible regions in division 8 ASC and positively correlate with predicted target gene expression, implicating these as a major contributor to the final reprogramming events. Collectively, studying chromatin accessibility and gene expression changes in discrete divisions during B cell differentiation, combined with genetic dissection of epigenetic enzymes and transcription factors through genetic deletion, has revealed the step-wise reprogramming events that occur during B cell differentiation. These data can be exploited to understand the timing, mechanism, and scope of reprogramming by factors that impact ASC differentiation.

While considerable progress has been made in understanding the reprogramming events required during B cell differentiation and the timing in which they are initiated, additional efforts are needed to better understand the molecular determinants that influence early cell fate decisions. The advent of scRNA-seq technologies can be leveraged to be more readily determine such factors. While IRF4 is one known component that contributes to such decisions, additional factors that act upstream / downstream remain to be determined. Furthermore, it remains largely unknown whether the same reprogramming events occur following memory B cell reactivation and differentiation of ASC. Although the use of LPS is a useful model to study the events leading to ASC formation, it will be critical to understand the reprogramming events that occur following TD stimulation, which results in a myriad of long-lived cell fates (memory B cells and LLPC). Such an analysis could uncover the timing of cell fate decisions and factors that influence cell outcomes, which may be exploited to influence a desired immune outcome.

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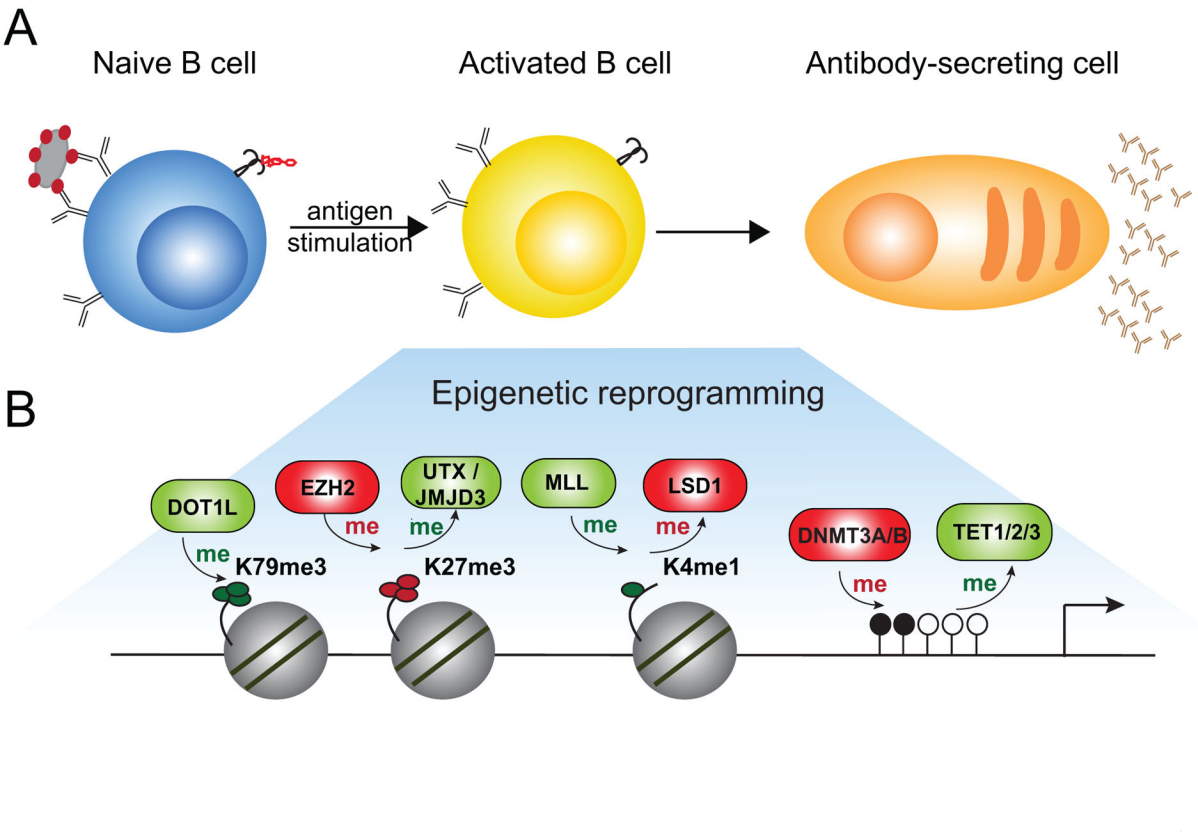

Figure 1. B cell differentiation to ASC requires epigenetic reprogramming.

(**A**) Following stimulation with T-cell independent or T-cell dependent antigen, naïve B cells become activated and differentiate into antibody-secreting cells. (**B**) The process of naïve B cell reprogramming requires substantial changes to the epigenome. Enzymes that catalyze the addition or removal of DNA methylation and various histone modification are depicted in the shaded box. Enzymes that promote gene expression are colored in green, while enzymes promoting gene repression are colored in red.

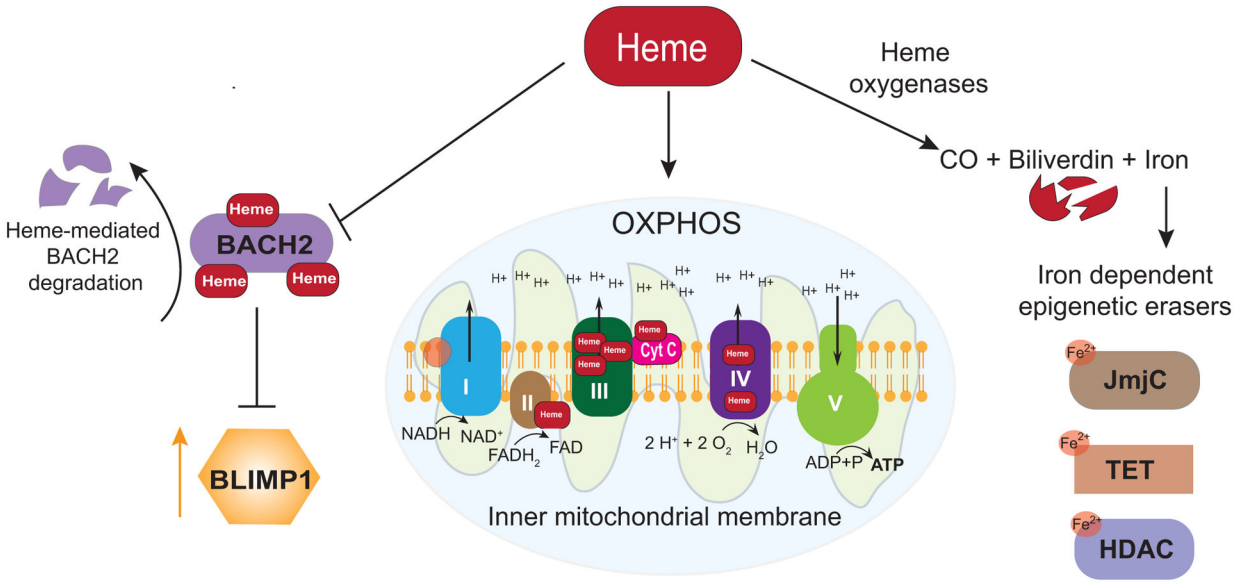

Figure 2. Heme regulates cell fate outcomes by various mechanisms.

Schematic of the processes regulated by heme that impact ASC formation is shown. Heme directly binds to BACH2 and promotes its degradation, thus relieving BLIMP1 repression (**left**). Heme is a component of number of cytochrome proteins involved in the electron transport chain of OXPHOS (**middle**). Heme oxygenase catalyzes heme degradation into biliverdin, carbon monoxide (CO), and free iron (Fe^{2+}). Iron serves as a cofactor for some enzymes that remove epigenetic modifications. Examples are depicted (**right**).

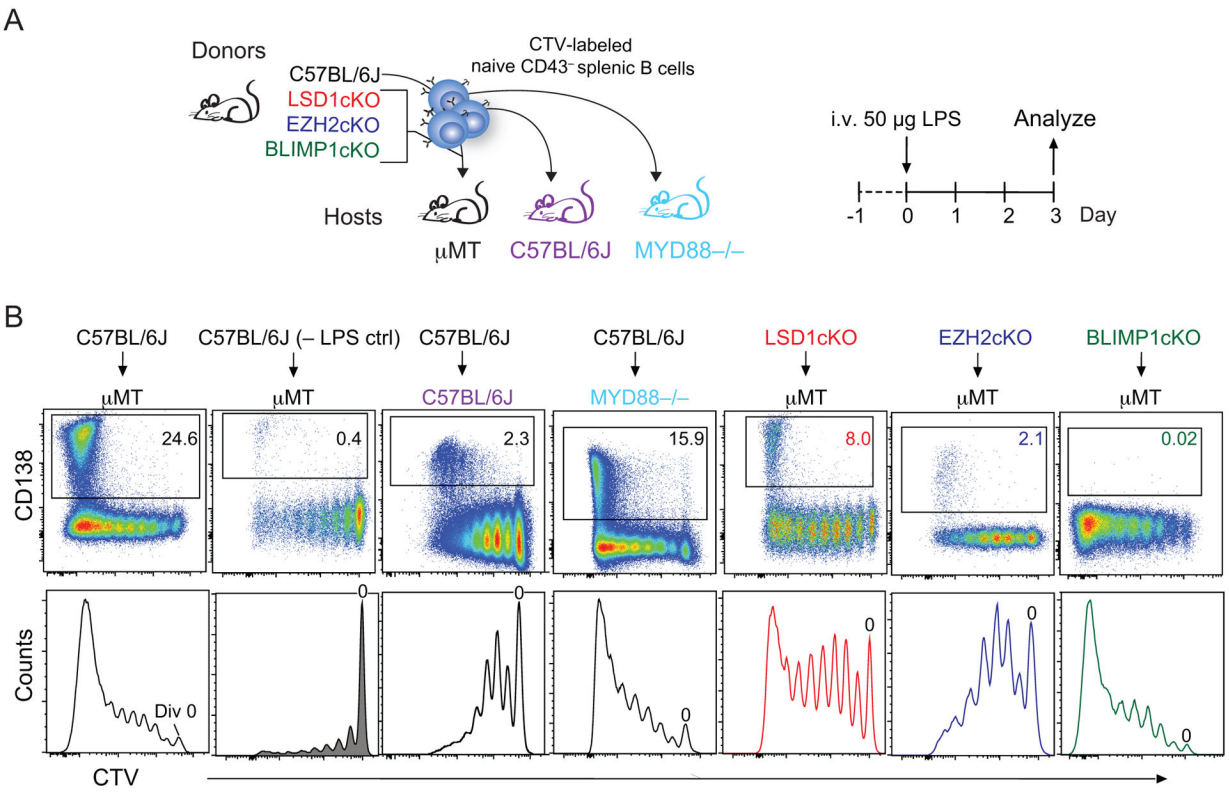

Figure 3. B cell differentiation is coupled to cell division and regulated by both epigenetic and transcription factors.

(A) Schematic of an adoptive transfer experimental design. Naïve B cells from the indicated mice were isolated, stained with CellTrace Violet (CTV), and transferred into B-cell deficient μMT, MYD88$^{-/-}$, or C57BL/6J hosts. After one day, host mice are stimulated with LPS or PBS (– LPS ctrl) and sacrificed three days later for analysis via flow cytometry. Transferred cells are recovered from host spleens. (**B)** Representative flow cytometry plots depicting CD138 expression versus CTV (top) and histograms of CTV (bottom) with division 0 indicated. The donor:host mouse combination is indicated. Representative examples were reformatted from previously performed and published experiments: C57BL/6J into μMT (from Figure 1, reference[107]), C57BL/6J into C57BL/6J or MYD88$^{-/-}$ (from Figure 2, reference[107]), LSD1cKO into μMT (from Figure 5 reference[157]), EZH2cKO into μMT (from Figure 7 reference[152]), and BLIMP1 cKO into μMT (from Figure 3 reference[107]). cKO, conditional knockout.

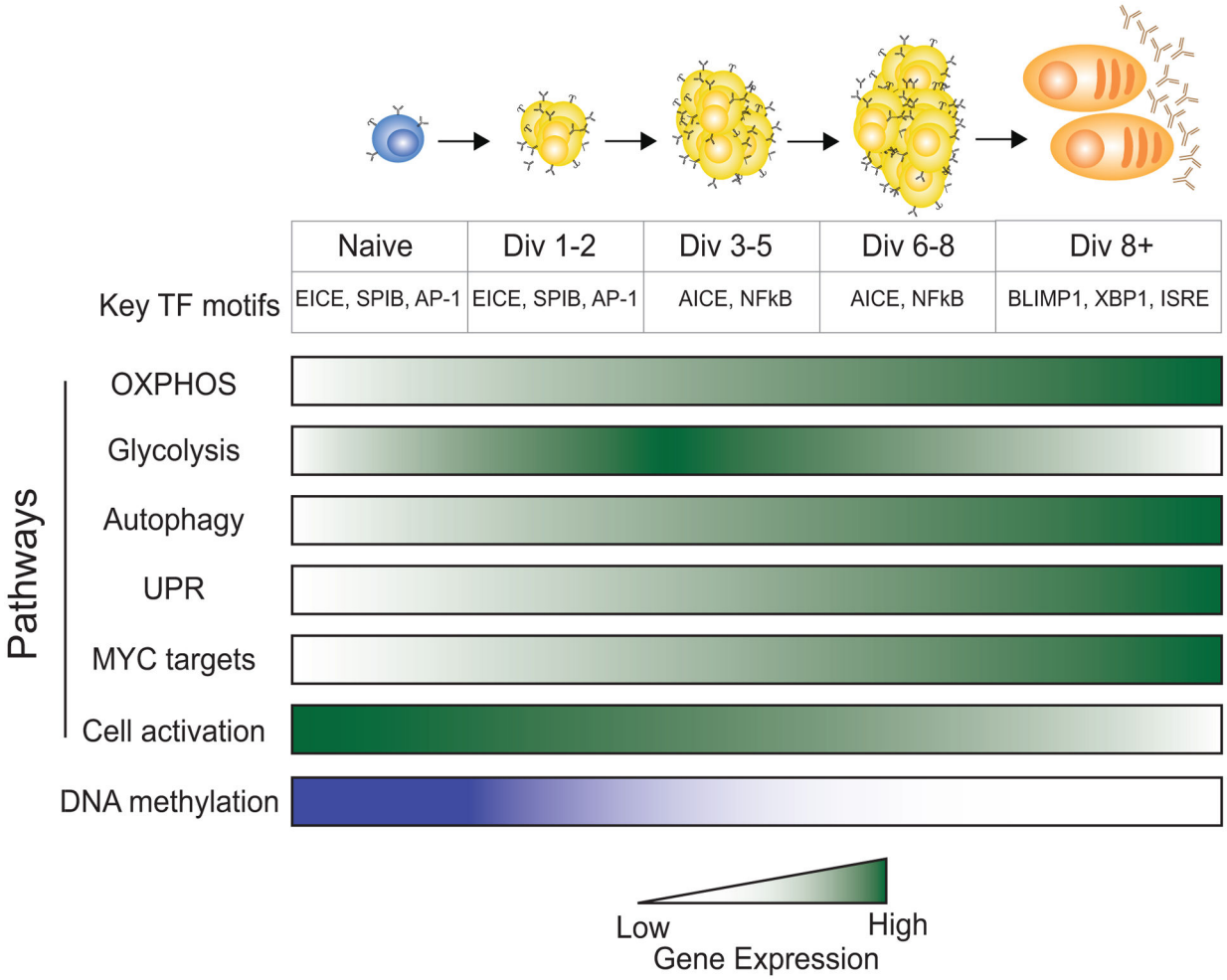

Figure 4. Cell divisions represent distinct stages during in vivo B cell differentiation in response to LPS.

Genome wide accessibility at discrete divisions revealed a hierarchy of transcription factor activity[106]. Transcription factor motifs enriched at each cell division stage are indicated. RNA-seq of cells in precise divisions revealed a progressive change of gene sets throughout differentiation[105]. Shaded boxes represent the expression level of genes in the indicated pathways that change as the cells divide during ASC differentiation. Darker color represents higher expression. DNA methylation levels are represented by color with the darker color indicating more methylation.