EPIGENETICS OF B CELLS AND ANTIBODY RESPONSES

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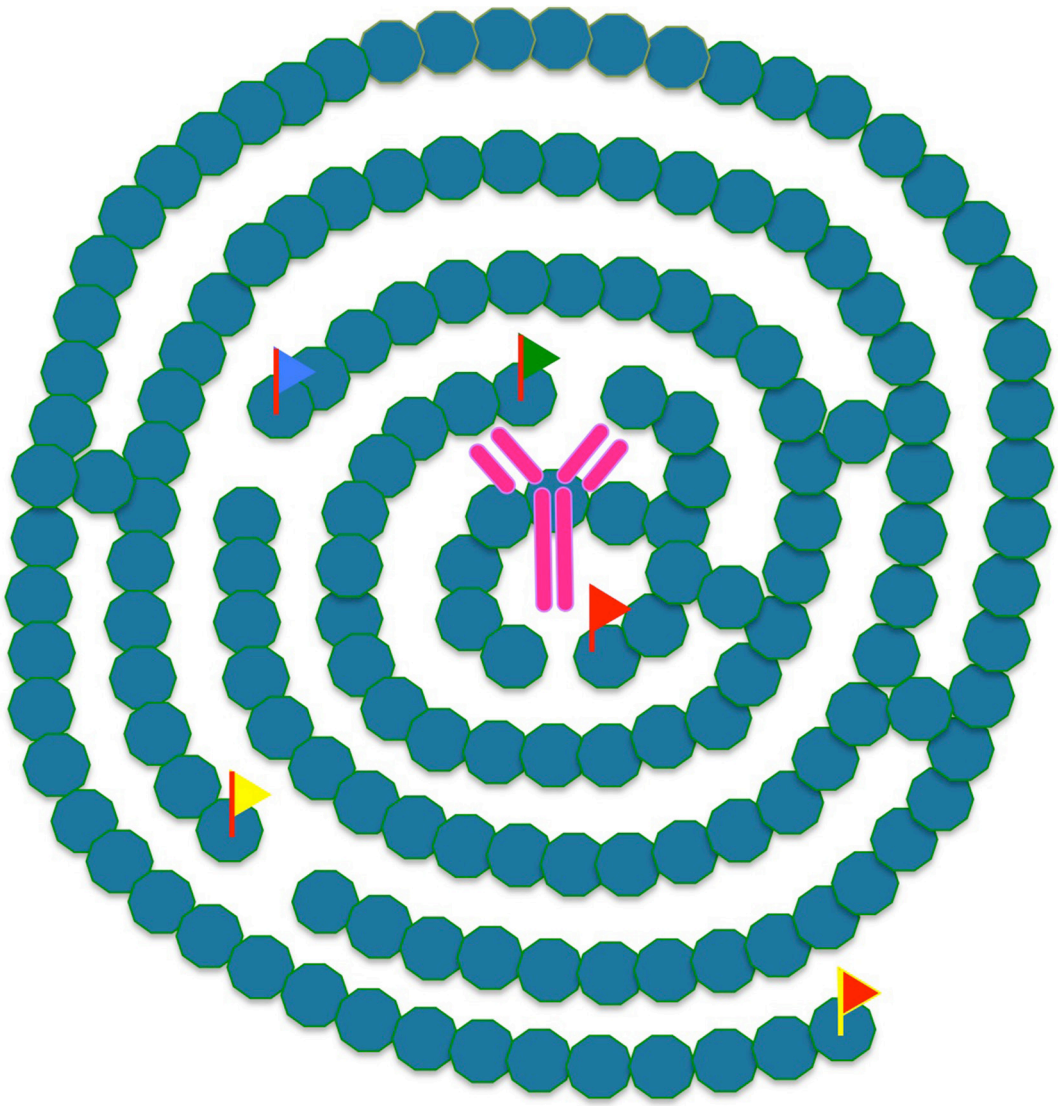

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EPIGENETICS OF B CELLS AND ANTIBODY RESPONSES

Topic Editor:
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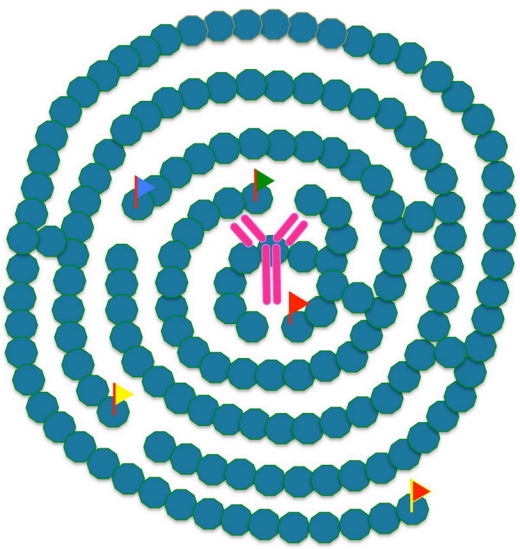

Epigenetics: road signs leading to B cell development, differentiation and antibody responses. Epigenetic marks/factors, including DNA methylation, histone posttranslational modifications and non-coding RNAs, particularly microRNAs, regulate cell functions by modulating gene activities. By acting in concert with networks of transcription factors, they modulate the activation of B cell lineage specific gene transcription programs at discrete B cell differentiation states. Epigenetic marks/factors interact with gene programs to regulate critical B cell functions, such as class switch DNA recombination, somatic hypermutation and differentiation to memory B cell or plasma cell, thereby informing the antibody response.

Image by Paolo Casali

Epigenetics is the study of changes in gene activity that are heritable but not caused by changes in the DNA sequence. By modulating gene activities, epigenetic changes regulate cell functions. They include DNA methylation, histone posttranslational modifications and gene silencing by the action of non-coding RNAs, particularly microRNAs. It is now clear that epigenetic changes regulate B cell development. By acting in concert with networks of transcription factors, they modulate the activation of B cell lineage specific gene programs and repress inappropriate gene transcription in particular B cell differentiation states.

A hallmark of B cell development in the bone marrow is the assembly of the B cell receptor (BCR) for antigen through rearrangement of immunoglobulin heavy (IgH) and light (IgL) chain V(D)J genes, as mediated by RAG1/RAG2 recombinases. Ig V(D)J rearrangement critically times the progression from pro-B cell to pre-B cell and, finally, mature B cell. Such progression is modulated by epigenetic marks, such as DNA methylation and histone posttranslational modifications, that increase chromatin accessibility and target RAG/RAG2 to V, D and J DNA. It is also dependent on the expression of multiple microRNAs. Mice deficient

in Ago2, which is essential for microRNA biogenesis and function, have B cell development blocked at the pro-B cell stage. In agreement with this, B cell specific ablation of microRNA by B cell-specific knockout of Dicer virtually blocks B cell differentiation at the pro-B to pre-B cell transition.

After mature B cells encounter antigen, changes of the epigenetic landscape are induced by the same stimuli that drive the antibody response; such epigenetic changes underpin the maturation of the antibody response itself. They instruct those B cell differentiation processes, somatic hypermutation (SHM), class switch DNA recombination (CSR) and plasma cell differentiation, that are central to the maturation of the antibody response as well as differentiation of memory B cells. Inducible histone modifications, together with DNA methylation and microRNAs modulate the transcriptome, particularly the expression of activation-induced cytidine deaminase (AID), central to SHM and CSR, and B lymphocyte-induced maturation protein-1 (Blimp-1), which is central to plasma cell differentiation.

Combinatorial histone modifications also function as histone codes in the targeting of the CSR and, possibly, the SHM machinery to the Ig locus by recruiting specific adaptors (histone code readers) that can in turn target and/or stabilize CSR/SHM factors. Epigenetic alterations in memory B cells contribute to their functionally distinction from their naive counterparts. Memory B cells inherit epigenetic information from their precursors and acquire new epigenetic marks, which make these resting B cells poised to promptly respond to antigen. The cross/feedback regulation of different epigenetic modifications/elements further increases the complexity of the B cell epigenome, which interacts with the genetic information for precise modulation of gene expression. It is increasingly evident that epigenetic dysregulation in B cells, including aberrant expression of microRNAs, can result in aberrant antibody responses to microbial pathogens, emergence of pathogenic autoantibodies or B cell neoplastic transformation. Epigenetic marks are potential targets for new therapeutics in autoimmunity and B cell malignancy.

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EDITORIAL
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Editorial: Epigenetics of B Cells and Antibody Responses

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Keywords: AID, B cell, class switch DNA recombination, epigenetics, immunoglobulin, memory B cell, plasma cell, somatic hypermutation, V(D)J recombination

The Editorial on the Research Topic

Epigenetics of B Cells and Antibody Responses

Epigenetics is the study of changes in gene activity that are heritable but not caused by changes in the DNA sequence. By modulating gene activities, epigenetic changes regulate cell functions. They include DNA methylation, histone post-translational modifications, and gene silencing by the action of non-coding RNAs, particularly microRNAs. It is now clear that epigenetic changes regulate B cell development. By acting in concert with networks of transcription factors, they modulate the activation of B cell lineage-specific gene programs and repress inappropriate gene transcription in particular B cell differentiation states (1).

The hallmark of B cell development in the bone marrow is the assembly of the B cell receptor (BCR) for antigen through rearrangement of immunoglobulin heavy (IgH) and light (IgL) chain V(D)J genes, as mediated by RAG1/RAG2 recombinases. Ig V(D)J rearrangement critically times the progression from pro-B cell to pre-B cell and, eventually, transitional and mature B cell. Such progression is modulated by epigenetic marks, such as DNA methylation and histone posttranslational modifications, which increase chromatin accessibility and target RAG1/RAG2 to V, D, and J DNA (2). It is also dependent on the expression of epigenetic factors, such as microRNAs. In mice deficient in Ago2, which is essential for microRNA biogenesis and function, B cell development is arrested at the pro-B cell stage (Danger et al.). Accordingly, B cell-specific ablation of microRNAs by B cell-specific knockout of Dicer virtually blocks B cell differentiation at the pro-B to pre-B cell transition (Danger et al.).

After mature B cells encounter antigen, changes of the epigenetic landscape are induced by the same stimuli that drive the antibody response. Such epigenetic changes underpin the maturation of the antibody response itself. They instruct those B cell differentiation processes, somatic hypermutation (SHM), class-switch DNA recombination (CSR), and plasma cell differentiation that are central to the maturation of the antibody response as well as the differentiation of memory B cells. Inducible histone modifications, together with DNA methylation and microRNAs, modulate the transcriptome, particularly the expression of activation-induced cytidine deaminase (AID), central to SHM and CSR, and B lymphocyte-induced maturation protein-1 (Blimp-1), the master transcription factor in plasma cell differentiation (1, 3–5) (Shen et al. and Zan and Casali).

Combinatorial histone modifications also function as histone codes in targeting the CSR and, possibly, the SHM machinery to the Ig locus by recruiting specific adaptors (histone code readers) that can in turn target and/or stabilize CSR/SHM factors (6). Epigenetic alterations in memory B cells contribute to their functional distinction from their naive counterparts. Memory B cells inherit epigenetic information from their precursors and acquire new epigenetic marks, which make these resting B cells poised to promptly respond to antigen. The cross/feedback regulation of different epigenetic modifications/elements further increases the complexity of the B cell epigenome, which

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interacts with the genetic information for precise modulation of gene expression. It is increasingly evident that epigenetic dysregulation in B cells, including aberrant expression of microRNAs, can result in aberrant antibody responses to microbial pathogens, emergence of pathogenic autoantibodies or B cell neoplastic transformation. Epigenetic marks are potential targets for new therapeutics in autoimmunity and B cell malignancy. The collection of experimental and review articles in this research topic addresses from different perspectives the role of epigenetic mechanisms in B cell function and antibody responses.

Zan and Casali presented an overview of the role of epigenetics in CSR, SHM, and B cell differentiation to plasma cells and memory B cells, and therefore, the regulation and maturation of the antibody response. They also highlight our current understanding of epigenetic modulations of CSR, SHM, and plasma cell differentiation by HDAC inhibitors. By performing genome-wide analysis of B cells that are induced to undergo CSR and plasma cells differentiation, Shen et al. further extended their recent discovery showing that HDAC inhibitors modulate the expression of AID and Blimp-1 by regulating the microRNAs that silence *Aicda* and *Prdm1*. This work reveals selective modulation of microRNAs and mRNAs by HDAC inhibitors.

During B cell development, only one allele of the BCR H chain and κ chain or λ chain locus undergoes V(D)J rearrangement at a time, and once productive rearrangement is sensed, rearrangement of the second allele is prevented. Levin-Klein and Bergman summarized recent advances in our understanding of the mechanisms specifying allelic exclusion of antigen receptor genes. They discussed the epigenetic processes, including asynchronous replication, nuclear localization, chromatin condensation, histone modifications, and DNA methylation, which appear to regulate the primary rearrangement of a single allele, while blocking the rearrangement of the second allele.

In immunoglobulins, juxtaposition of the three complementary-determining regions (CDRs) of the L chain and the three of the H chain creates the site that binds antigen. While the CDR1 and the CDR2 are encoded by germline sequences and the CDRL3 is largely so, the CDRH3, which provides the most critical structure basis for antigen binding, is the product of multiple VDJ rearrangements and multiple unencoded N nucleotide insertions. This makes CDRH3 the most diverse component of pre-immune Ig repertoire. The diversity of CDRH3 is constrained by natural selection of Ig D_H sequence. Restricted D gene usage leads to defective T-dependent immune responses. By utilizing elegant genetic mouse models and defined B cell response assays, Trad et al. showed that T-dependent B cell responses can be heavily influenced by the effects of natural selection of the D on CDRH3 repertoire diversity. They provided strong evidence that the antigen-independent usage of the D gene segments during VDJ recombination plays a role in modulating the affinity maturation in antigen-dependent B cell responses.

Class-switch DNA recombination is a tightly controlled multistep process involving transcription through switch regions, the DNA cytidine deaminase AID and the participation of several general DNA repair pathways. Vaidyanathan and Chaudhuri reviewed the multilevels of epigenetic regulation that orchestrate CSR, and thoroughly discussed epigenetic controls of switch region accessibility, the epigenetic regulation of AID expression and targeting, as well as of subsequent events of DNA repair.

Expression of AID is tightly regulated due to its mutagenic and recombinogenic potential. AID is known to target not only Ig genes but also non-Ig genes, thereby contributing to lymphomagenesis. In addition to the essential role of AID in CSR and SHM, a new epigenetic function of AID and its link to DNA demethylation has came to light in several developmental systems. Dominguez and Shaknovich summarized existing evidence linking deamination of unmodified and modified cytidine by AID to base-excision repair and mismatch repair machinery resulting in passive or active removal of DNA methylation mark, with the focus on B cell function. They also discuss potential contribution of AID-dependent DNA hypomethylation to B lymphomagenesis.

Transitional B cells (T1 and T2) are selected to avoid self-reactivity and to safeguard against autoimmunity, then differentiate into mature follicular (FO-I and FO-II) and marginal zone (MZ) B cells. To understand how gene expression coordinates transitional B cell tolerance and mature B cell fate, Kleiman et al. performed a comprehensive transcriptome analysis of T1, T2, FO-I, FO-II, and MZ B cell subsets by next-generation RNA-Seq. They identified several genes and gene clusters that are likely linked to specific B cell subsets and transitions of different B cell differentiation stages and their functions.

Understanding the regulation of antibody production and B cell memory formation and function is core to find new treatments for antibody-mediated autoimmune diseases, immunodeficiencies, and B cell-derived cancers. Progression from a small number of antigen-specific B cells to the production of a large number of antibody-secreting cells is tightly regulated. Although much progress has been made in revealing the transcriptional regulation of B cell differentiation that occurs during antibody responses, there are still many questions that remain to be answered. Recent work on the expression and roles of histone modifications in lymphocytes has begun to shed light on this additional level of regulation. Good-Jacobson discussed the recent advancements in understanding how antibody responses, in particular germinal centers and memory B cells, are modulated by histone modifications.

Heterochromatin protein 1γ (HP-1γ) is a highly conserved component of chromatin. It recognizes H3K9 methylation through a chromo-domain and is involved in transcription elongation. *In vitro* observations suggest a role for HP-1γ in the immune system. However, it has not been shown if and how HP-1γ contributes to immunity *in vivo*. Ha et al. uncovered a novel molecular pathway that regulates the adaptive immune response to T-dependent antigens. They demonstrated that HP-1γ positively controls the germinal center reaction and maturation of the high-affinity antibody response by modulating the size of the $CD8^+$ T_{reg} compartment.

Eukaryotic cells contain a variety of intracellular membranes, including endosomes, lysosomes, autophagosomes, Golgi network, and endoplasmic reticulum. It is becoming increasingly clear that proteins associated with these membrane compartments play important roles in B cell activation and differentiation. These processes can be regulated by microRNAs, which are important regulators of a wide range of cellular processes.

Lou et al. discussed the regulation of B cell differentiation by intracellular membrane-associated proteins and their modulation by microRNAs.

Altogether, these articles provide new and important information for the understanding of how epigenetic marks/factors contribute to B cell development and differentiation, as well as the maturation of the antibody response. We are grateful for the efforts that the authors have made to help us compile this ebook for *Frontiers in Immunology*.

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AUTHOR CONTRIBUTIONS

HZ and PC worked together to prepare this editorial.

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REVIEW
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Epigenetics of Peripheral B-Cell Differentiation and the Antibody Response

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Epigenetic modifications, such as histone post-translational modifications, DNA methylation, and alteration of gene expression by non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), are heritable changes that are independent from the genomic DNA sequence. These regulate gene activities and, therefore, cellular functions. Epigenetic modifications act in concert with transcription factors and play critical roles in B cell development and differentiation, thereby modulating antibody responses to foreign- and self-antigens. Upon antigen encounter by mature B cells in the periphery, alterations of these lymphocytes epigenetic landscape are induced by the same stimuli that drive the antibody response. Such alterations instruct B cells to undergo immunoglobulin (Ig) class switch DNA recombination (CSR) and somatic hypermutation (SHM), as well as differentiation to memory B cells or long-lived plasma cells for the immune memory. Inducible histone modifications, together with DNA methylation and miRNAs modulate the transcriptome, particularly the expression of activation-induced cytidine deaminase, which is essential for CSR and SHM, and factors central to plasma cell differentiation, such as B lymphocyte-induced maturation protein-1. These inducible B cell-intrinsic epigenetic marks guide the maturation of antibody responses. Combinatorial histone modifications also function as histone codes to target CSR and, possibly, SHM machinery to the *Ig* loci by recruiting specific adaptors that can stabilize CSR/SHM factors. In addition, lncRNAs, such as recently reported lncRNA-CSR and an lncRNA generated through transcription of the S region that form G-quadruplex structures, are also important for CSR targeting. Epigenetic dysregulation in B cells, including the aberrant expression of non-coding RNAs and alterations of histone modifications and DNA methylation, can result in aberrant antibody responses to foreign antigens, such as those on microbial pathogens, and generation of pathogenic autoantibodies, IgE in allergic reactions, as well as B cell neoplasia. Epigenetic marks would be attractive targets for new therapeutics for autoimmune and allergic diseases, and B cell malignancies.

Keywords: AID, B cell, Blimp-1, class switch DNA recombination, epigenetics, histone post-translational modification, memory B cell, microRNA, plasma cell differentiation, somatic hypermutation

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INTRODUCTION

Epigenetic changes brought about by genetic susceptibility and/or environmental exposure can modulate gene expression and alter cellular functions without altering genomic sequences (1). Epigenetic modifications and factors, such as DNA methylation, histone post-translational modifications and non-coding RNAs, such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), comprise the epigenome and interact with genetic programs to regulate immune responses. Immunoglobulin (Ig) class switch DNA recombination (CSR) and somatic hypermutation (SHM) are critical for the production of protective antibodies against microbial pathogens, IgE in allergic responses, as well as pathogenic autoantibodies in autoimmune diseases. CSR recombines S region DNA located upstream of constant heavy-chain (C_H) region exons, thereby encoding new Ig C_H regions that endow antibodies new biological effector functions (2). SHM introduces mostly point mutations in variable regions of Ig heavy and light chains, thereby providing the structural substrate for antigen-mediated selection of B cell mutants with higher affinity B cell receptors (BCRs) (3–5). CSR and SHM occur mainly in germinal center and require activation-induced cytidine deaminase (AID, encoded by *AICDA* in humans and *Aicda* in mice), which is expressed in a differentiation stage-specific fashion in B cells (2–4). Class switched and hypermutated B cells further differentiate into long-lived memory B cells, which can react quickly to a recurrent antigenic challenge, or antibody-secreting plasma cells in a fashion critically dependent on B lymphocyte-induced maturation protein 1 (Blimp-1, encoded by *PRDM1* in humans and *Prdm1* in mice) (6, 7). Epigenetic modifications and factors influence gene expression and modulate critical B cell processes, such as CSR, SHM, and differentiation to memory B cells or plasma cells, thereby informing the antibody response (4, 8–10). Epigenetic dysregulation can result in aberrant antibody responses to exogenous antigens or self-antigens, such as chromatin, histones, and double-strand DNA in lupus.

B cell development and differentiation occur in two sequential stages. The initial, antigen-independent stage occurs in the bone marrow and involves recombination activating gene (RAG)1/RAG2-dependent V-(D)-J DNA rearrangement, which produces clonally unique Ig variable regions that specifically bind antigen. This stage generates mature, immunocompetent B cells that can bind to a unique antigen. The B cells move into the periphery and complete further, antigen-independent maturation into immunocompetent naïve mature B cells. In the periphery lymphoid organs, B cell undergoes the antigen-dependent stage of development or differentiation, upon activation by antigen binding and co-stimulation (5). In this stage, resting naïve mature B cells are induced to undergo cell proliferation, CSR, as well as SHM-mediated antibody affinity maturation, and differentiate into memory B cells, or short- or long-lived antibody-secreting plasma cells (6, 7). Multiple epigenetic changes are associated with each B cell development and differentiation stage. Resting, naïve B cells undergo V_HDJ_H-Cμ transcription, which initiates at the V_H promoter and runs through the intronic Sμ region and Cμ/Cδ exon clusters. This encodes the surface BCR, which comprises *Igμ* and *Igδ* heavy chain genes. These resting B cells display low levels of overall histone acetylation and genome-wide DNA hypermethylation, therefore most regions within the Ig heavy chain (*IgH*) locus are in a closed chromatin state (11), enriched in repressive histone post-translational modifications (e.g., H3K9me3 and H3K27me3) but lacking of activated histone modifications (12, 13). In B cells, epigenetic marks, such as DNA methylation, histone modifications, and miRNAs, are induced by the same stimuli that drive the antibody response, and modulate the transcriptome, especially the expression of AID, which is essential for SHM and CSR, and factors critical for plasma cell differentiation, such as Blimp-1 (4). By functioning as histone codes, combinatorial histone modifications also play a role in the targeting of the CSR and, possibly, SHM machinery to the *Ig* loci through recruiting specific scaffold proteins that stabilize CSR/SHM factors (8). These inducible B cell-intrinsic epigenetic marks control transcription programs that distinguish individual stages of B cell differentiation and underpin the molecular changes that are necessary for antibody response.

In this review, we provide a conceptual framework to understand how epigenetic modifications/factors modulate CSR and SHM, and the generation of plasma cells and memory B cells, with focus on AID-dependent peripheral B cell differentiation into memory B cells and long-lived plasma cells (but not differentiation of naïve B cells to short-lived plasma cells). We also highlight our current understanding of epigenetic modulations of CSR, SHM, and plasma cell differentiation by histone deacetylases (HDACs) inhibitors (HDIs). Finally, we summarize recent discoveries that indicate the importance of B cell epigenetic dysregulation in autoimmunity and B cell neoplasia.

EPIGENETIC REGULATION OF AID INDUCTION

Somatic hypermutation and CSR are initiated by transcription through V(D)J and the donor/acceptor S regions that will undergo recombination, respectively, and are mediated by AID, a 198 amino acid protein, which is structurally and functionally similar to apolipoprotein B RNA-editing cytidine deaminases (APOBEC enzymes) (2, 3). AID shares a conserved catalytic domain with other members of the APOBEC family of cytosine or cytidine deaminases (3). It deaminates deoxycytidines (dCs) into deoxyuracils (dUs) yielding dU:dG mismatches. These mismatches can be repaired by an error-prone DNA repair pathway, which introduce somatic mutations, or processed by uracil DNA glycosylase (Ung), which is recruited to and stabilized on S regions by the scaffold functions of 14-3-3 adaptors, the translesion DNA synthesis (TLS) polymerase Rev1 and replication protein A (RPA), or elements of the mismatch repair (MMR) machinery, such as Msh2 and Msh6, which trigger DNA repair processes leading to introduction of point mutations (SHM) or double-strand DNA breaks (DSBs) (CSR) (2).

As a potent mutator, AID can effectively introduce mutations in not only *Ig* loci but also a variety of non-*Ig* genes, thereby causing genome instability in both B cells and non-B cells, including non-lymphoid cells, and contributing to tumorgenesis (3). Tight regulation of AID expression and function is necessary to maintain genomic stability in both B cells and non-B cells,

and to avoid damages, such as chromosomal translocations, resulting from its dysregulation (14–18). This is achieved through fine control of transcription, post-transcription and post-translation regulation, nuclear/cytoplasmic distribution, stability, and activity (3) (**Figure 1**). AID express at a very low level (mostly undetectable) in naïve B cells, it is greatly induced in B cells undergoing SHM/CSR, and repressed in memory B cells and plasma cells to preserve the specificity, affinity, and isotype of antibody and BCR. *Aicda* transcription is under the control of multiple elements, particularly Homeobox protein C4 (HoxC4) (**Figure 1**). HoxC4 is a highly conserved helix-loop-helix homeodomain-containing transcription factor. As we have shown, HoxC4 directly binds to the *Aicda* promoter through an evolutionarily conserved 5′-ATTT-3′ site embedded within a conserved binding site for POU domain-containing transcription factors Oct1 and Oct2 (5′-ATTTGAAT-3′) (19). Sp1/Sp3 and NF-κB also bind the same promoter core and synergize with HoxC4 for *Aicda* induction (19, 20).

Regulation of AID Expression by DNA Modifications and Histone Modifications

The expression of *Aicda* is modulated by changes of *Aicda* epigenetic status. Suppression of *Aicda* expression in naïve B cells is mediated by promoter DNA hypermethylation (21). In naive B cells, in which AID is not expressed, histone H3 acetylation occurs in the *Aicda* gene at low levels comparable to the overall H3 acetylation in the genome and neighboring genes, such as *Mfap5*, which is not

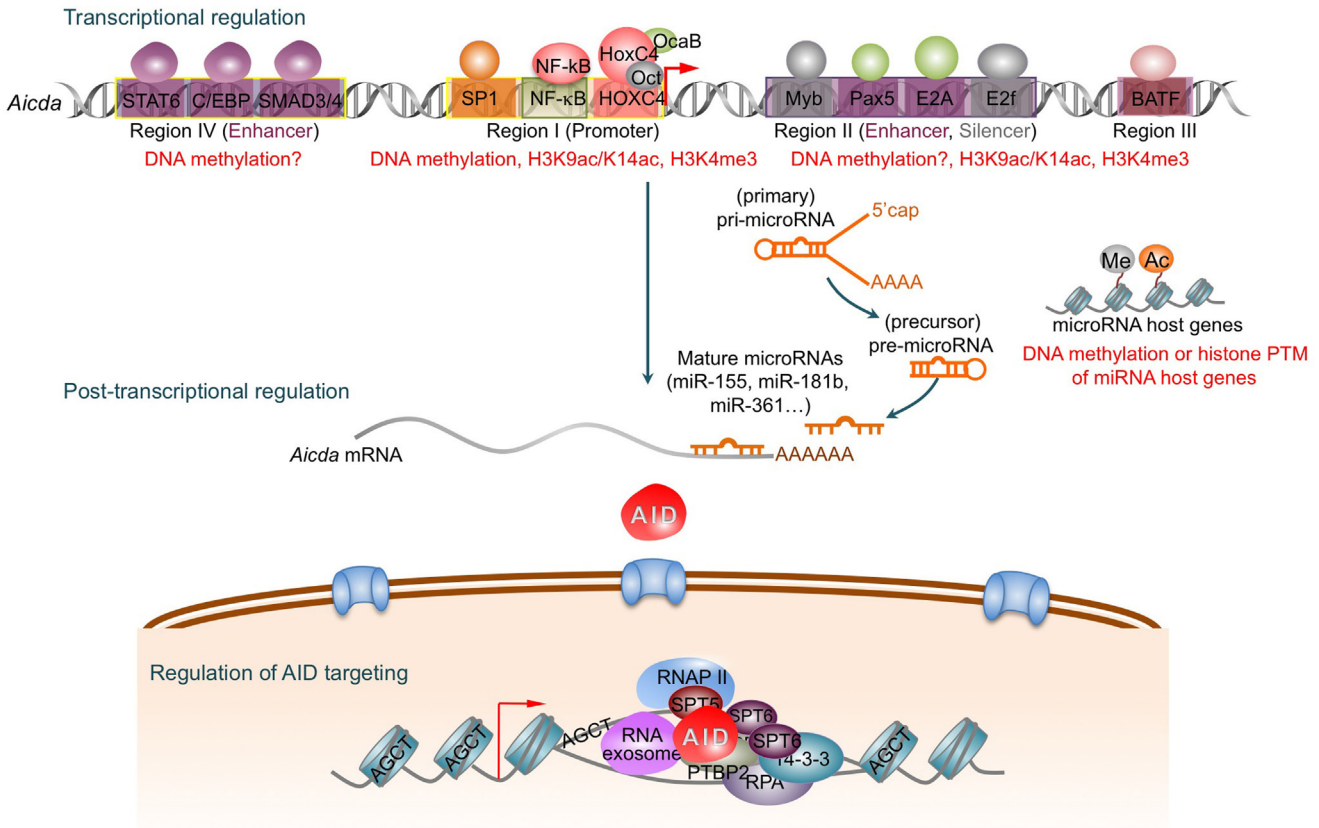

FIGURE 1 | AID is tightly regulated at the transcriptional, post-transcriptional, and post-translational levels. Four distinct DNA regions (regions I–IV) of the AID gene (*Aicda*) locus possess binding sites for multiple transcription factors to regulate *Aicda* expression. Region I functions as promoters containing the binding sites for HoxC4/Oct and NF-κB/Sp1/Sp3, which can be induced by activating the *Aicda* promoter. In resting naive and memory B cells, and non-B cells, silencer elements in region II bind the repressor proteins E2f and c-Myb to counter the activity of the transcriptional activators. Stimulation of B cells with the primary inducing stimuli and cytokines that promote CSR induce activation signals through region IV enhancer in collaboration with the intronic enhancer in region II can overcome the effect of the region II silencer. After transcription, the *Aicda* mRNA can be negatively regulated by miR-155, miR-181b, and miR-361, which specifically bind to the conserved target sites on the 3′ UTR of *Aicda* mRNA. Nuclear AID is either degraded or exported back to the cytoplasm. Only a small proportion of AID molecules are targeted onto DNA at *Ig* or non-*Ig* loci by its co-factors. AID preferentially deaminates single-strand DNA, which emerges from transcription by RNA Pol II and depends on histone modifications in the transcribed locus. AID can be recruited to the open DNA before or during transcription. In CSR, AID recruitment to S regions occurs with interaction of RNA Pol II and AID-interacting factors, such as Spt5, Spt6, PTBP2, RNA exosome, lncRNAs, and 14-3-3 adaptor proteins, which would form a macromolecular complex. AID is enriched and stabilized on the targeted DNA by 14-3-3 adaptor proteins, which access the same S regions as the transcription machinery owing to their open chromatin state. These 14-3-3 adaptors are recruited and/or stabilized through interactions with 5′-AGCT-3′ repeats and possibly by H3K9acS10ph. The RNA exosome also interacts with AID and allows AID to deaminate both the transcribed and the non-transcribed DNA strand in the S regions undergoing transcription. AID deaminate dCs into dUs to yield dU:dG mismatches. Resolution of these lesions can lead to different physiological or pathological outcomes.

significantly expressed in B cells (22). Upon activation of B cells, DNA of the *Aicda* gene is demethylated and the locus becomes enriched in H3K4me3 and H3K9ac/K14ac. B cell activation by LPS plus IL-4, which induces *Aicda* transcription, greatly increases H3 acetylation at the *Aicda* locus, particularly in the *Aicda* regulatory regions (22). These epigenetic modifications, together with induction of nuclear factor NF-κB, homeobox protein HoxC4 and other transcription factors, activate *Aicda* transcription. Transcription elongation depends on the H3K36me3 posttranslational modicification, an intragenic mark of gene activation in the *Aicda* gene body, as suggested by AID down-regulation following depletion of the H3K36me3 methyltransferase Setd2 (3, 23). Post-CSR/SHM down-regulation of *Aicda* transcription probably results from remethylation of the *Aicda* DNA.

Regulation of AID Expression by miRNA

In addition to DNA methylation of the *Aicda* promoter and histone acetylation of the *Aicda* locus, selected miRNAs provide a more important mechanism of modulation of AID expression. miRNAs are small (~21–23 nt), evolutionarily conserved noncoding RNAs derived from much larger primary transcripts encoded by their "host genes." miRNAs bind to complementary sequences within the 3′ untranslated region (3′ UTR) of their target mRNAs and negatively regulate protein expression at the post-transcriptional level. miRNA can recognize its target mRNA through the short (as few as 6–8 nt) "seed sequence" at the 5′ end of the miRNA. miRNA repress gene expression by accelerating mRNA degradation and inhibiting mRNA translation. A given miRNA can potentially target different mRNAs, and a given mRNA can be targeted by multiple miRNAs. The mammalian genome encodes hundreds of miRNAs that collectively impact the expression of about one-third of all genes. miRNAs are transcribed from intergenic genomic DNA by RNA polymerase (Pol) II or Pol III as primary transcripts (pri-miRNAs) that are then cleaved by the nuclear ribonuclease Drosha to generate an about 70 bp long characteristic stem-loop structure, known as a pre-miRNA, and exported to the cytoplasm (24). The pre-miRNA is further processed by cytoplasmic enzyme Dicer into mature miRNA (24), which forms complexes with ribonucleoproteins (RNPs) and expresses regulatory effects (25, 26).

microRNAs control various biological processes by fine-tuning gene expression at the post-transcriptional level. They can modulate a wide range of physiological and pathological processes by regulating cellular function at every aspect, from proliferation and apoptosis to differentiation. miRNAs are present in all human and mouse cells, in which they each modulate the expression of a few to hundreds of target genes. miRNAs likely play important roles in B cell development and peripheral differentiation, as well as T cell stage-specific differentiation and autoimmunity. Naïve B cells, germinal center B cells, memory B cells, and plasma cells show distinct miRNA expression profiles (27, 28). Deletion of Dicer, which is critical for miRNA maturation, in activated B cells resulted in defective generation of germinal center B cells, memory B cells, and plasma cells (29). Indeed, Dicer-deficient B cells showed impaired biogenesis of many miRNAs, including miR-155 and miR-125b, which regulate expression of genes that modulate B cell germinal center reaction and plasma cell differentiation (30, 31). A single miRNA, such as miR-155, can target multiple genes, including *Myd88*, *Pu.1*, and *Aicda*, and regulate sequential stages of B cell differentiation (30, 32–34). By contrast, multiple miRNAs, such as miR-15a and miR-16, can cooperatively repress one critical gene in germinal center B cells, *Bcl2* (27).

miR-155, miR-181b, and miR-361 modulate AID expression by binding to the evolutionarily conserved target sites in the 3′ UTR of *AICDA/Aicda* mRNA, thereby reducing both *AICDA/Aicda* mRNA and AID protein levels (33–37). These miRNAs could suppress AID expression in naïve B cells and in B cells that completed SHM/CSR. miR-155 is the most abundant miRNA that has been shown to silence AID expression. miR-155 is processed from an RNA sequence encoded by miR-155 host gene (*miR155HG*). This was originally identified as a gene transcriptionally activated by promoter insertion at a common retroviral integration site in B-cell lymphomas (*Bic*, B-cell integration cluster). *Bic* RNA is a spliced and polyadenylated but non-protein-coding RNA that accumulates in lymphoma cells (32) and is induced along with AID in B cells activated by CSR-inducing stimuli (33, 34). The sequences of pre-miRNA-155 and mature miR-155 are highly conserved across more than 22 different organisms, including mammals, amphibians, birds, reptiles, sea squirts, and sea lampreys (38).

miR-155 and miR-361 are directly repressed by BCL-6, a transcriptional repressor required for germinal center formation. miR-361 is embedded in the *CHM* gene, which encodes a subunit of a Rab geranylgeranyl transferase and is known for its genetic inactivation in choroideremia (37). BCL-6 display a coordinated activity in sustaining high levels of AID expression in germinal center B cells undergoing CSR and SHM. By direct repressing miR-155 and miR-361, BCL-6 upregulates the expression of the target genes of these miRNAs, including *AICDA* and other elements involved in the maintenance of the germinal center B cell centroblast phenotype (37). The specific effect of miR-155 in the regulation of AID expression was demonstrated by the findings that disruption of the miR-155 binding site in the 3′ UTR of *Aicda* mRNA in B cells led to an increase in *Aicda* mRNA and AID protein by increasing the half-life of *Aicda* mRNA, resulting in increased CSR and *c-Myc/IgH* translocations (33, 34). Nevertheless, the role of miR-155 in regulating B cell function and antibody response is much more than modulating the expression of AID. miR-155 is expressed at a high level in germinal center B cells and plays an important role in germinal center formation and subsequent antibody response following antigen challenge (30, 32, 39). miR-155 deficiency in B cells resulted in reduced extra follicular and germinal center responses, decreased numbers of $IgG1^+$ plasma cells and memory B cells, and failed production of high-affinity IgG1 antibodies (30). In B6/$Fas^{lpr/lpr}$ mice, deficiency of miR-155 results in a reduced autoantibody production and autoimmunity (40). This is likely stemmed from dysregulation of a variety of genes in multiple immune cells, including derepressed expression of SHIP-1 in B cells, which lead to mitigation of B-cell activation, proliferation and autoantibody production (40).

The 3′ UTR of *Aicda* mRNA contains multiple putative binding sites for miR-181b, which is predominantly expressed in lymphoid cells (10, 41). miR-181b is expressed at the highest levels in resting B cells and is downregulated upon B cell activation

by CSR-inducing stimuli (34, 42). Expression of miR-181b in B cells leads to down-regulation of AID at both the transcript and protein levels. It has been suggested that miR-181b and miR-155 have non-overlapping functions in controlling AID expression. miR-181b may inhibit premature AID activity but allows proper AID transcriptional activation at early time points, while miR-155 could narrow AID function at a later stage of activation (34). By controlling AID expression, miR-155 and miR-181b protect resting B cells and non-B cells from AID-mediated mutagenesis. Accordingly, in Burkitt's lymphoma B cells, deficiency of miR-155 expression is associated with high levels of somatic mutations and inter-chromosomal translocations (43).

EPIGENETIC REGULATION OF AID TARGETING IN CSR AND SHM

One fundamental question for B cell biology remains to be answered is how CSR and SHM machineries are targeted to the *Ig* locus. For CSR to take place, the *IgH* genes are subjected to transcriptional activation, RNA splicing, AID-mediated cytidine deamination, as well as DNA cleavage, repair, and recombination. Each of these events is likely associated with, and possibly regulated by specific changes in chromatin structure. Histone modifications in S and V(D)J regions are critical for targeting of the CSR and SHM machinery, respectively (**Table 1**). Chromatin structure that impacts on and likely regulate most aspects of gene expression, also contributes to the regulation of CSR and SHM. In B cells poised to undergo CSR, the *IgH* genes are in an "accessible" chromatin conformation before CSR (44). Upon induction of germ-line transcription, histones H3 and H4 have been shown to be acetylated at the I_H exon promoters and S regions (45, 46).

Epigenetic Targeting of the CSR Machinery

Histone Modifications in CSR Targeting

Histone post-translational modifications are important for targeting of the CSR machinery to the upstream donor and the downstream acceptor S regions that are involved in CSR (**Table 1**). The significant levels of germline Iμ-Sμ-Cμ transcription and activating histone marks, such as H3K4me3, H3K36me3, H2BK5ac, H3K9ac/K14ac, H3K27ac, and H4K8ac, are observed in the donor Sμ region even in resting naïve B cells, suggesting that Sμ is in a constitutively open state and poised for switch recombination (8). CSR induction requires both primary and secondary (CSR-inducing) stimuli. Primary stimuli, such as engagement of B cell CD40 by CD154 expressed on activated T cells, and T-independent dual toll-like receptor (TLR) and BCR engagement, induce B cell activation, proliferation, and differentiation. In conjunction with primary stimuli, secondary stimuli, which comprise cytokines (e.g., IL-4, TGF-β, and IFN-γ), direct class switching to IgG, IgA, or IgE by selecting the acceptor S region, through activation of S region histone modifications and inducing specific germline I_H-S-C_H transcription (46–49). Primary stimuli induce histone-modifying enzymes and trigger chromatin decondensation in downstream S regions by removing repressive H3K9me3 and H3K27me3. These allow histone-modifying enzymes to ride on the RNA Pol II to reach S regions during germline I_H-S-C_H transcription elongation (46, 47, 50–52). Upon RNA Pol II stalling, caused by complex secondary DNA structures, such as cruciform-like structures and R-loop, in S regions (2, 53), histone-modifying enzymes catalyze histone modifications in these regions. DNA transcription together with modified histones opens the chromatin in S regions, thereby allowing for access of CSR factors. The role of histone-modifying enzymes in CSR is further emphasized by the reduced S region histone modifications and CSR in B cells knockdown H3K4 methyltransferase Set1 (48), or H3K9 acetyltransferases Pcaf and Gcn5 (8).

In the donor and acceptor S regions, activating histone modifications, such as H3K9ac and H3K4me3, are enriched at levels much higher than those in the associated I_H promoter and C_H regions (46, 47). These histone marks are read by CSR factors, including 14-3-3 adaptors, which, such as AID and histone-modifying enzymes, are induced by primary CSR stimuli (54). 14-3-3 adaptors directly interact with AID and target it to the upstream and downstream S regions that undergo recombination (2), thereby transducing the epigenetic code. 14-3-3 adaptors specifically bind to the combinatorial histone H3K9acS10ph modification in S regions and 5′-AGCT-3′ (2, 54) repeats. These are characteristic motifs of all *IgH* locus S regions, but not I_H promoters, C_H regions or other genome areas (54). Due to their high affinity for 5′-AGCT-3′ repeats, 14-3-3 can potentially bind all S regions. Nevertheless, these adaptors are recruited only to the S regions that undergo recombination (54). This is due to the open chromatin state of such regions as well as specific binding of 14-3-3 to H3K9acS10ph (8). The specificity of 14-3-3 adaptors for H3K9acS10ph and 5′-AGCT-3′ repeats are evocative of RAG1/RAG2 complex specificity for H3K4me3 and V, D and J gene recombination signal sequences (RSSs) (55).

H3K4 methylation, particularly H3K4me3, plays a critical role in AID-mediated DNA cleavage in S regions during CSR (48). The formation of H3K4me3 at AID target loci is dependent on the histone chaperone complex, facilitates chromatin transcription (FACT), a chromatin-modifying complex during RNA processing (48, 56). We have shown that specific H3K4 methyltransferases and H3K9 acetyltransferases can be induced by TLR or CD40 signaling and catalyze histone H3K4me3 and H3K9ac/K14ac modifications. These are decorated S regions, regardless of whether they are targets of CSR (8). Conversely, the combinatorial histone H3K9acS10ph modification specifically marks the S regions set to recombine and directly recruits 14-3-3 adaptors for AID stabilization (8). 14-3-3 adaptors, which possess no enzymatic activity, function as histone code readers to recruit/stabilize downstream effector molecules, which *per se* cannot read histone codes, consistent with the "histone code hypothesis" (57, 58). Inhibition of the enzymatic activity of Gcn5/Pcaf histone acetyltransferases leads to decreased H3K9acS10ph, 14-3-3 recruitment and AID stabilization in S regions, and CSR.

H3K9me3 is also present, although at a relatively low level, in Sμ but not downstream S regions. In the Sμ region, H3K9me3 recruits the KAP1-HP1γ complex to stabilize AID (13). Accordingly, CSR to IgA can be impaired by deletion of the H3K9 methyltransferase Suv39 (59). Histone modification readers that function as scaffolds, such as 14-3-3 adaptors, translesion DNA

synthesis polymerase Rev1 (60) and RPA (61), act as core for the assembly of macromolecular complexes on S region DNA, to stabilize AID and/or Ung for generation of DNA lesions. Histone modifications are also recognized by DNA repair factors, such as p53-binding protein 1 (53BP1), which may functions as a scaffold to recruit/stabilize additional DNA repair factors for CSR. Abrogation of histone methyltransferase MMSET expression impairs H4K20me2 enrichment and 53BP1 recruitment in S regions, and results in reduced CSR (62).

Suppressor of Ty6 (Spt6), a RNA Pol II-interacting histone H3-H4 chaperone, also plays a role in the regulation of H3K4me3 for SHM and CSR (23). Depletion of Spt6 impaired H3K4me3 and AID-mediated DSBs in the S regions in CH12 B cells, which can otherwise be induced to express AID and CSR to IgA (23). In addition, knockdown Spt6 in human Burkitt's lymphoma BL2 cells overexpressing mutant AID (JP8Bdel) that lacks C-terminal 16 aa residues, which are critical for CSR but not to SHM (63), abolished SHM and H3K4me3 in *Ig* V_H region and non-*Ig* AID target genes (23). Thus, activating histone modifications are induced in S regions and create an open chromatin state, which allows for the access of the CSR machinery. Epigenetic specification of CSR targeting entails reading of histone codes by scaffold proteins, which orchestrate the assembly of macromolecular complexes in the sequential DNA lesion and repair stages.

Long Non-Coding RNA and CSR Targeting

Long non-coding RNAs are evolutionarily conserved non-coding RNA molecules that are longer than 200 nt and located within the intergenic loci or regions overlapping antisense transcripts of protein coding genes (64–66). Their expression can be tissue- and cell-type specific. lncRNAs are involved in numerous cellular functions, such as transcriptional regulation, RNA processing, RNA modification and epigenetic silencing. lncRNAs have been recently shown to play an important role in the targeting of the CSR machinery (**Table 1**). They target AID to divergently transcribed loci in B cells (67). In B cells undergoing CSR, the RNA exosome, a cellular

TABLE 1 | Epigenetic marks/factors, and their functions in CSR and SHM.

Target(s)	Epigenetic mark(s)	(Putative) functions	Modulate		Reference
			CSR	SHM	
microRNAs					
Aicda	miR-93 miR-155 miR-181b miR-361	Decrease expression of AID	+	+	(9, 33–37)
lncRNAs					
S regions	Germline I_H-S-C_H transcripts	Increase S region accessibility	+	–	(2)
S regions	Intronic switch RNA	Recruit AID to S region	+	–	(69)
S regions	Antisense S region transcripts	Increase S region accessibility	+	–	(173)
V_HDJ_H	Antisense V_HDJ_H transcripts	Increase V_HDJ_H region accessibility	–	+	(173)
IgH 3′ RR super-enhancer	lncRNA-CSR	Regulate IgH 3′ regulatory region super-enhancer function	+	–	(70)
IgH and other AID target regions	xTSS-RNAs	Recruit AID to ssDNA-forming site	+	+	(67)
DNA METHYLATION					
V(D)J	DNA hypomethylation	Increases V(D)J region accessibility	–	+	(84)
Igh 3′-LCR	DNA hypomethylation	Mediates germline V_HDJ_H and I_H-S-C_H transcription	+	+	(174)
HISTONE MODIFICATIONS					
V(D)J	H3K4me2/3, H3K9ac/K14ac, H4K8ac	Increase V(D)J region accessibility and transcription	–	+	(23, 72, 85)
iEμ	H3K4me3, H3K9ac/K14ac	Activate iEμ and enhance germline VDJ transcription and I_H-S-C_H transcription	+	+	(46, 47)
Igh 3′-LCR	H3K4me1/2 H3K9ac, H3K27ac, H4K8ac, H2BK5ac	Mediate VDJ and germline transcription	+	+	(175) (47)
S region(s)	H3K27me3	Decreases S region(s) accessibility	+	–	(12)
	H3K4me3, H3K9ac/K14ac, H3K27ac, H4K8ac	Increase S region(s) accessibility	+	–	(12, 13, 45–49, 61, 176)
	H3K9me3	Recruits the HP1γ-KAP1 complex and AID to Sμ region	+	–	(13)
	H3K9acS10ph	Recruits 14-3-3 adaptors and AID to S region(s)	+	–	(8)
	H4K20me2	Recruits 53BP1 to S region(s) in the DNA repair stage	+	–	(62)
Aicda	H3K4me3, H3K9ac/K14ac, H3K36me3	Increase transcription of *Aicda*	+	+	(22, 47)

Repressive histone methylation: H3K9me3, H3K27me3.

Activating and recruiting histone modifications include histone acetylation: H3K9ac, H3K14ac, H3K27ac, H4K8ac, H2BK5ac, and H3K9ac; histone methylations: H3K4me2/3, H4K20me2, and H3K36me3; combinatorial histone H3K9acS10ph modification; histone phosphorylation: H2BS14ph; histone ubiquitination: H2AK119ub and H2BK120ub.

RNA-processing/degradation complex is required for optimal CSR (68). The RNA exosome associates with AID, accumulates on S regions in an AID-dependent fashion. Both the cellular RNA exosome complex and a recombinant RNA exosome core complex inform robust transcription-dependent DNA deamination by AID in both strands of transcribed SHM substrates *in vitro*. In B cells, deficiency of *Exosc3* or *Exosc10*, the essential subunits of the RNA exosome complex, impairs CSR and SHM (67, 69). Many novel RNA exosome substrate lncRNAs have been identified by transcriptome analysis of *Exosc3*- or *Exosc10*-deficient B cells. RNA exosome-regulated, antisense-transcribed regions accumulate single-strand DNA structures containing RNA-DNA hybrids and recruit AID in B cell. RNA exosome regulation of lncRNA has been suggested to recruit AID to single-strand DNA-forming sites of antisense and divergent transcription in the B cell genome (67). In addition, a recent study has shown that an lncRNA generated by S region transcription followed by lariat debranching can fold into G-quadruplex structures, which can be directly bound by AID, thereby targeting of AID to S region DNA (70).

Epigenetic Targeting of the SHM Machinery

Activation-induced cytidine deaminase initiates SHM by deaminating cytosine residues in Ig V(D)J genes. It also introduces DNA damages, including point-mutations, in non-*Ig* loci at a lower frequency. How AID is recruited to the target sites is not fully understood. AID has been suggested to target a specific microenvironment rather than a defined set of genes (71). The SHM machinery is targeted to the V(D)J region through unique targeting sequences, transcription, and possibly, DNA demethylation and histone modifications (**Table 1**) (72). Like CSR, SHM requires not only the expression of AID, but also transcription in the target regions. Alterations in chromatin structure at *IgH* may also play an important role in promoting and/or stabilizing AID targeting (73). AID targets a distinct set of hotspots, which are concentrated in genes that are highly transcribed but frequently stalled genes (74). AID associates with RNA Pol II (75). RNA Pol II transcription, as well as several chromatin alterations could facilitate the access of AID to *Ig* loci and give use to single-stranded DNA, a preferential substrate for AID deamination. However, transcription alone is insufficient to recruit AID activity (74). AID targets are predominantly grouped within topological complex, highly transcribed superenhancers and regulatory clusters, which are enriched in chromatin modifications associated with active enhancers (such as H3K27Ac), they are also and marks of active transcription (such as H3K36me3), indicating that these features are universal mediators of AID recruitment (71, 74, 76). In both human and mouse B cells, there is a strong overlap between hypermutated genes and superenhancer domains (71).

The phosphorylated histone H2B (H2BSer14P) correlates tightly with SHM and CSR. In Ig V(D)J and S regions, H2B phosphorylation requires AID and may be mediated by the histone kinase Mst1 (72, 77). It has been suggested that SHM and CSR trigger distinct DNA damage responses and identify a novel histone modification pattern for SHM consisting of H2B (Ser14P) in the absence of γH2AX (72, 77). The non-Ig AID targets share important characteristics with Ig genes, namely, repetitive sequences that can form non-B DNA structures upon efficient transcription, and the accumulation of chromatin H3K4me3 histone marks (78).

The FACT complex may also promote SHM (56). FACT, a histone chaperone-type elongation factor, was originally discovered for its biochemical activity to promote transcription elongation of RNAPII on the nucleosomal DNA template (79). It has been suggested to remove nucleosomal histones and deposit them at the RNAPII of transcription site, and this allows the RNA polymerase to be proceed beyond the nucleosomes (80). FACT is important for inducing H3K4me3, which can be recognized by a protein complex with DNA cleaving activity and accumulates at SHM-targeted genomic regions (48). Furthermore, FACT and histone variant H3.3, a hallmark of replication-independent histone turnover, are enriched at the heavy and light chain V(D)J regions, the light chain Jκ5 region and the Sμ region 5′ flanking sequence (48). The importance of the chromatin histone-exchanging dynamics in SHM target regions, especially Ig genes, is emphasized by high abundant FACT and H3.3 deposition in the most efficient targets of SHM (56, 81). Histone post-translational modifications would also mediate recruitment of DNA repair factors, such as error-prone TLS DNA polymerases, in SHM at the DNA repair stage. H2AK119 ubiquitination (ub) and H2BK120ub are enriched in V_HDJ_H but not C_H regions and colocalize with DNA Pol η, an important TLS polymerase for SHM, in AID nuclear foci (82). Pol η is likely recruited to those loci by H2AK119ub and H2BK120ub, as well as by monoubiquitinated PCNA scaffold, through its ubiquitin-binding domain (83). As mentioned before, AID-dependent histone H2BSer14P mark in the V(D)J region (72) may also contributes to recruitment of DNA repair factors. The role of DNA hypomethylation in SHM has been suggested by the finding that only the hypomethylated allele is hypermutated in B cells carrying two nearly identical pre-rearranged Igκ transgenic alleles, even though transcription of both alleles are comparable (84).

It is possible that DNA demethylation facilitates SHM targeting by promoting histone modifications H3K4me3, H3K9ac/K14ac and H4K8ac, which are enriched in the V(D)J region and associated with an open chromatin state (23, 72, 85). Both H3K4me3 and H4K8ac are involved in SHM. Decreased H3K4me3 in V_HDJ_H regions in human BL2 cells, a Burkitt's lymphoma cell line that can be induced to undergo SHM, upon knockdown of histone chaperone Spt6 is associated with reduced V_HDJ_H mutations (23). In BL2 cells, H4K8ac increases concomitantly with V_HDJ_H mutations upon treatment with HDI trichostatin A (TSA) (85). Persistent H4K8ac in VλJλ region required E2A, whose inactivation result in decreased mutations (72, 86).

Some *cis*-acting regulatory regions, such as *Ig* enhancer and *Ig* enhancer-like sequences, are important for targeting SHM to *Ig* loci (87, 88). In chicken, mouse and human B cells that *Ig* locus enhancers and enhancer-like elements function as core diversification activator (DIVAC) sequences that work together to target SHM (88). In chicken DT40 B cells, short mammalian *Igλ* and *IgH* enhancer fragments can increase mutation rates by more than 20-fold (88). lncRNAs, which are likely to regulate many biological functions, have been recently shown to be link

to enhancer activity. lncRNAs are expressed in a lineage-specific fashion and function through RNA–protein, RNA–DNA, or RNA–RNA target interactions. They are induced to modulate innate and adaptive immunity (66). Many regulatory lncRNAs can be categorized as DNA accessibility modulators, and likely play a role in SHM targeting, especially in conjunction with the function of *Ig* enhancers (67). Thus, activating histone modifications, DNA hypomethylation, and possibly lncRNAs increase V(D)J region chromatin accessibility to the SHM machinery, including AID and error-prone DNA repair factors, which can be stabilized by modified histones.

EPIGENETIC REGULATION OF PLASMA CELL DIFFERENTIATION

B cell differentiation is initiated by extracellular stimuli that bind to cellular receptors and trigger a signaling cascade resulting in the induction of transcription factors that reprogram B cells to secrete antibodies. The function of transcription factors is controlled by the accessibility to DNA through epigenetic modifications. Little is known about how the epigenetic mechanisms direct B cell differentiation into antibody-secreting plasma cells (6). Plasma cells are terminally differentiated elements in the B cell lineage that mostly have undergone SHM and CSR. These cells do not proliferate, but secrete large volumes (10^7 molecules/h) of clone-specific antibodies. Plasma cells are derived from either germinal center or memory B cells. Although some memory B cells are IgM^+, most memory B cells are class-switched and express mutated V(D)J gene segments. Upon reactivation by specific antigen, memory B cells differentiate into antibody-secreting plasma cells to mediate an anamnestic humoral response (89). Reactivated memory B cells can also reenter into the germinal center reaction and undergo further CSR and/or SHM before differentiating into plasma cells or reverting back to memory B cells (90, 91).

Plasma cells display a transcriptional signature that is distinct from B cells (92). Their changes in gene expression correlated with the acquisition of permissive histone modifications, including H3K4me1 and H3K4me4, which are enriched in active promoters and distal enhancers and play an important role in B cell development (92, 93). Upregulation of Blimp-1, a transcriptional repressor, is central to plasma differentiation (6). Blimp-1 down-regulates the expression of *Bcl6*, *Pax5*, and *Spib,* all of which inhibit B cell differentiation into plasma cells by binding to the promoters of these genes (6), and, possibly, deacetylating their promoters. Indeed, *Pax5, Spib*, and perhaps *Bcl6* promoters display decreased histone acetylation in plasma cells (94, 95). Furthermore, Blimp-1 can interact with HDACs that remove acetyl groups on a histone (94). In addition, in plasma cells, Blimp-1 down-regulates *c-Myc* expression through a similar epigenetic mechanism (94), thereby maintaining the terminal differentiation state of these cells (6). Finally, Blimp-1 can interact with H3K9 methyltransferase G9a and likely recruits this enzyme to the *Pax5* and *Spib* promoters, thereby increasing H3K9me3 and repressing activation of these promoters (95, 96). Thus, epigenetic induction of Blimp-1 and Blimp-1-mediated epigenetic inhibition of target genes drives plasma cell differentiation, and possibly maintains plasma cell identity. Down-regulation of Pax-5 and Pax-5-driven Bcl-6 lead to derepression of the *Prdm1* promoter from Bcl-6-mediated epigenetic silencing. This is associated with increased histone acetylation in the *Prdm1* promoter, likely resulting from release of Bcl-6-bound HDACs (97, 98). Reduction of Blimp-1 in a plasmacytoid cell line by enforced expression of Bcl-6 resulted in re-expression of B cell markers, including CD19 (98).

Prdm1 mRNA contains a long (>2,000 nt) 3′ UTR, which can be potentially targeted by multiple miRNAs, including miR-9, miR-23b, miR-30, miR-125b, miR-127, and let-7 (10, 27, 28, 31, 99–102). Overexpression of miR-125b in B cells impairs expression of Blimp-1 and inhibits B cell differentiation into plasma cells (31). In addition, miR-125b can downregulate IFN regulatory factor (Irf)-4, which reciprocally regulates Blimp-1 and is required for the generation of plasma cells (27, 31, 103). Furthermore, X-box binding protein (Xbp)-1 that governs late events of plasma cell differentiation can be downregulated by miR-127 (102).

EPIGENETIC REGULATION OF MEMORY B CELL DIFFERENTIATION

B cell memory is a hallmark of adaptive immunity. Memory B cells are antigen-experienced quiescent B cells, which can be generated in response to both T-dependent antigens (usually proteins) and T-independent antigens (usually carbohydrates) (7). B cells quickly react to a second challenge with the same antigen, thereby providing humoral immune protection. While inherit epigenetic information from their active B cell precursors, memory B cells acquire new epigenetic marks, which make these resting B cells poised to quickly respond to experienced antigen. Expression of memory B cell hallmark genes, such as *CD27* (in humans) and *Cd38* (in mice), is likely mediated by histone modifications induced during B cell activation (4). Genes that control B cell identity and function, including *Pax5* and *Spib*, are also expressed in memory B cells, likely reflecting the epigenetic state that originated in naïve B cells and led them to memory B cells differentiation. Post-recombined S–S regions in the *IgH* locus show constitutive (upstream Sμ portion) or induced (downstream Sγ, Sϵ, or Sα portion) histone modifications, which could be transferred to memory B cells and result in comparable epigenetic landscapes in class-switched memory B cells (4). The functional distinction between memory B cells and their naïve counterparts could at least partially result from the epigenetic alterations.

Quiescent and activated B cells display different histone marks (104). In resting cells, histone lysine methylation was reduced as compared to activated cells (105). Enhancer of zeste homolog 2 (Ezh2) catalyzes H3K27me3, which is enriched at transcription start sites of repressed genes, through its SET domain. EZH2 is highly expressed in human germinal center B cells. Inactivation of Ezh2 in mouse germinal center B cells resulted in a profound reduction of germinal center reactions, memory B cell formation, and antibody response (106). Ezh2 protected germinal center B cells against AID mutagenesis and facilitated cell cycle progression. Repression of Blimp-1 and Irf4 expression in germinal center B cells is also necessary to limit plasma cell differentiation (106). In *Ezh2$^{fl/fl}$Cγ1-Cre* mice, the B cell differentiation stage-specific

Ezh2 deficiency resulted in profound impairment of germinal center reactions and memory B cell formation, suggesting that the methyltransferase activity of *Ezh2* is essential for not only germinal center B cell functions but also generation of memory B cells (106). Furthermore, it has been recently shown that histone acetyltransferase monocytic leukemia zinc finger protein (MOZ), which specifically targets H3K9 and plays a role in stem cell self-renewal, regulates B cell memory formation, controlling memory compartment composition (104). This activity of MOZ is B cell-intrinsic and is required for establishing the germinal center gene expression program. B cell stage-specific deletion of MOZ alters fate decisions in both primary and secondary antibody responses. The lack of MOZ affected the functional outcome of antibody responses, with an increase in secondary germinal centers and a corresponding decrease in secondary high-affinity antibody-secreting cell formation (104).

The differentiation of naïve B cells to germinal center B cells and then to plasma cells or memory B cells would also be associated with changes in DNA methylation. Several genes can be silenced by DNA methylation catalyzed by DNA methyltransferases (DNMTs), such as DNMT3a, which is highly expressed in memory B cells (107). In memory B cells, different expression, as compare to naïve B cells, of the immune activation related elements is likely concomitant with distinctive DNA methylation (108). This supports the concept that the memory B cell epigenome is poised to facilitate a more rapid and robust activation response than that of its naive counterparts.

As demonstrated by concomitant miRNA and mRNA profiling, miRNAs play a regulatory role at every stage of the B cell peripheral differentiation process (28). Selected miRNAs, including miR-125b and let-7, negatively regulate *Prdm1* (10, 27, 28, 101). Down-regulation of miR-15a and miR-16, which target *Bcl2*, likely contributes to memory B cell survival (27, 109) and re-expression of Krüppel-like factors (KLFs), which can bind HDACs, mediates memory B cell quiescence (110). miR-223 is enriched in human memory B cells and down-regulates the expression of LMO2, a key transcription factor in B cell differentiation (28). In addition to function as a negative regulator for CSR and SHM by silencing AID, miR-155 also plays an important role in memory B cell responses. miR-155 deficiency greatly reduced memory B cells (111). Several lncRNAs have been shown to be preferentially expressed in human memory B cells, but not in naïve B cells or B1 cells (112), suggesting that lncRNA also play a role in memory B cell differentiation. Thus, DNA methylation, histone modifications and non-coding RNAs, especially miRNAs, control gene expression programs that lead to B cell differentiation into plasma cells and memory B cells, and maintain the identity of these differentiated cells.

EPIGENETIC MODULATION OF AID AND Blimp-1 EXPRESSION, CSR, SHM, AND PLASMA CELL DIFFERENTIATION BY HDAC INHIBITORS

Histone deacetylases are a class of enzymes that remove the acetyl groups from the lysine residues on a histone leading to the formation of a condensed and transcriptionally silenced chromatin. HDIs block this action and can result in histone hyperacetylation, thereby affecting gene expression. HDIs have been shown to alter gene expression by altering chromatin accessibility (113–115). In immune cells, these epigenetic modifiers exert modulatory effects even at moderate concentrations. By using well-characterized short-chain fatty acid (SCFA) HDIs, valproic acid (VPA) (116), and butyrate (117), we have shown that HDIs regulate intrinsic B cell functions that are critical in shaping effective antibody and autoantibody responses. Our findings were further supported by a recent publication showing that HDIs Panobinostat (a novel broad-spectrum HDI) and Vorinostat (suberanilohydroxamic acid or SAHA) significantly impair antibody and autoantibody responses (118). VPA is an FDA-approved drug, widely used as an anticonvulsant and a mood-stabilizer. It selectively inhibits class I HDACs, particularly, HDAC1 and HDAC2, and, less effectively, class IIa HDACs, of the four HDAC classes identified in mammals (116, 119). Butyrate is a major metabolite in the digestive tract, arising from bacterial fermentation of dietary fibers, mainly "resistant" starch (120, 121), and it is widely available as a dietary supplement. Butyrate modulates gene expression by selectively inhibiting HDAC1 and HDAC3, and, less effectively, other members of class I and class IIa HDACs (117). SCFA HDIs have been suggested to display significant selectivity for different HDACs (122). HDAC activity is mostly associated with multiprotein complexes, the role and composition of which are often cell type-specific. HDAC-associated proteins would specify the selectivity of HDI, which display different affinities for different HDAC/co-factor complexes. HDIs with diverse chemical properties target different HDACs and HDAC/co-factor complexes, thereby regulating gene expression in a locus- and cell type-specific fashion (122). In B cells, HDIs would modulate miRNAs selectively, possibly as a result of HDACs existing in unique contexts of HDAC/co-factor complexes, as occurring in these lymphocytes, particularly when in an activated state (10).

Although HDIs may also indirectly modulate antibody responses or diminish autoimmunity by affecting elements other than B cells, such as innate immune cells (123) and T cells (Treg, T_H1, and T_H17 cells), or inhibit proinflammatory cytokines (115, 124–126), HDIs would directly regulate B cell genes that are central to the peripheral differentiation of these lymphocytes and the maturation of antibody and autoantibody responses (**Figure 2**) (10). Silencing of *AICDA/Aicda* and *PRDM1/Prdm1* (and *XBP1/Xbp1*) by HDIs has been found to be intrinsic to B cells and independent of other cellular elements, as shown by our *in vitro* experiments using purified human and mouse B cells, as well as our *in vivo* studies of the T-dependent response to NP-CGG and the T-independent response to NP-LPS. In both *in vivo* and *in vitro*, HDI-mediated down-regulation of *AICDA/Aicda* and *PRDM1/Prdm1* expression was associated with a concomitant increase of the respective B cell targeting miRNAs (miR-155, miR-181b, and miR-361 for *AICDA/Aicda*; miR-23b, miR-30a, and miR-125b for *PRDM1/Prdm1*), in a tight dose-dependent fashion (10). HDI-induced down-regulation of *XBP1/Xbp1* could be secondary to decreased Blimp-1 expression.

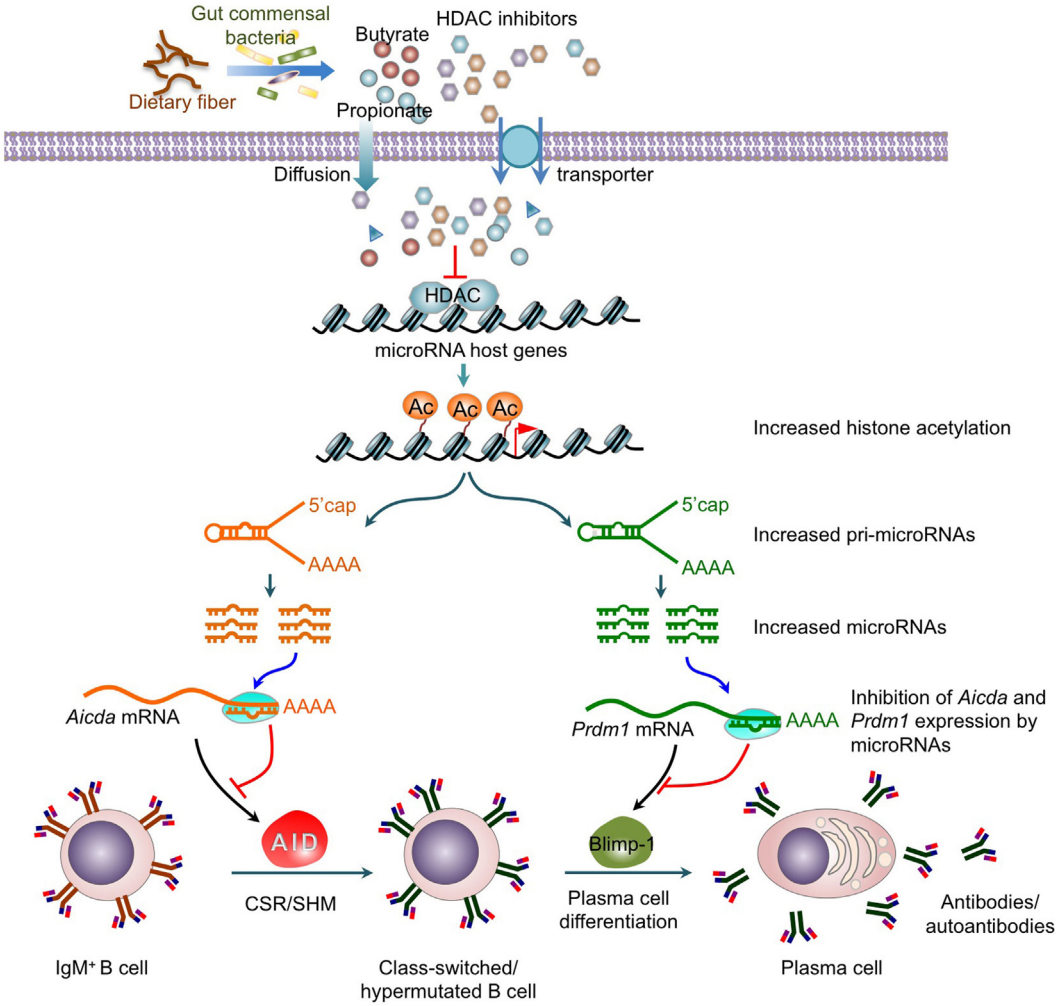

FIGURE 2 | Histone deacetylase inhibitors upregulate selected B cell miRNAs that silence AID and Blimp-1 expression to epigenetically modulate CSR, SHM, plasma cell differentiation and antibody/autoantibody responses. HDAC inhibitors, including short-chain fatty acid butyrate and propionate produced by gut commensal bacteria through fermentation of dietary fiber, epigenetically modify CSR and SHM by upregulating miRNAs, which silence *AICDA/Aicda* mRNA and *PRDM1/Prdm1* mRNA. The upregulation of miRNA expression results from an increase in the histone acetylation of the host genes of these miRNAs. This leads to down-regulation of AID and Blimp-1 expression, and the dampening of CSR, SHM, and plasma cell differentiation.

The selectivity of HDI-mediated silencing of *AICDA/Aicda* and *PRDMI/Prdm1* in B cells that were induced to undergo CSR and plasma cell differentiation was demonstrated by genome-wide mRNA-Seq and further emphasized by the unchanged expression of *Ung*, *Irf4*, *HoxC4*, *Rev1*, and *Bcl6*, as well as the unchanged expression of miR-19a/b, miR-20a, and miR-25, which are not known to regulate *AICDA/Aicda* or *PRDMI/Prdm1* (10, 99). This, however, cannot rule out the possibility that HDI regulated other B cell factors (e.g., NF-κB, Id2/3, or Pax5), which contributed to the reduction of AID or Blimp-1. Nevertheless, relief of the HDI-mediated repression of luciferase activity under the control of *Aicda* and *Prdm1* mRNA 3′ UTRs bearing mutated miR-155, miR-181b, miR-23b, miR-30a, and miR-125b target sites demonstrated that miRNAs are indeed direct effectors of the HDI-mediated repression of such selected genes in B cells (10).

Potential Role for Gut Microbiota-Derived Short-Chain Fatty Acid HDAC Inhibitors in the Modulation of Antibody Response

At ant time, the human body carries 10^{13}–10^{14} microorganisms, a number 10-fold more than the total number of human cells in the body. Human gastrointestinal tract microbiota composed of up to 1,000–1,150 bacterial species, which play an important role in nutritional, metabolic and physiological processes that are crucial for the maintenance of human health. Gut commensal bacteria are critical regulators of health and disease by protecting against pathogen while also maintaining immune tolerance to allergens (127–131). Commensal bacteria may modulate host immunity through metabolite-dependent mechanisms (129, 131). SCFAs, such as acetic acid, propionic

acid, and butyric acid, which are generated in the colon by commensal bacteria through digestion of dietary fiber, are among the most abundant of these dietary metabolites. They are important for gut motility and colonocyte development. SCFAs function through binding to host cell surface receptors, such as GPR41, GPR43, and GPR109A, and through their HDI activity (132). It has been suggested that SCFAs produced in the gut could distribute systemically and shape the immunological environment in the respiratory system, thereby influencing the severity of allergic inflammation (132).

Mice fed a low-fiber diet displayed decreased serum levels of SCFAs and increased IgE-mediated allergic inflammation in the lung, while a high-fiber diet increased levels of SCFAs and were protected against allergic airway disease (132). Butyrate and propionate, which are potent HDIs, modulate the function of intestinal macrophages and naive T cells to promote epigenetic changes that regulate the expression of genes responsible for differentiation into regulatory T cells and IL-10-producing T cells (121, 132, 133). Our recent findings that butyrate modulates AID expression and CSR to IgG, IgA, and IgE, as well as plasma cell differentiation through its direct HDI activity on B cells (10), indicates that this SCFA can play an important role in modulating antibody responses of gut lymphoid organs. A diverse microbial population, which would produce an appropriate amount of SCFA HDIs, particularly, butyrate, is required to maintain a baseline immune-regulatory state, including IgG, IgA, and IgE levels. Elevated serum IgE and CSR to IgE in B cells at mucosal sites in the absence of microbial colonization in germ-free mice and in mice with low-diversity gut microbiota (134) further emphasize the important role for gut commensal bacteria-produced butyrate in modulating IgE production. Altered composition and decreased bacterial diversity of gut microbiota would lead to changes in absolute and relative levels of SCFA HDIs and, therefore, changes in systemic IgG, IgA and IgE levels and specificities, which contribute to altered immunity and increased susceptibility to immune-mediated diseases.

B CELL EPIGENETIC DYSREGULATION IN AUTOIMMUNITY AND LYMPHOMAGENESIS

Epigenetic factors also play an important role in the pathogenesis of B cell-related immune disorders, such as autoimmunity, allergic states and B cell malignancies, by integrating the effects of genetic makeup and the environment, two major disease-causing factors. Epigenetic dysregulation would compound genetic susceptibility in the generation of autoantibodies and autoimmunity, as suggested by alterations of histone modifications and DNA methylation in patients with lupus, autoimmunity in mice with miRNA dysregulation (9), and low penetrance (25–45%) of lupus in monozygotic twins (135). The development of allergic diseases has been associated with environmental conditions, such as diet, drugs, toxins, sex hormones, and microbiota, all of which can impact the epigenetic profile (136, 137). Aberrant AID expression that can result from epigenetic dysregulation can lead to autoantibody-mediated autoimmunity, IgE-mediated allergic responses and tumorigenesis (**Figure 3**).

Epigenetic Changes and Autoimmunity

Systemic autoimmune diseases, such as SLE, rheumatoid arthritis, systemic sclerosis, and dermatomyositis, are associated with overall DNA hypomethylation (1, 138, 139). Autoreactive

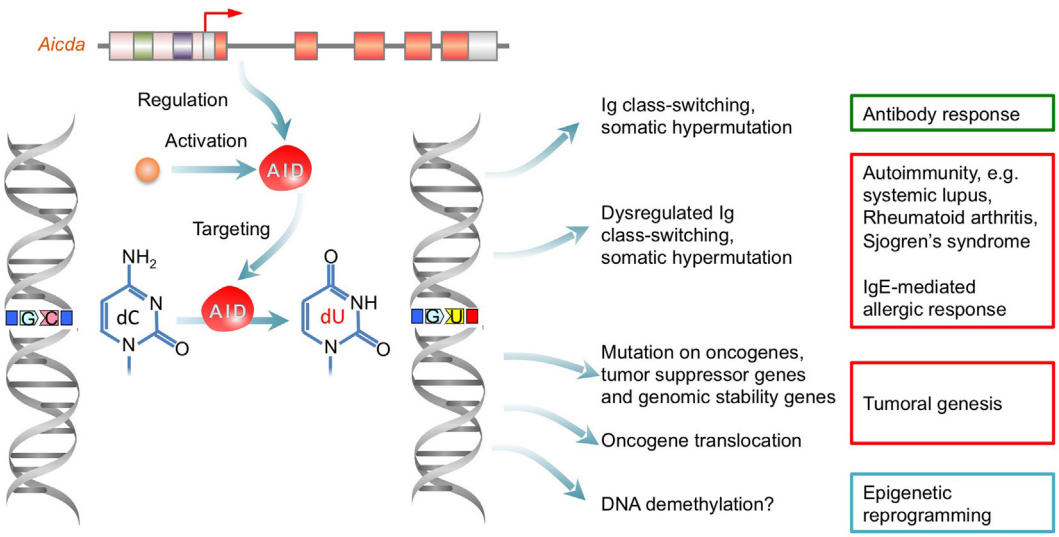

FIGURE 3 | Dysregulation of AID result in autoantibody-mediated autoimmunity, IgE-mediated allergic response and tumoral genesis. AID initiates CSR and SHM by deaminating dCs into dUs yielding dU:dG mismatches, which lead to point-mutations in Ig V(D)J regions and DSBs in S regions. Aberrant AID expression result from epigenetic dysregulation can lead to a dysregulated antibody/autoantibody response and AID-mediated DNA mutagenesis, which can cause autoimmunity, allergic response, or tumoral genesis.

B cells in lupus patients are characterized by lack of ability to induce DNA methylation that extends their survival (140). In mice, prolonged treatment with DNA methylation inhibitors, or adaptive transfer of B cells treated with DNMT inhibitors, resulted in autoantibody production and lupus-like disease (141, 142). The role of DNA methylation in autoimmune diseases is further emphasized by the discordance of SLE in monozygotic twins. B cells isolated from SLE patients displayed profound defects in DNA methylation, as compared to those from healthy monozygotic siblings (135). The decreased DNA methylation in autoreactive B cells likely resulted from reduced DNMT1 and DNMT3b expression; it could also result from active DNA demethylation mediated by AID-mediated cytosine deamination (143). Indeed, AID is upregulated in B cells of lupus patients or lupus-prone mice (3, 9, 144). The contribution of aberrant histone modifications to lupus development has been strongly suggested by the increased histone acetylation and reduced autoantibody production in lupus-prone mice treated with HDIs (1, 10, 145).

Dysregulation of miRNAs has been associated with autoimminty. miRNAs are aberrantly expressed in different cell types and tissues in patients with autoimmune disease. B cell-specific deletion of Dicer, which is critical for miRNA maturation, resulted in a distorted BCR repertoire with increased of autoreactivity, suggesting a role for miRNAs in preventing the generation of self-reactive antibodies (9, 146, 147). In lupus-prone MRL/$Fas^{lpr/lpr}$ mice, miR-150 is downregulated in spleen B cells, as compared to that in MRL/$Fas^{+/+}$ mice (148), possibly as a result of decreased acetylation and transcription of the miR-150 host gene due to defective HDAC activity. Indeed, specific knock-in dominant negative mutant of histone acetyltransferase p300 in B cells resulted in production of class-switched anti-double-stranded DNA autoantibodies and development of lupus-like symptoms (149). Conversely, elevated expression of some miRNAs can also contribute to the development of autoimmunity. In lupus mice, expression miR-21 is upregulated in B cells, silencing of this miRNA ameliorates autoimmune splenomegaly (150). Constitutively expression of miR-17 ~ 92 in transgenic mice results in an increased numbers of germinal center B cells in the spleen and peripheral lymph nodes that may lead to autoimmune response (151).

TABLE 2 | B cell epigenetic dysregulation and lymphomagenesis.

Neoplasm	Epigenetic change	Potential impacts of epigenetic changes	Reference
DLBCL, FL	Loss-of-function mutation of HAT genes *CREBBP* and *EP300*	Reduce P53 and BCL6 acetylation	(177, 178)
HD, NHL	Deregulation of the H3K27 methyltransferase EZH2	Malignant GC B cell transformation	(179)
Multiple myeloma	Overexpression of H3K36 methyltransferase *MMSET/NSD2*	Alter H3K36 and H3K27 methylation, upregulate *c-MYC* expression by reducing *c-MYC* targeting miR-126	(180)
NHL, DLBCL, FL, MLL	Dysregulation of the H3K4 methyltransferase MLL2/MLL3/KMT2D	Modulate cell-type- and stage-specific transcriptional programs by regulating chromatin accessibility at enhancer regulatory sequences	(181)
NHL, DLBCL	Aberrant DNA methylation	Promote aberrant gene expression	(155, 182, 183)
B cell CLL	miR-15a/16-1 depletion	Aberrant expression of cell cycle regulators Ccnd2, Ccnd3, Cdk4, Cdk6 and Chk1, and anti-apoptotic protein BCL2	(171, 184)
NHL, HD, BL	miR-155 deregulation	Enhance/sustain AID mutagenic activity	(37, 185)
DLBCL, BL	Overexpression of the miR-17-92 cluster	Downregulate inhibitors of the PI3K (*Pten*), NF-κB (*A20, Cyld*) and the intrinsic apoptotic (*Bim*) pathways; alter MYC-centered regulatory network	(151, 186, 187)

Epigenetics Changes and Lymphomagenesis

At all stages of cancer development, inappropriate epigenetic marks interact with genetic alterations to promote neoplastic transformation and tumor cell progression (152, 153). The epigenome of B cell lymphomas is characterized by global changes in DNA methylation and histone modification patterns, which varies with chromosomal regions, local gene density, as well as DNA and histone modification status of neighboring genes (**Table 2**) (154, 155). Aberrant DNA hypomethylation of promoters can lead to increased transcription of genes with oncogenic potential, and aberrant DNA hypermethylation can lead to decreased transcription of genes with tumor suppressor function. For example, mantle B cell lymphomas display DNA hypomethylation in promoters of genes that are involved in pathways controlling cell cycle or apoptosis, such as *Cdk5*, and aberrant hypermethylation in the promoter of tumor suppressor genes, such as *Cdkn2b* (156). Hypermethylation is likely associated with increased expression of DNMTs, such as DNMT3b, which is upregulated in diffuse lager B cell lymphomas (157). Accordingly, *Dnmt3b* transgenic mice develop mediastinal B cell lymphomas, which display significantly altered methylation patterns (157). Aberrant hypomethylation could involve AID, which can mediate active DNA demethylation (158) and is upregulated in human B cell non-Hodgkin lymphomas (NHL) (159).

Active DNA demethylation can also be initiated by the 10–11 translocation (Tet) family of proteins Tet1, Tet2, and Tet3, which catalyze the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), a critical step for ultimate removal of a methyl mark (160, 161). Reduced expression of Tet proteins that lead to decreased 5hmC has been shown to associate with tumor development, suggests an important role of Tet proteins and 5hmC in normal cellular function (162). Tet1 is required for maintaining the normal abundance and distribution of 5hmC, which prevented hypermethylation of DNA in B cells (163). It is important for regulation of the B cell lineage and of genes encoding molecules

involved in chromosome maintenance and DNA repair. Tet1 may function as a tumor suppressor of B cell malignancy. Deletion of *Tet1* in mice promotes the development of B cell lymphoma (163).

Altered expression or mutation of the histone-modifying enzymes promotes aberrant gene expression, which is responsible for many tumor changes (164). Aberrant germinal center development is common in many B cell malignancies. EZH2, a histone methyltransferase component of polycomb repression complex (PRC)2 that catalyzes H3K27me3 and promotes tumor growth, is highly expressed in germinal center B cells and is often constitutively activated in germinal center-derive NHLs. EZH2 prevents apoptosis caused by DNA damage, including that generated by AID, facilitated cell cycle progression, and silenced Blimp-1, which can function as tumor suppressor (106). Inhibition of EZH2 in NHL cells induces Blimp-1, which impairs tumor growth. Overexpression of EZH2 is associated with B lymphomagenesis (165). Somatic mutations at Y641 and A677 residues within the catalytic domain of EZH2 have been found in diffuse large B cell lymphoma and follicular lymphoma (166). These mutations promote EZH2 activity and increase H3K27me3 levels in those cells (165). Furthermore, other histone-modifying enzymes, such as histone acetyltransferases CBP and p300, histone methyltransferases MLL2, and histone demethylases UTX and JMJD2C, are frequently mutated in B cell lymphomas (157). Mutations in these enzymes are likely mediated by AID, which is highly expressed in those neoplastic B cells, and result in aberrant patterns of histone modifications and disruption of chromatin structure, ultimately leading to dysregulated gene transcription programs.

Dysregulated miRNA expression also contributes to B lymphomagenesis (28, 167). The miR-17 ~ 92 cluster, which consists of six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1) that target the tumor repressor genes *Bim* and *Pten*, is often increased in human lymphomas (151, 168). Accordingly, ectopic expression of the miR-17 ~ 92 cluster in lymphocytes leads to development of lymphoproliferative disease (151, 168). Conversely, expression of miR-15 and miR-16-1, which target anti-apoptotic oncogene *Bcl-2*, is downregulated in chronic lymphocytic leukemia B cells (169, 170). Knockout miR-15a and miR-16-1 in B cells results in clonal lymphoproliferative disorders (171). B cell lymphomas often express significant amounts of mRNA isoforms with shorter 3′ UTRs, which lack miRNA-binding sites, thereby escaping miRNA-directed silencing (172). For example, a short *Cyclin D1* mRNA isoform that lacks part of the 3′ UTR is expressed in a subset of mantle cell lymphomas (172). These cells have increased expression of Cyclin D1, an important regulator of cell cycle progression, and B cell proliferation. Thus, by allowing selected oncogenes to escape regulation by their modulatory miRNAs, the shortening of 3′ UTRs may provide an important mechanism in B cell neoplastic transformation.

CONCLUDING REMARKS

Epigenetic changes are critical in shaping B cell differentiation functions, such as CSR, SHM, generation of plasma cells as well as memory B cells, for the production of class-switched and high affinity antibodies. In addition, a growing body of evidence implicates the involvement of epigenetic mechanisms in immune programing and development of allergic and autoimmune diseases. Nevertheless, important questions on the nature and role of such epigenetic changes remain to be answered. These include the mechanisms by which histone posttranslational modifications and non-coding RNAs target the CSR and SHM machineries, particularly the selective recruitment of AID, to the *Ig* locus and how the dynamics of these epigenetic modifications orchestrate AID-mediated DNA lesion and DNA repair processes. High-affinity antibodies are generated in germinal centers through SHM and selection of higher affinity B cell submutants for survival and expansion. These B cells will then undergo differentiation to plasma cells or memory B cells. Much needs be understood on the role of epigenetic modifications in the selection of germinal center B cells and in what determines whether a germinal B cell becomes a memory B cell or a plasma cell. These processes would involve unique epigenetic and transcriptional changes. Furthermore, altered DNA methylation, histone methylation and acetylation, and miRNA expression, resulting in immune imbalance, have been shown to be associated with the onset and progression of allergic and autoimmune diseases, as well as B cell lymphomagenesis. Thus, knowledge of the epigenetic profiles associated with B cell development and peripheral differentiation, and molecular mechanisms that cause and result from disease-associated epigenetic patterns in B cells is required to understand the pathophysiology of allergic and autoimmune diseases, as well as B cell malignancies.

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Genome-Wide Analysis Reveals Selective Modulation of microRNAs and mRNAs by Histone Deacetylase Inhibitor in B Cells Induced to Undergo Class-Switch DNA Recombination and Plasma Cell Differentiation

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As we have suggested, epigenetic factors, such as microRNAs (miRNAs), can interact with genetic programs to regulate B cell functions, thereby informing antibody and autoantibody responses. We have shown that histone deacetylase (HDAC) inhibitors (HDI) inhibit the differentiation events critical to the maturation of the antibody response: class-switch DNA recombination (CSR), somatic hypermutation (SHM), and plasma cell differentiation, by modulating intrinsic B cell mechanisms. HDI repress the expression of AID and Blimp-1, which are critical for CSR/SHM and plasma cell differentiation, respectively, in mouse and human B cells by upregulating selected miRNAs that silenced *AICDA/Aicda* and *PRDM1/Prdm1* mRNAs, as demonstrated by multiple qRT-PCRs (*J Immunol* 193:5933–5950, 2014). To further define the selectivity of HDI-mediated modulation of miRNA and gene expression, we performed genome-wide miRNA-Seq and mRNA-Seq analysis in B cells stimulated by LPS plus IL-4 and treated with HDI or nil. Consistent with what we have shown using qRT-PCR, these HDI-treated B cells displayed reduced expression of *Aicda* and *Prdm1*, and increased expression of miR-155, miR-181b, and miR-361, which target *Aicda,* and miR-23b, miR-30a, and miR-125b, which target *Prdm1*. In B cells induced to undergo CSR and plasma cell differentiation, about 23% of over 22,000 mRNAs analyzed were expressed at a significantly high copy number (more than 20 copies/cell). Only 18 (0.36%) of these highly expressed mRNAs, including *Aicda*, *Prdm1*, and *Xbp1*, were downregulated by HDI by 50% or more. Further, only 16 (0.30%) of the highly expressed mRNAs were upregulated (more than twofold) by HDI. The selectivity of HDI-mediated modulation of gene expression was emphasized by unchanged expression of the genes that are involved in regulation, targeting, or DNA repair processes of CSR, as well as unchanged expression of the genes encoding epigenetic regulators and factors that are important

for cell signaling or apoptosis. Our findings indicate that, in B cells induced to undergo CSR and plasma cell differentiation, HDI modulate selected miRNAs and mRNAs, possibly as a result of HDACs existing in unique contexts of HDAC/cofactor complexes, as occurring in B lymphocytes, particularly when in an activated state.

Keywords: AID, Blimp1, B cell, class-switch DNA recombination, epigenetics, HDAC, HDAC inhibitor, histone acetylation, microRNA, mRNA, mRNA-Seq, miRNA-Seq, plasma cell differentiation, somatic hypermutation

INTRODUCTION

Epigenetic markers or factors, such as DNA methylation, histone posttranslational modifications, and microRNAs (miRNAs), dynamically regulate gene activities. As we have contended, epigenetic markers/factors "interact" with genetic programs to regulate B cell functions, such as class-switch DNA recombination (CSR), somatic hypermutation (SHM), and differentiation to memory B cell or plasma cell, thereby informing antibody and autoantibody responses (1). CSR and SHM are B cell-intrinsic differentiation processes that underpin the generation of class-switched and high-affinity antibodies, such as those that clear microbial pathogens or kill tumor cells. CSR and SHM critically require activation-induced cytidine deaminase (AID, encoded by *AICDA* in humans and *Aicda* in mice), which is specifically and highly induced in B cells in both T-dependent and T-independent antibody responses (2). Class-switched and hypermutated B cells further differentiate into antibody-secreting plasma cells in a fashion critically dependent on B lymphocyte-induced maturation protein 1 (Blimp1, encoded by *PRDM1* in human beings and *Prdm1* in mice) (3), or transition to long-lived memory B cells, which can differentiate into plasma cells upon reactivation by antigen to mediate an anamnestic response (4). Pathogenic autoantibodies, including those to nuclear components in systemic lupus erythematosus (SLE) patients (5, 6), are also class-switched and hypermutated (7, 8). Thus, epigenetic dysregulation of B cells can result in aberrant antibody responses to exogenous antigens, such as those on viruses and bacteria, or self-antigens, such as chromatin, histones, and dsDNA in lupus (1, 7).

The chromatin structure is comprised of DNA and histones. The basic repeating unit of chromatin is the nucleosome, a 147 bp of DNA chain wrapped around one histone octamer composed of two copies of each of four histones: H2A, H2B, H3, and H4. Histone posttranslational modifications include phosphorylation of serine or threonine residues, methylation of lysine or arginine, acetylation and deacetylation of lysines, and ubiquitylation and sumoylation of lysines. All these posttranslational modifications play an important role in regulating gene expression (9, 10). Histone acetylation and deacetylation, which are essential for gene regulation, are typically modulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) (9, 10). Histone acetylation catalyzed by HAT will result in a loose chromatin structure, which enables DNA binding proteins to activate gene transcription, while histone deacetylation catalyzed by HDAC will result in a condensed chromatin structure, which prevents binding of transcription factors or proteins to DNA and silence gene expression. HDAC inhibitors (HDI) alter gene expression by altering chromatin accessibility (11, 12).

MicroRNAs also play an important role in regulation of the genes involved in CSR, SHM, and plasma cell differentiation (1, 7, 13). miRNAs are small (~22 nucleotides), evolutionarily conserved non-coding RNAs derived from much larger primary transcripts encoded by their "host genes." miRNAs bind to complementary sequences within the 3′ untranslated region (3′ UTR) of their target mRNAs and negatively regulate protein expression at the posttranscriptional level through inhibition of translation and/or reduction of mRNA stability (14, 15). The mammalian genome encodes thousands of miRNAs that collectively affect the expression of more than half of protein-coding genes. In addition, miRNAs have been implicated as fine-tuning regulators controlling diverse biological processes at posttranscriptional level. They can potentially regulate every aspect of cellular activity, from proliferation and differentiation to apoptosis, as well as modulate a large range of physiological and pathological processes. miRNAs likely play important roles in B cell development and peripheral differentiation, as well as T cell stage-specific differentiation and autoimmunity. Some miRNAs, including miR-155, miR-181b, and miR-361, can silence AID expression, whereas miR-30a and miR-125b can silence Blimp-1 expression (16). These miRNAs bind to evolutionarily conserved miRNA target sites in the 3′ UTR of *Aicda* and *Prdm1* mRNAs and cause degradation of the mRNA transcripts and/or inhibit their translation.

We have recently shown that HDI, such as short-chain fatty acid valproic acid and butyrate, inhibit the expression of AID and Blimp-1 in human and mouse B cells *in vivo* and *in vitro* and regulate intrinsic B cell functions that are critical in shaping effective antibody and autoantibody responses (16). Valproic acid or sodium valproate (VPA, 2-propyl-pentanoic acid sodium) is widely used to treat epilepsy and mood disorders. VPA can selectively inhibits class I HDACs, particularly, HDAC1 and HDAC2, and less effectively, class IIa HDACs among the four HDAC classes identified in mammals (17, 18) to alter gene expression by changing chromatin accessibility. We have further shown that HDI, such as VPA and butyrate, inhibit AID and Blimp1 expression by upregulating miR-155, miR-181b, and miR-361, which silenced *AICDA/Aicda* mRNA, and miR-23b, miR-30a, and miR-125b, which silenced *PRDM1/Prdm1* mRNA (16). The selectivity of HDI-mediated silencing of *AICDA/Aicda* and *PRDM1/Prdm1* was emphasized by unchanged expression of HoxC4 and Irf4 (important inducers/modulators of *AICDA/Aicda*), Rev1 and Ung (central elements for CSR/SHM), and Bcl6, Bach2, or Pax5 (repressors of *PRDM1/Prdm1* expression), as well as unchanged expression of miR-19a/b, miR-20a, and miR-25, which are not known to regulate *AICDA/Aicda* or *PRDM1/Prdm1*. Epigenetic modulations always display a cell type- and cell stage-specific regulation pattern of gene expression (19). To extend our findings

and further define the selectivity of HDI-mediated modulation of miRNAs and gene expression, we performed genome-wide miRNA-Seq and mRNA-Seq analysis in B cells induced to undergo CSR and plasma cell differentiation in the presence of VPA. Here, we showed that this HDI modulated selected miRNAs and mRNAs, possibly as a result of HDACs existing in unique contexts of HDAC/cofactor.

MATERIALS AND METHODS

Stimulation of Mouse B Cells for CSR and Plasma Cell Differentiation, and HDI Treatment

C57BL/6 mice were purchased from The Jackson Laboratory and maintained at the University of Texas Health Science Center at San Antonio (UTHSCSA) animal facility. The Institutional Animal Care and Use Committee of UTHSCSA approved all animal protocols. Naïve IgD$^+$ B cells were isolated from 8-week-old C57BL/6 mice as described (16, 20). B cells were resuspended in RPMI 1640 medium with 10% FBS, 50 mM β-mercaptoethanol, and 1× antibiotic–antimycotic mixture (15240-062; Invitrogen) (FBS-RPMI) at 37°C and stimulated with LPS (3 μg/ml) from *Escherichia coli* (055:B5; Sigma-Aldrich) plus IL-4 (5 ng/ml; R&D Systems) for CSR to IgG1/IgE and plasma cell differentiation. HDI [VPA 500 μM, a concentration comparable to serum concentration of VPA-treated mice (21)] or nil were also added to the cultures. Cells were collected 60 h later for qRT-PCR, mRNA-Seq, and miRNA-Seq, or 96 h later for surface Ig analysis by flow cytometry (16, 22).

RNA Extraction and High Throughput mRNA-Seq and miRNA-Seq

Total RNA was extracted from 2×10^6 cells using miRNeasy® Mini Kit (Qiagen), as previously described (16). RNA integrity was verified using an Agilent Bioanalyzer 2100 (Agilent). Next generation mRNA-Seq and small RNA-Seq were performed by the Genome Sequencing Facility (Greehey Children's Cancer Research Institute, GCCRI), UTHSCSA. High-quality RNA (RNA Integrity number or RIN.9.0) was processed using an Illumina TruSeq RNA sample prep kit v2 or TruSeq Small RNA Sample Prep kit following the manufacturer's instructions (Illumina). Clusters were generated using TruSeq Single-Read Cluster Gen. Kit v3-cBot-HS on an Illumina cBot Cluster Generation Station. After quality control procedures, individual mRNA-Seq or small RNA-Seq libraries were then pooled based on their respective 6-bp index portion of the TruSeq adapters and sequenced at 50 bp/sequence, read using an Illumina HiSeq 2000 sequencer. The barcode combinations were further crosschecked by Illumina Experiment Manager software. Sequence data were checked by assurance (QA) pipeline and initial genome alignment (Alignment). Approximately 33 million and 5 million reads per sample were generated in mRNA-Seq and miRNA-Seq, respectively. After the sequencing run, demultiplexing with CASAVA was employed to generate the fastq file for each sample. All sequencing reads were aligned with their reference genome (UCSC mouse genome build mm9) using TopHat2 default settings and the Bam files from alignment were processed using HTSeq-count to obtain the counts per gene in all samples. Quality control statistical analysis of outliers, intergroup variability, distribution levels, PCA, and hierarchical clustering analysis were performed for statistical validation of the experimental data.

Quantitative RT-PCR (qRT-PCR) of mRNAs and miRNAs

For mRNA quantification, post-recombination Iμ-C_H and mature V_HDJ_H-C_H transcripts. cDNA was synthesized from total RNA with the SuperScript™ III First-Strand Synthesis System (Invitrogen) using oligo-dT primer. Transcript expression was measured by qRT-PCR using the appropriate primers, as previously reported (16) using a Bio-Rad MyiQ™ Real-Time PCR Detection System (Bio-Rad Laboratories) to measure SYBR Green (IQ™ SYBR® Green Supermix, Bio-Rad Laboratories) incorporation with the following protocol: 95°C for 15 s, 40 cycles of 94°C for 10 s, 60°C for 30 s, 72°C for 30 s. Data acquisition was performed during the 72°C extension step. Melting curve analysis was performed from 72 to 95°C. For quantification of mature miRNA transcripts, RNA was extracted from $0.2–5 \times 10^6$ cells using miRNeasy® Mini Kit (Qiagen) and then reverse-transcribed with miScript II RT Kit (Qiagen) using the miScript HiSpec buffer. A Bio-Rad MyiQ™ Real-Time PCR Detection System was used to measure SYBR Green (miScript SYBR Green PCR Kit; Qiagen) incorporation according to manufacturer's instructions. Mature miRNA forward primers were used at 250 nM in conjunction with the Qiagen miScript Universal Primer and normalized to expression of small nuclear/nucleolar RNAs Rnu6/RNU61/2, Snord61/SNORD61, Snord68/SNORD68, and Snord70/SNORD70. The ΔΔCt method was used for qRT-PCR data analysis with Microsoft Excel.

Statistical Analysis

Statistical analysis was performed to determine p values by paired and unpaired Student's t-test, and p values <0.05 were considered significant.

RESULTS

HDI Inhibit CSR and Plasma Cell Differentiation

We have shown that HDI repress the expression of AID and Blimp-1, which are critical for CSR/SHM and plasma cell differentiation, respectively, in mouse and human B cells by upregulating selected miRNAs that silenced *AICDA/Aicda* and *PRDM1/Prdm1* mRNAs, as demonstrated by multiple qRT-PCRs (16). To further define the selectivity of HDI-mediated modulation of miRNA and gene expression, we stimulated purified mouse B cells with LPS plus IL-4, which induce B cells to undergo CSR to IgG1 or IgE and differentiate to plasma cells in the presence of HDI (VPA, 500 μM) or nil. Consistent with our previous findings (16), HDI significantly inhibited CSR and plasma cell differentiation, as shown by greatly reduced surface IgG1$^+$ B cells and B220^{low} CD138$^+$ plasma cells (**Figure 1**). HDI inhibition of CSR was

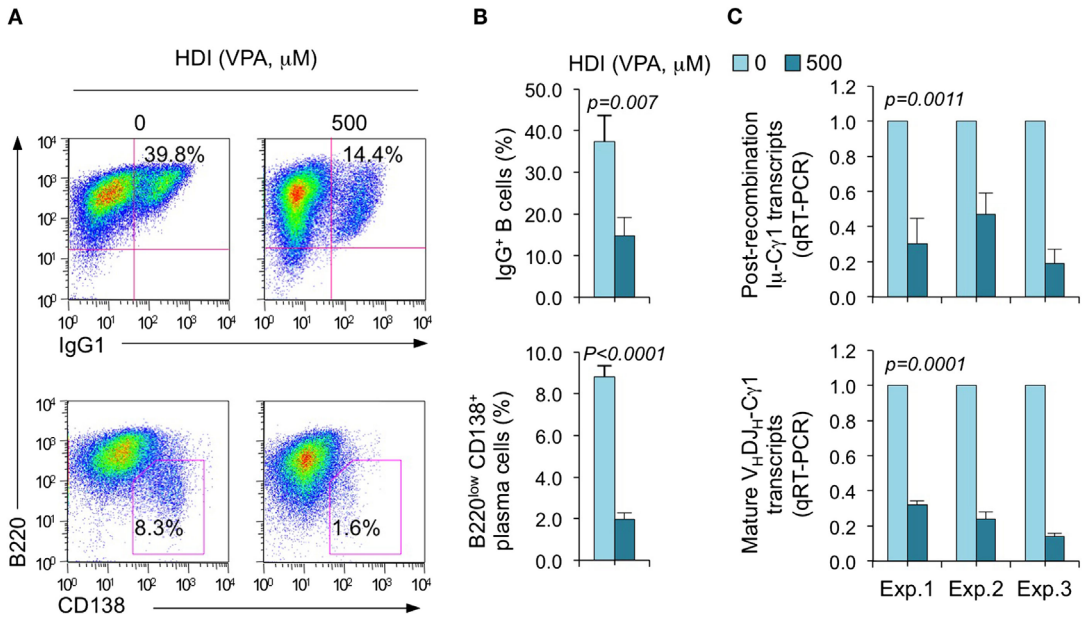

FIGURE 1 | HDI inhibits B cell CSR and plasma cell differentiation. Purified spleen B cells were stimulated with LPS plus IL-4 in the presence of HDI (VPA, 500 μM) or nil. **(A)** Surface expression of B220 and IgG1 was measured by flow cytometry 96 h after the stimulation, Data are one representative of three independent experiments. **(B)** The average percentage of IgG1$^+$ B cells and B220^{low} CD138$^+$ plasma cell in nil or HDI-treated cells in the three experiments. **(C)** Mature V_HDJ_H-Cγ1 transcripts and post-recombination Iμ-Cγ1 transcripts, both hallmarks of completed CSR, were analyzed by qRT-PCR and normalized to *Cd79b* transcripts in B cells stimulated for 60 hours, measured by qRT-PCR and normalized to *Cd79b* transcripts. Data are from three independent experiments. *p* values, paired *t*-test.

further confirmed by decreased numbers of post-recombination Iμ-Cγ1 and mature V_HDJ_H-Cγ1 transcripts.

HDI-Mediated Modulation of mRNA Expression in B Cells is Highly Selective

Histone deacetylases remove the acetyl groups from the histone lysine residues leading to the formation of a condensed and transcriptionally silenced chromatin. HDI block this activity, thereby increasing histone acetylation and alteration of gene expression. It has been shown that HDI, such as TSA, suberoylanilide hydroxamic acid (SAHA), MS-275, and FK228, could alter the expression of 5–20% of genes (23). To further define the modulation of gene expression by HDI in B cells that undergo CSR and plasma cell differentiation, we performed high throughput mRNA-Seq to analyze the transcriptome in B cells stimulated by LPS plus IL-4 and treated with HDI (VPA, 500 μM) or nil. In general, one RPKM (read per kb per million reads) of mRNA represents approximately one copy of transcript per cell (24). In three independent experiments, HDI did not significantly alter overall mRNA expression ($p = 0.99925$, 0.74835, and 0.39640, respectively), although the average PRKM was slightly reduced from 42.71 to 38.49 ($p = 0.3506$) (**Figure 2**). Among over 22,000 genes analyzed, about 5,000 of them were significantly expressed (more than 20 RPKM, average more than 20 transcripts per cells) in B cells stimulated by LPS plus IL-4. Upon treatment with HDI, only 18 (0.36%) of the "highly" expressed genes, including *Aicda, Prdm1, Xbp1, Bhlha15, RRhob, Soat2, Hist1h2bg, Fut1, Cxcr3, Eaf2, Fkbp11, Ppapdc1b, Il10, Rcbtb2, Ly6c2, Saa3, Txndc5*, and *IgJ*, were downregulated, on average, by 50% or more. The mRNA of Cxcr3 (C-X-C motif chemokine receptor 3), which is highly expressed in IgG1$^+$ memory B cells and can promote the production of IgG1 autoantibodies (25, 26), was reduced by about 70% by HDI. The mRNA of Saa3 (Serum amyloid A3), which can interact with Tlr4 and induce Tlr4-mediated NF-κB activation (27), was reduced by over 51%. Sixteen (0.30%) of the "highly" expressed (more than 20 RPKM) genes, including *Gstk1, Gstm1, Zbtb20, Gapt, Ift57, Slc16a3, Ptprs, Atp11a, Kctd17, Itm2a, Syt11*, and *Ifit1*, were upregulated by HDI by more than twofold (**Figures 2A,B**). Downregulation or upregulation of the above genes by more than twofold was consistent in all the three experiments, suggesting that the modulation of gene expression by HDI is highly selective.

Aicda, Prdm1, and *Xbp1* are Selectively Silenced by HDI

Consistent with our real-time qRT-PCR results (16), the mRNA-Seq experiments further demonstrated that *Aicda*, *Prdm1*, and *Xbp1* transcripts were significantly downregulated by HDI (**Figures 2** and **3A–C**). In all three independent experiments, *Aicda*, *Prdm1*, and *Xbp1* were consistently reduced by HDI by more than 57, 48, and 47%, respectively, ranking 7th, 21st, and 24th, of the most downregulated genes among the total of more than 5,000 genes that were highly expressed (more than 20 RPKM) in B cells stimulated by LPS plus IL-4. Thus, these deep sequencing experiments showed that *Aicda*, *Prdm1*, and *Xbp1* are selectively inhibited by HDI in B cells undergoing CSR and plasma cell differentiation.

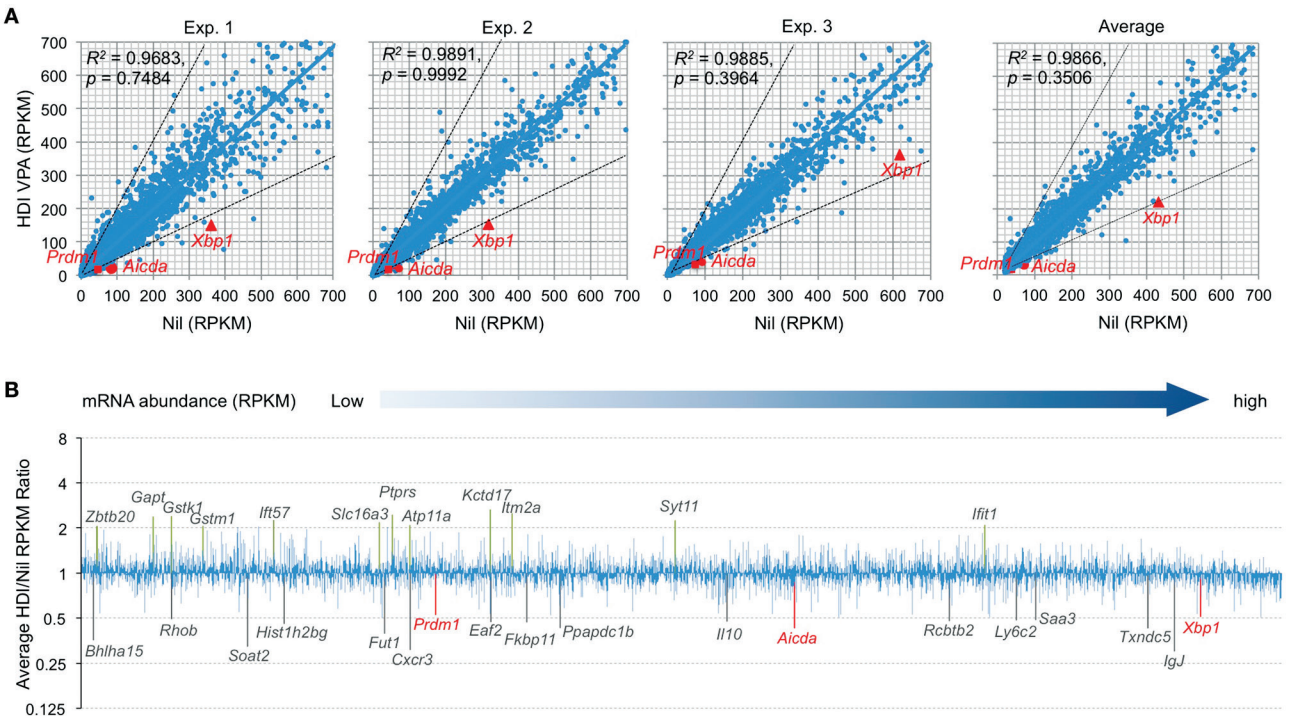

FIGURE 2 | HDI selectively inhibits mRNA expression in B cells induced to undergo CSR and plasma cell differentiation. B cells were cultured with LPS plus IL-4 in the presence of HDI (VPA, 500 μM) or nil for 60 h before preparation of RNA for mRNA-Seq. **(A)** Scatter plots of average mRNA expression levels in three independent experiments (reads per kilobase per million mapped reads, RPKMs), in LPS plus IL-4-stimulated B cells treated with VPA versus those in LPS plus IL-4-stimulated B cells treated with nil. Each plot corresponds to one individual mRNA expression level. **(B)** Bar graphs depict the average folds of changes of mRNA expression levels (average RPKMs from three independent experiments) in LPS plus IL-4-stimulated B cells treated with HDI, as compared to those in B cells treated with nil. Only the mRNAs at average RPKM >20 in LPS plus IL-4-stimulated B cells treated with nil are included. *Aicda, Prdm1*, and *Xbp1* mRNAs and the mRNAs that were increase or decreased or increased in average by more than twofold are indicated. *p* values, paired *t*-test.

HDI Does Not Alter the Expression of 14-3-3 Adaptors or Rab7

The 14-3-3 adaptor family consists of a class of phosphoserine/phosphothreonine (pSer/Thr)-binding proteins, which include seven isoforms (14-3-3β, 14-3-3ε, 14-3-3γ, 14-3-3η, 14-3-3σ, 14-3-3τ, and 14-3-3ζ) encoded by different genes. 14-3-3 proteins are involved in a variety of cellular processes, including gene regulation, differentiation, and cell cycle progression (28). As we have shown, 14-3-3 adaptors play an important role in targeting the CSR machinery to S regions by virtue of their ability to bridge proteins with DNA or other proteins (29, 30). They directly interact with AID, protein kinase A catalytic subunit-α (PKA-Cα), regulatory inhibitory subunit-α (PKA-RIα), and uracil DNA glycosylase (Ung) to function as scaffolds to stabilize these enzymatic CSR elements on S regions. 14-3-3 expression has been suggested to be regulated by posttranscriptional modulation. 14-3-3 can interact with the phosphorylation sites of HDAC4, 5, and 7 and regulate cellular localization of these HDACs (31). As we have shown, the expression of most 14-3-3 proteins is significantly upregulated by the induction of CSR in B cells (2, 29, 30, 32). To determine whether potential alterations of 14-3-3 proteins in B cells could contribute to the HDI-mediated inhibition of CSR, we analyzed our mRNA-Seq data on the seven 14-3-3 isoforms. None of the seven 14-3-3 isoforms were significantly altered by HDI in B cells stimulated by LPS plus IL-4 (**Figure 3D**). 14-3-3ζ expression can be modulated by miR-193b and miR-375, which target 3′ UTR of 14-3-3ζ mRNA, in cancer cells (33, 34). However, these two miRNAs were not expressed in LPS plus IL-4-stimulated B cells (not shown).

We have recently demonstrated that Rab7, a small GTPase, plays an important role in CSR, through activation of the canonical NF-κB pathway and induction of AID expression (35). Like 14-3-3 adaptors, Rab7 expression is upregulated by the stimuli that induce CSR in B cells. To analyze whether the HDI-mediated downregulation of AID was at least partially due to a potential alteration of Rab7 expression, we analyzed Rab7 mRNA levels in B cells treated with HDI or nil. As shown by mRNA-Seq, HDI did not significantly alter Rab7 expression (**Figure 3E**). In one of the three experiments, Rab7 mRNA levels were virtually the same in B cells treated with HDI or nil. In the other two experiments, Rab7 mRNA was even slightly ($p = 0.20$) increased by HDI. Thus, downregulation of the AID-targeting 14-3-3 adaptors or downregulation of the CSR-regulating Rab7 small GTPase play no role in HDI-mediated modulation of CSR.

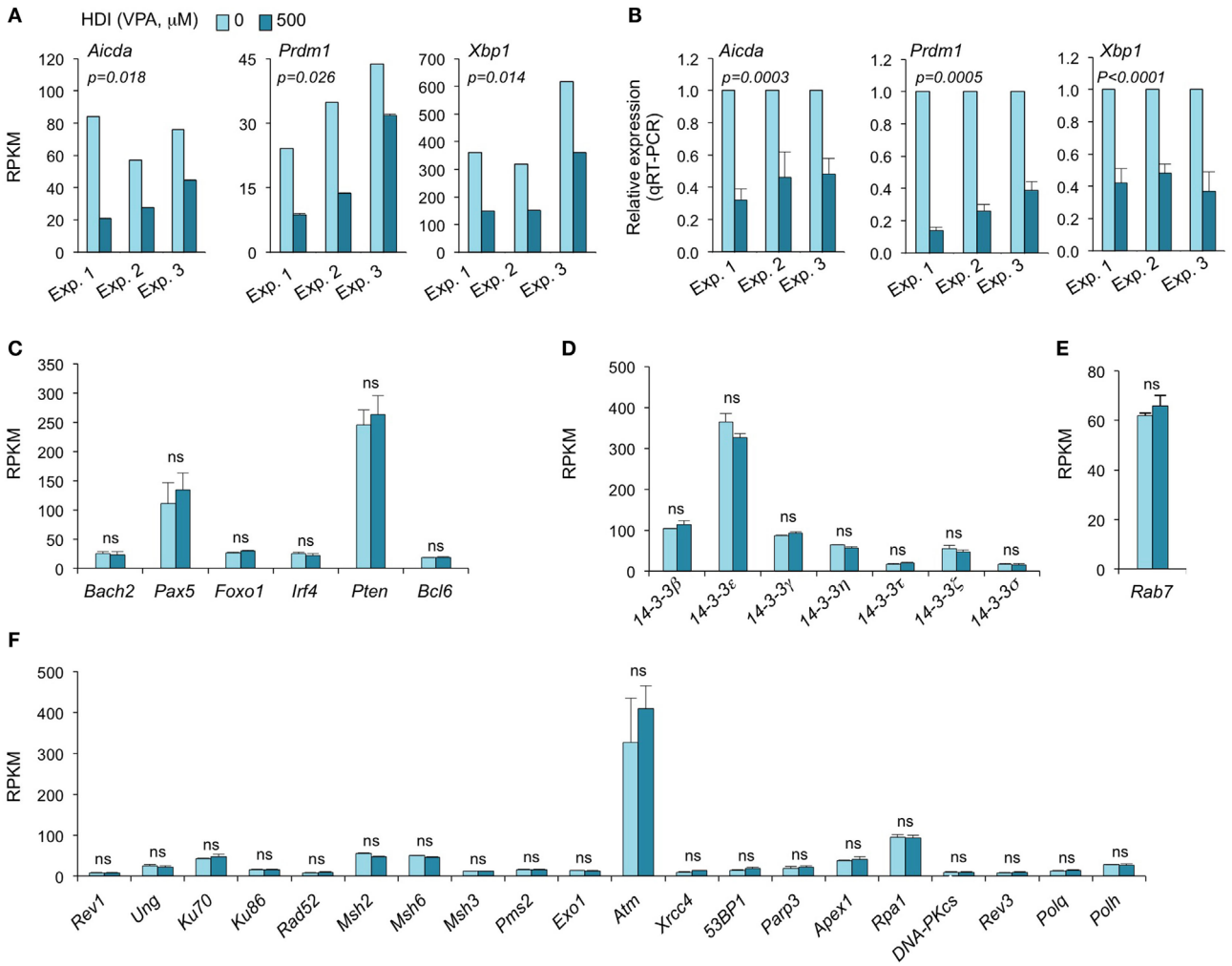

FIGURE 3 | HDI inhibits the expression of *Aicda*, *Prdm1*, and *Xbp1*, but not other elements that are important for CSR/SHM and plasma cell differentiation. B cells were stimulated with LPS plus IL-4 for 60 h, in the presence of HDI (VPA, 500 μM) or nil. **(A)** Expressions of *Aicda, Prdm1*, and *Xbp1* were mRNAs analyzed by mRNA-Seq. Data are from three independent experiments. **(B)** qRT-PCR validation of *Aicda, Prdm1*, and *Xbp1* expressions. Data are from three independent experiments. *p* values, paired *t*-test. Expressions of **(C)** *Bach2, Pax5, Foxo1, Irf4, Pten*, and *Bcl6,* **(D)** the seven 14-3-3 isoforms, **(E)** Rab7, and **(F)** the genes that encode the proteins that have been shown to play important roles in CSR and/or SHM were analyzed by mRNA-Seq and depicted as RPKM. Data are from three independent experiments. ns, not significant, paired *t*-test.

HDI Does Not Alter the Expression of DNA Repair Factors that Are Important for CSR and SHM

Class-switch DNA recombination and SHM are tightly regulated and both are effected by a two-step process: (i) DNA lesions initiated by AID and (ii) lesion repair by the combined intervention of DNA replication and repair factors (2, 36, 37). Many DNA repair factors, including the base excision repair factor Ung, mismatch repair factors Msh2, Msh3, Msh6, Pms2, and Exo1 nuclease, translesion synthesis (TLS) DNA polymerases Rev3, Rev1, Polθ, and Polη, as well as DSB repair factors Ku70/Ku80, Rad52, RPA, and DNA-PK play important roles in CSR and/or SHM (2, 37–41). Dysregulation of these DNA repair factors can result in altered CSR/SHM, and thereby the antibody response. To define whether the HDI-mediated reduction of CSR and plasma cell differentiation was associated with any alteration in these factors, we analyzed the mRNA-Seq data for the expression of these factors. None of them was significantly altered by HDI (**Figure 3F**).

HDI Does Not Significantly Alter the Expression of Epigenetic Regulators HATs, HDACs and Tet Proteins

In addition to inhibiting catalytic activity of HDACs, HDI have been suggested to selectively change the expression of some epigenetic regulators in certain type of cells. SAHA, a pan HDAC inhibitor, has been shown to downregulate HDAC7 expression in fibroblast cell lines (42). MS-275, TSA, and VPA downregulate

DNMT1 protein expression in testis and embryonal carcinoma, as butyrate, SAHA, and PD98059 do in LNCaP prostate cancer cells (43). To determine whether HDI alter the expression of HDACs or DNMTs, as well as HATs and Tet1/Tet2/Tet3, which also mediate histone acetylation and DNA methylation, respectively, we analyzed the mRNA levels of these genes in our mRNA-Seq data (**Figure 4**). Thirty-six out of the 42 gene transcripts analyzed were not significantly altered by HDI, while *HDAC1, HDAC6, Clock*, and *Tet2* were marginally increased (by 24.63, 36.64, 9.57, and 23.19%, respectively), and *Sirt1* and *Crebbp* were slightly reduced (by 16.54 and 10.11%, respectively) by HDI. Thus, epigenetic regulator genes are not significantly modulated by HDI in B cells undergoing CSR and plasma cell differentiation.

HDI Does Not Alter the Genes that Are Important in Cell Apoptosis

We have recently found that HDI inhibit, in a dose-dependent fashion, CSR and plasma cell differentiation without altering B cell proliferation or B cell and plasma cell viability (16). We have shown by qRT-PCR that the expression of the anti-apoptotic genes *Bcl2*, *Mcl1*, and *Bcl2l1*, which enhance B cell and plasma cell survival, was unaltered or increased by HDI *in vivo* and *in vitro* (16). Consistent with these findings, our mRNA-Seq data show that the expressions of all these genes were not altered by HDI ($p = 0.28$, 0.21, or 0.27) (**Figure 5A**). In addition, other 19 anti-apoptotic genes and 22 pro-apoptotic genes were also unchanged by HDI (**Figures 5A,B**). Thus, these findings further demonstrate that HDI significantly reduce CSR and plasma cell differentiation, without altering cell viability.

HDI Does Not Significantly Alter TLR Expression and the Genes Involved in NF-κB Signaling

Toll-like receptors (TLRs) are a family of conserved pattern recognition receptors that sense diverse types of microbe-associated molecular patterns (MAMPs). Engagement of B cell TLRs by MAMPs not only induces T-independent antibody responses but also plays an important role in the early stages of T-dependent antibody responses, before specific T cell help becomes available (44). We have shown that TLR1/2-, TLR4-, TLR7-, or TLR9-signaling synergizes with BCR-signaling, which enhances TLR-dependent activation of the canonical NF-κB pathway, to induce AID and enable CSR (20). Given the important role of TLR-signaling and NF-κB pathway in the induction of AID, an alteration in the expression of TLR and the factors that are involved in NF-κB pathway would result in a change of AID expression and CSR. As shown by mRNA-Seq, the expression of 13 TLRs and 27 genes that are involved in NF-κB-signaling pathway was not altered by HDI (**Figures 5C,D**).

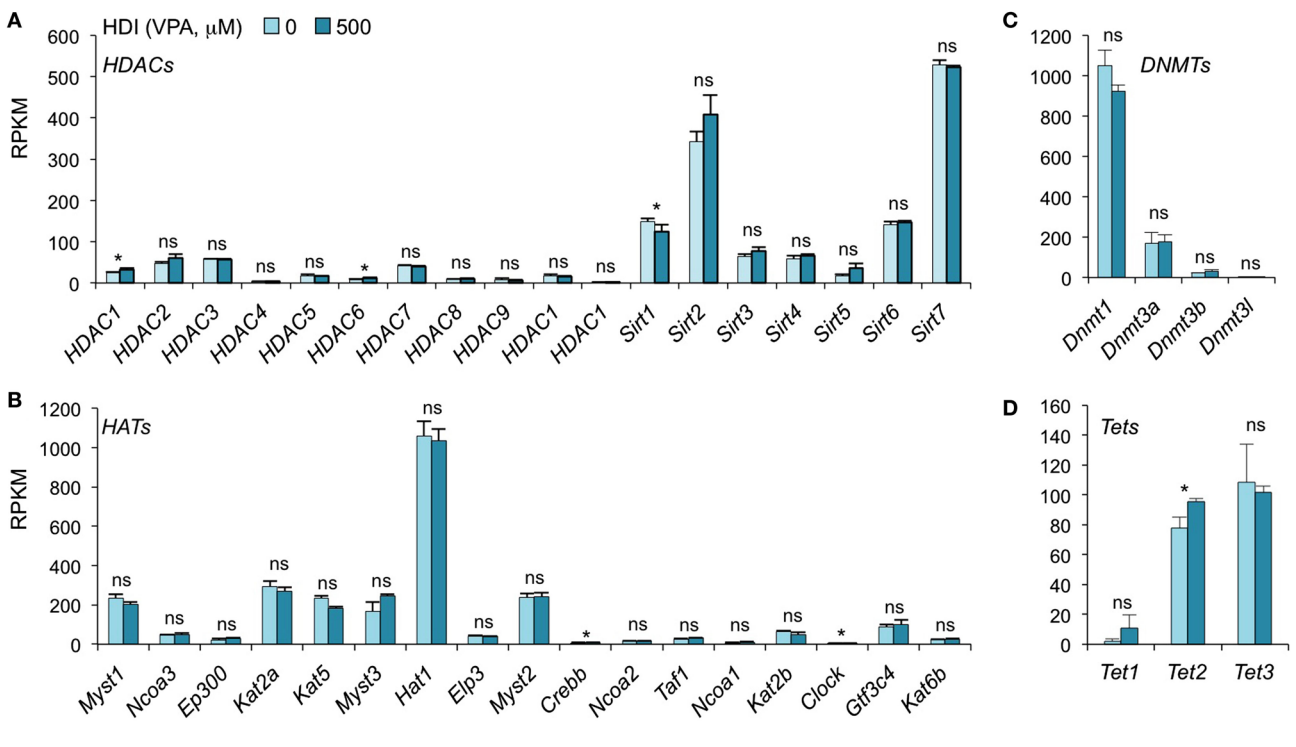

FIGURE 4 | HDI does not alter the expression of the epigenetic regulators HDACs, HATs, DNMTs, and Tet proteins. B cells were stimulated with LPS plus IL-4 for 60 h, in the presence of HDI (VPA, 500 μM) or nil. mRNA expressions of the genes that encode **(A)** HDACs and **(B)** HATs, which modulate histone acetylation, and **(C)** DNMTs, as well as **(D)** Tet1, Tet2, and Tet3, which modulate DNA methylation, were analyzed by mRNA-Seq and depicted as RPKM. Data are from three independent experiments. *$p < 0.05$; ns, not significant, paired *t*-test.

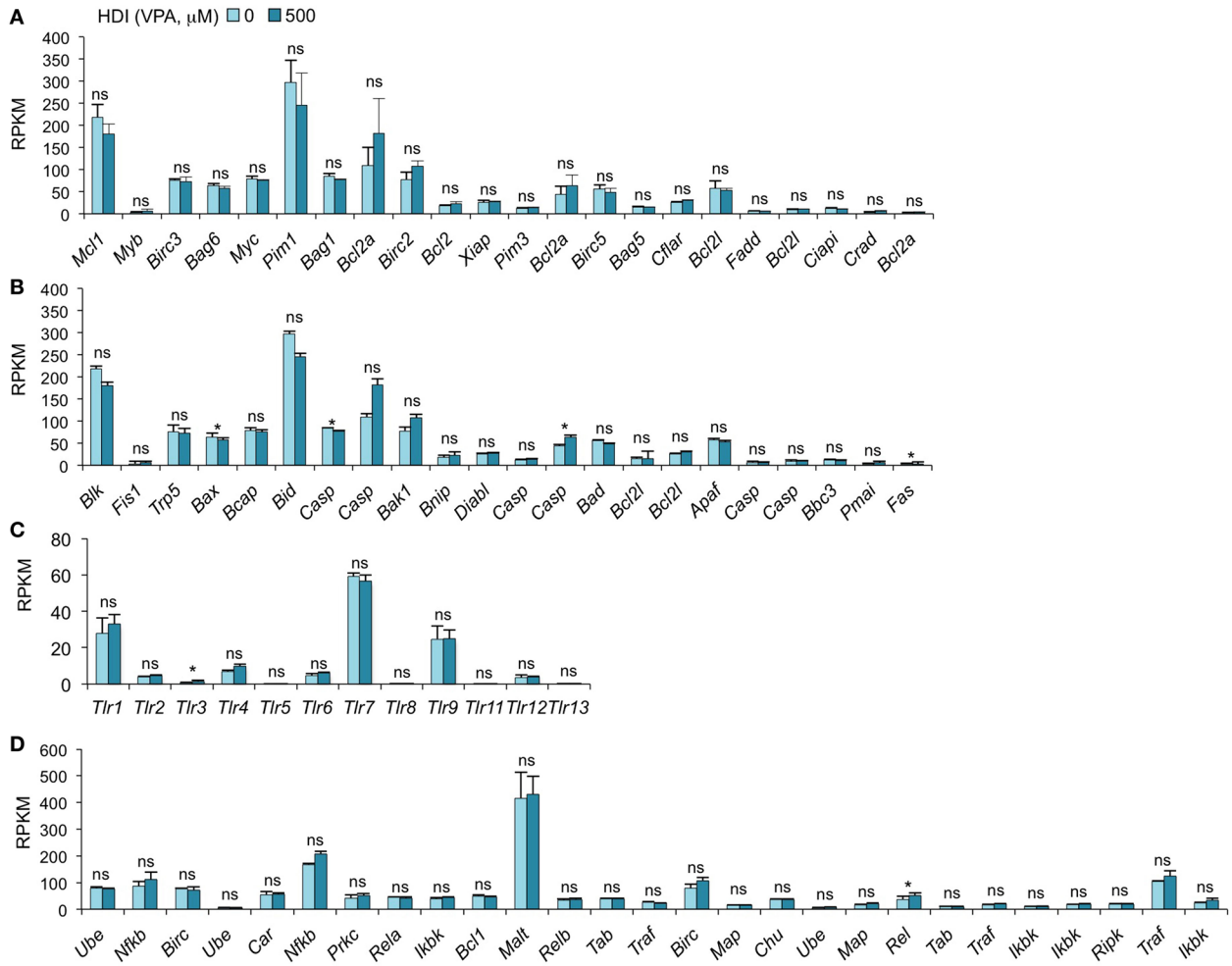

FIGURE 5 | HDI does not significantly alter the expression of the genes that are important for apoptosis, the genes that encode TLRs and the genes that are involved in NF-κB signaling. B cells were stimulated with LPS plus IL-4 for 60 h, in the presence of HDI (VPA, 500 μM) or nil. mRNA expressions of the genes that encode **(A)** anti-apoptotic factors, **(B)** pro-apoptotic factors, **(C)** Toll-like receptors, and **(D)** the factors that are involved in NF-κB-signaling pathway were analyzed by mRNA-Seq and depicted as RPKM. Data are from three independent experiments. *$p < 0.05$; ns, not significant, paired *t*-test.

HDI Inhibits Expression of Selected mRNAs in B Cells

In B cells induced to undergo CSR and plasma cell differentiation, the number of genes that were downregulated by HDI nearly equated that of genes that were upregulated by HDI (**Figure 2**), suggesting that HDI can modulate gene expression by a mechanism other than directly increasing histone acetylation. Indeed, generally, only a small number of genes are thought to be directly modulated by changes in histone acetylation. Thus, it is possible that HDI upregulate the expression of genes, which negatively regulate the expression of other genes. HDI can modulate the expression of miRNAs, which silence target mRNAs by inducing their degradation and/or reducing their translation. We have recently shown that HDI downregulated the expression of AID and Blimp-1 by upregulating miR-155, miR-181b, and miR-361, which silence *Aicda* mRNA, and miR-23b, miR-30a, and miR-125b, which silence *Prdm1* mRNA, but not miR-19a/b, miR-20a, and miR-25, which are not known to regulate *Aicda, Prdm1*, or *Xbp1* (16). To further define the modulation of miRNA by HDI, we analyzed microRNAome in LPS and IL-4 stimulated B cells treated with HDI or nil by miRNA-Seq (**Figure 6**). In stimulated B cells, the average RPM of 520 miRNAs analyzed was 26.9 (10.75, if one excludes the three most abundant miRNAs miR-21a, miR-191, and miR-146a). Upon HDI treatment, the average RPM of all the miRNAs were slightly increased to 32.19 (13.63, if we exclude the three most abundant miRNAs miR-21a, miR-191, and miR-146a) ($R^2 = 0.9811$, $p = 0.5134$). A total of 185 of these miRNAs had, in average, 0.5 copy per cell. Among these 185 miRNAs, only 6 of them were reduced by 50% or more, and 26 of them were upregulated by more than twofold by HDI (**Figure 6**). Thus, the HDI-mediated modulation of miRNA expression in B cells undergoing CSR and plasma cell differentiation is selective.

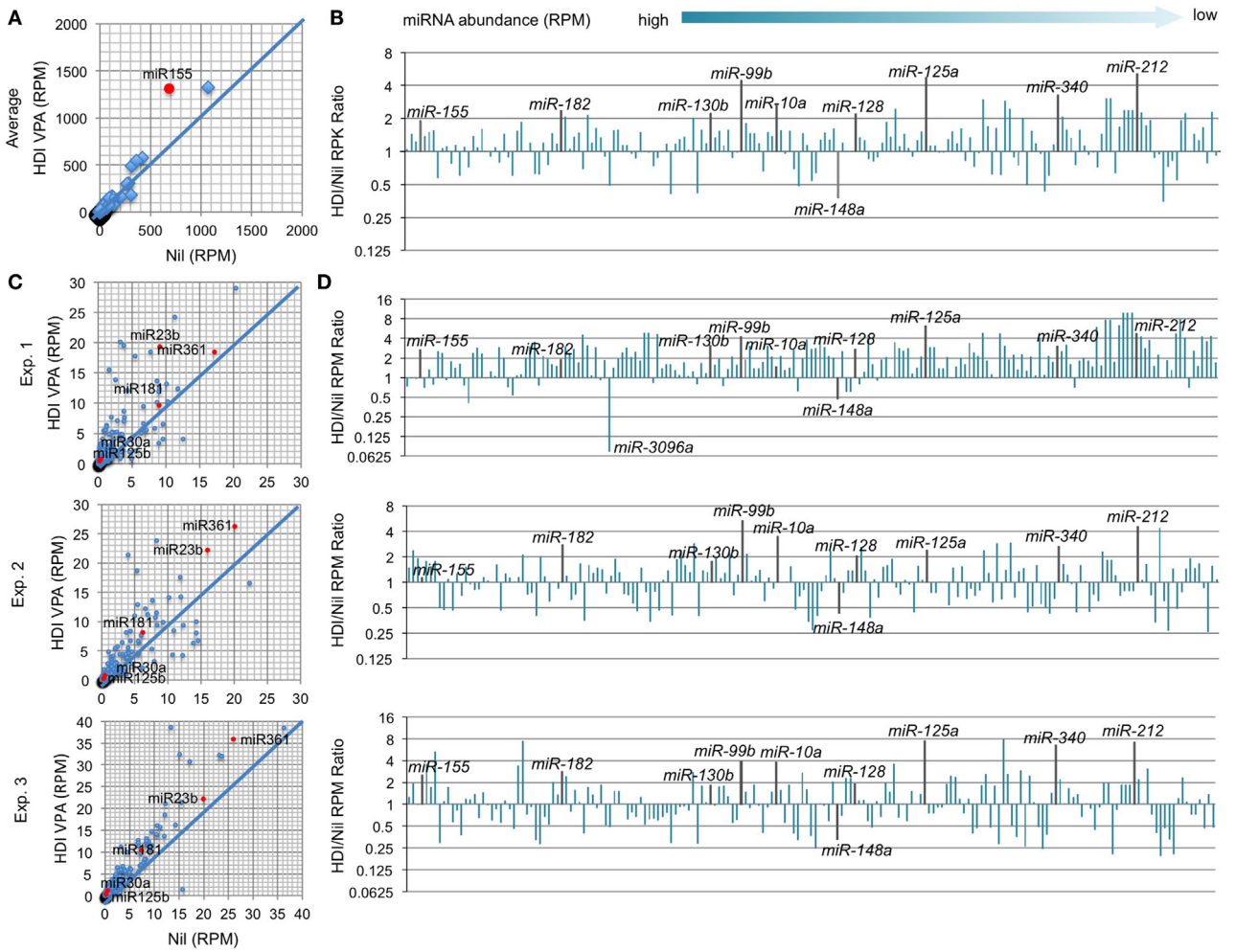

FIGURE 6 | HDI selectively inhibits miRNA expression in B cells induced to undergo CSR and plasma cell differentiation. B cells were cultured with LPS plus IL-4 in the presence of HDI (VPA, 500 μM) or nil for 60 h before preparation of RNA for miRNA-Seq. **(A)** Scatter plots of average (in three independent experiments) miRNA expression levels (RPMs) in B cells treated with HDI versus that in B cells treated with nil. *P* values, paired *t*-test. **(B)** Bar graphs depict the changes of folds of the average miRNA expression levels (RPMs) in B cells treated with HDI as compared to that in B cells treated with nil. **(C)** Bar graphs depict the changes of folds of miRNA expression levels (RPMs) in B cells treated with HDI as compared to that in B cells treated with nil, in three independent experiments. **(D)** Scatter plots of miRNA expression levels in B cells treated with HDI versus those in B cells treated with nil, in three independent experiments. Only the miRNAs at average RPM > 0.5 in LPS plus IL-4-stimulated B cells treated with nil are included.

HDI Upregulates Selected miRNAs that Target *Aicda*

We have shown by qRT-PCR that miR-155, miR-181b, and miR-361, which silence AID by targeting *Aicda* 3′ UTR, were significantly upregulated by HDI (16). The HDI-mediated upregulation of these miRNAs, particularly, miR-155 was validated by miRNA-Seq in three independent experiments (**Figure 7**). miR-155 targets a highly conserved site in the 3′ UTR of *Aicda* mRNA in several different species (45, 46). As shown by miRNA-Seq analysis, miR-155 is one of the most abundant miRNAs expressed in B cells after stimulation by LPS plus IL-4 (**Figure 6**). In such B cells, the average RPM of miR-155 was 684.9 (572.0, 981.7, and 501.0 in three independent experiments, respectively), which is more than 25 times higher than the average RPM of all the 520 miRNAs analyzed. Upon treatment with HDI, miR-155 expression was increased by more than 1.9-fold ($p = 0.032$) (**Figure 7**). miR-155 is encoded by the miR155 host gene *miR155HG*. *miR155HG* was originally identified as a gene that was transcriptionally activated by promoter insertion at a common retroviral integration site in B cell lymphomas and was formerly referred to as *Bic* (*B* cell *i*ntegration *c*luster) (13). Consistent with our qRT-PCR results (16), the mRNA-Seq data showed that HDI-mediated upregulation of miR-155 was associated with an increase of primary *miR-155HG* transcript (**Figure 7C**). In addition to the targeting sites for miR-155, miR-181b, and miR-361, the 3′ UTR of mouse *Aicda* mRNA also contains the putative target sites for miR-125a, miR-351, miR-92b, miR-26a, and miR-103 (identified by using miRNA-targeting

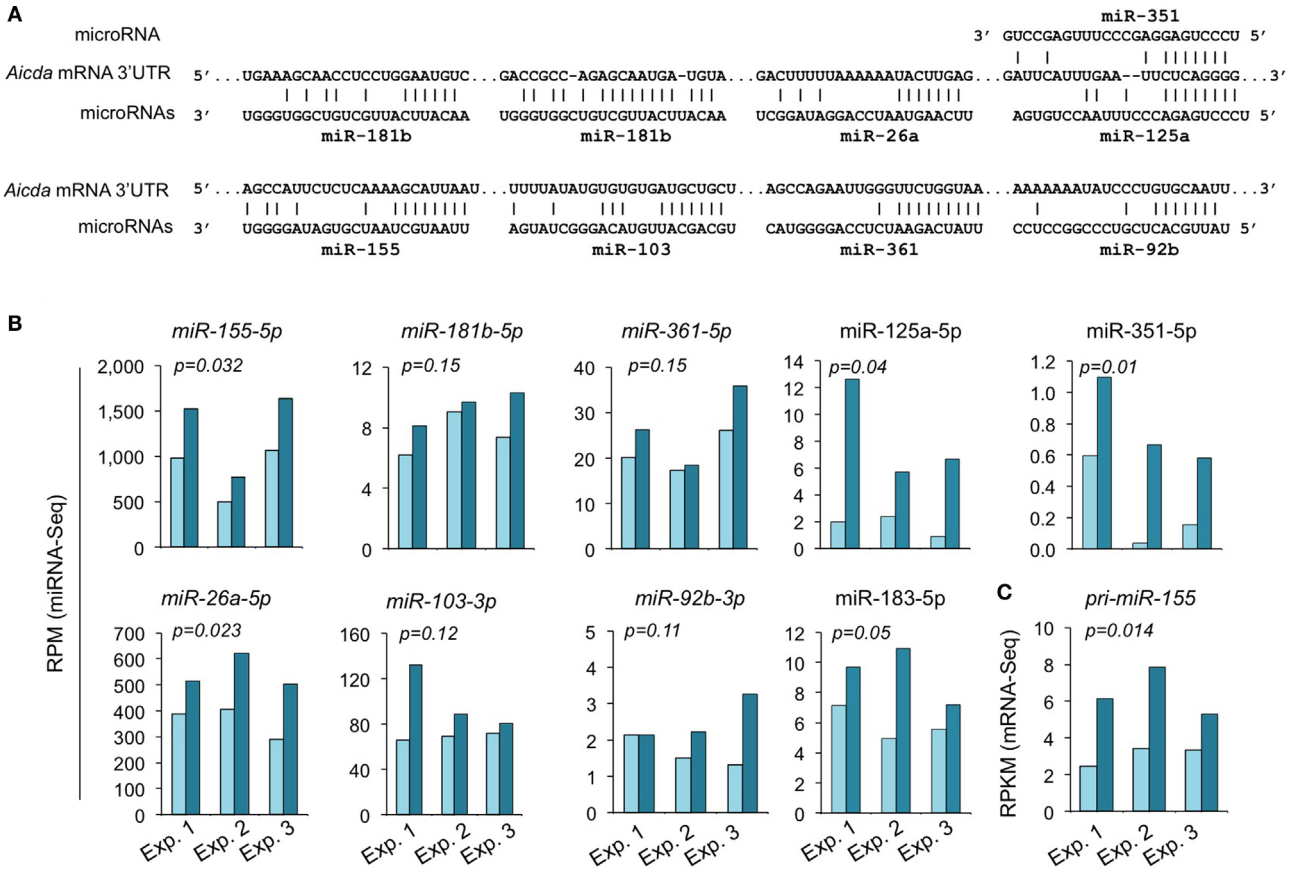

FIGURE 7 | HDI upregulates the miRNAs that target *Aicda*. **(A)** Alignment of the miRNAs and their target sites in the 3′ UTR of *Aicda* mRNAs. **(B)** Expression of miRNAs that modulate AID or are predicated to modulate AID in B cells cultured with LPS plus IL-4 in the presence of HDI (VPA, 500 μM) or nil for 60 h were analyzed by miRNA-Seq and depicted as RPM. **(C)** Primary (pri-) miRNA transcripts of miR-155 in B cells cultured for 60 h with LPS plus IL-4 in the presence of nil or HDI were analyzed by mRNA-Seq and depicted as RPKM. Data are from three independent experiments. *p* values, paired *t*-test.

prediction tools: TargetScan.org, miRNA.org, and miRbase.org). These miRNAs were also upregulated by HDI. Thus, HDI upregulate miRNAs that silence *Aicda* and do so by increasing the primary transcripts of host genes of these miRNAs.

HDI Upregulate miRNAs that Target *Prdm1*

We have shown that HDI significantly downregulated *Prdm1* expression, plasma cell differentiation, and antibody/autoantibody production (16). The greatly reduced plasma cell differentiation was associated with downregulation of the Ig J chain (*IgJ*) gene, which is expressed only after B cells terminal differentiation into plasma cells (47). IgJ is incorporated into an IgM pentamer or an IgA dimer and is necessary for both the cellular and mucosal secretion of antibodies. In B cells stimulated by LPS plus IL-4 for 60 h, the average RPKM of *IgJ* mRNA was 344.6 (395.1, 175.6, and 463.2 in three independent experiments, respectively). HDI reduced *IgJ* expression (RPKM) by more than 3.3-fold to average 103.6 (77.3, 46.2, and 187.3, in three independent experiments, respectively) (**Figure 2**). Both human *PRDM1* and mouse *Prdm1* mRNA have a long (2,453 bp) 3′ UTR, including putative miRNA-targeting sites, which are evolutionary conserved. By using miRNA-targeting prediction tools (TargetScan.org, miRNA.org, and miRbase.org), we identified miR-125a, miR-125b, miR-96, miR-351, miR-30, miR-182, miR-23a, miR-23b, miR-200b, miR-200c, miR-33a, miR-365, let-7, miR-98, miR-24, miR-9, miR-223, and miR-133 as *PRDM1/Prdm1* targeting miRNAs in both the human and the mouse. With exception of miR-33a, miR223, miR-9, miR-24, and miR-429, whose expression level was low in activated B cells, such *Prdm1*-targeting miRNAs were significantly upregulated by HDI. We have shown by qRT-PCR that miR-23b, miR-30a, and miR-125b, which silence Blimp-1 by targeting *Prdm1* 3′ UTR, were significantly upregulated by HDI (16). The upregulation of these miRNAs was further validated in B cells by miRNA-Seq in three independent experiments (**Figure 8**). This likely resulted from increased primary miRNA transcripts, as suggested by the upregulation of *pri-miR-23b*.

The miR-30 family consists of five miRNAs (miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e) encoded by different host genes. The miR-30 family members are similar to each other and have identical seed sequences. Like human *PRDM1* (48), the 3′ UTR of mouse *Prdm1* mRNA contains three highly conserved bindings sites complementary to the seed sequence of miR-30a

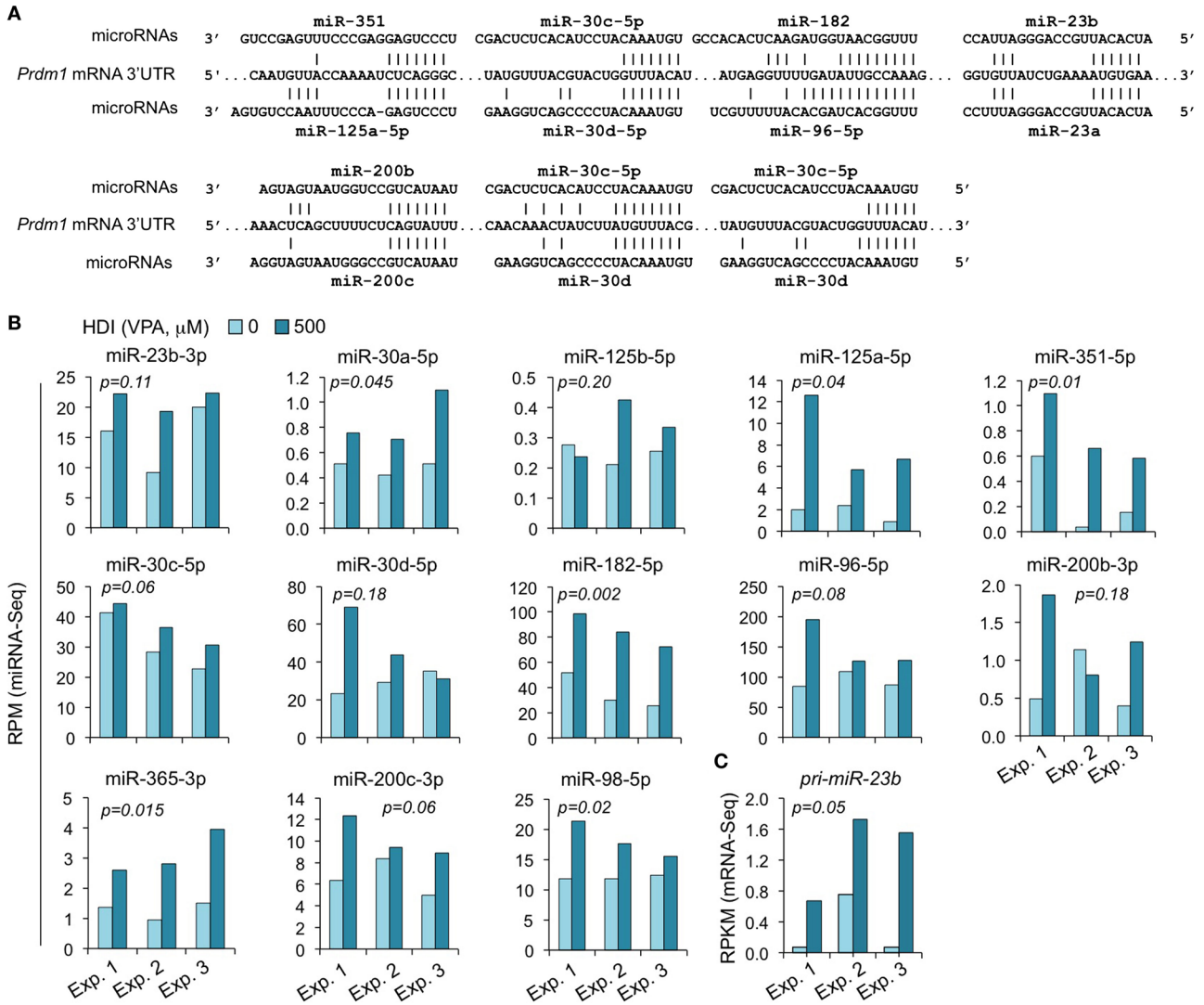

FIGURE 8 | The *Prdm1* targeting miRNAs miR-23b, miR-125a, miR-351, miR-30a/c/d, miR-182, miR-96, miR-98, miR-200b/c, and miR-365 are upregulated by HDI. **(A)** Alignment of the 3′ UTR of *Prdm1* mRNA and the targeting miRNAs. **(B)** Expressions of the miRNAs miR-23b, miR-125a, miR-351, miR-30a/c/d, miR-182, miR-96, miR-98, miR-200b/c, and miR-365 that target or are predicted to target *Prdm1* 3′ UTR, as well as miR-183, which is expressed in a same cluster with miR-182, in B cells cultured with LPS plus IL-4 in the presence of HDI (VPA, 500 μM) or nil for 60 h were analyzed by miRNA-Seq and depicted as RPM. **(C)** Primary (pri-) miRNA transcripts of miR-23b in B cells cultured with LPS plus IL-4 in the presence of nil or HDI for 60 h were analyzed by mRNA-Seq and depicted as RPKM. Data are from three independent experiments. *p* values, paired *t*-test.

and other miR-30 family members (**Figure 8**). All the five miR-30 miRNAs were expressed in B cells stimulated by LPS plus IL-4. The abundance of miR-30b, miR-30c, miR-30d, and miR-30e were greater than that of miR-30a (**Figure 8**). miR-30c was upregulated by HDI in all the three experiments, miR-30d was upregulated in two of the three experiments, while miR-30b and miR-30e were upregulated in one of the three experiments but were downregulated in the other two experiments.

miR-125 is a an evolutionarily conserved miRNA family consisting of three paralogs, including miR-125a, miR-125b-1, and miR-125b-2 (miR-125b). Recent studies have presented strong evidence for a role of the miR-125 family in the immune response. miR-125a shares the same seed sequence with miR-125b. Like miR-125b, miR-125a also potentially targets *Prdm1* in both human being and mouse, as predicted by the sequences (**Figure 8**). The abundance of miR-125a in B cells induced to undergo CSR and plasma cell differentiation by LPS plus IL-4 was much greater than that of miR-125b, as in all three experiments. In the presence of HDI, miR-125a expression was increased by up to 7.5-fold, perhaps suggesting a more important role of this miRNA than miR-125b in modulating *Blimp1* expression. miR-125a and miR-351 contain the same seed sequence as miR-125b, and therefore potentially target *Prdm1* 3′ UTR at the same site as miR-125b. Likewise, HDI upregulated miR-351 expression by

up to 16.5-fold. miR-98 potentially target the same site as let-7 in *Prdm1* 3′ UTR. While let-7 miRNAs were not consistently altered by HDI, miR-98 was significantly upregulated by HDI in B cells in all the three experiments of CSR/plasma cell differentiation induction ($p = 0.02$).

miR-182, miR-96, and miR-183 belong to a polycistronic miRNA cluster that is located within a 4-kb area on mouse chromosome 6q. These miRNA siblings share similar seed sequences; in fact, the seed sequences of miR-96 and miR-182 are identical. As predicted using online miRNA analyze tools, TargetScan.org, miRNA.org, and miRbase.org, in both mouse and human, miR-182 and miR-96 can potentially target *Prdm1/PRDM1* 3′ UTR at the same site (**Figure 8**). In B cells stimulated with LPS plus IL-4, miR-182, miR-96, and miR-183 were all highly expressed. All these three miRNAs were upregulated by HDI. Because the precursors of miR-96, miR-182, and miR-183 are transcribed as a single transcript, these findings further support the contention that HDI modulate miRNA expression through regulation of their primary transcript (16). Thus, these experiments showed that HDI upregulate the miRNAs that target *Prdm1*, possibly by increasing the primary transcripts of host genes of these miRNAs.

DISCUSSION

Epigenetic marks/factors, such as histone posttranslational modifications, DNA methylation, and non-coding RNAs, including miRNAs, play important roles in the complex interplay between genes and environment. As we have suggested, they "interact" with genetic programs to regulate B cell functions, including CSR, SHM, and plasma cell differentiation, thereby informing the antibody response (1, 2, 7, 49). Epigenetic dysregulation can result in aberrant antibody responses and compound genetic susceptibility to mediate autoimmunity (7, 49). We have recently shown that HDI epigenetic modulators inhibited CSR, SHM, and plasma cell differentiation by modulating intrinsic B cell mechanisms (16). HDI repressed AID and Blimp-1 expression in human and mouse B cells by upregulating selected miRNAs that silenced *AICDA/Aicda* and *PRDM1/Prdm1* mRNAs, as demonstrated by multiple qRT-PCRs. In this study, we performed high throughput miRNA-Seq and mRNA-Seq to further define the HDI-mediated modulation of miRNA and gene expression. We showed here that HDI selectively upregulated miRNAs involved in targeting and modulating genes whose expressions are critical for B cells to undergo CSR and plasma cell differentiation. The selective upregulation of miRNAs and mRNAs by HDI was emphasized by unchanged expression of miRNAs, which are not known to regulate *Aicda* or *Prdm1*, the master genes for CSR/SHM or plasma cell differentiation, and unchanged expression of the genes that are involved in regulation, targeting, or DNA repair processes in CSR/SHM, as well as the genes of epigenetic regulators and the factors that are important for cell signaling and apoptosis. Consistent with the notion that HDI downregulate mRNA expression by upregulating selective miRNAs, HDI slightly reduced average RPKM of overall mRNA, in association with a slightly increased overall miRNA expression. This study further extends our previous findings and outlines more precisely epigenetic mechanisms that are critical to the B cell differentiation processes that underpin antibody and autoantibody responses.

In spite of the broad distribution of HDACs in chromatin, our findings showed that HDI-mediated modulation of miRNA and mRNA expression is very selective. Upon exposure to HDI, 18 genes were upregulated by more than twofold in B cells induced to undergo CSR and plasma cells differentiation. These genes, included *Zbtb20* (Zinc finger and BTB domain-containing protein 20), a Bcl6 homolog, which is highly expressed in activated B cells and memory B cells and has been shown to regulate long-term antibody production through a B cell-intrinsic mechanism (50, 51), and *Syt11* (synaptotagmin-11), which is specifically expressed in memory B cells (52), suggesting that HDI can modulate the memory B cell response. Sixteen genes were downregulated by HDI by more than 50%. More than half of these downregulated genes, including *Bhlha15, Rhob, Fkbp11, Ppapdc1b, Rcbtb2, Ly6c2, Txndc5*, and *IgJ*, are preferentially expressed in plasma cells rather than naïve, germinal center, or memory B cell (52) (http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS1695). This may imply that, in addition to the inhibition of AID and Blimp-1 expression, alteration of the expression of other genes that are involved in B cell differentiation could also contribute to HDI-mediated modulation of the antibody response. Alternatively, the reduction of these "plasma cell specific" genes may simply result from the inhibition of plasma cells differentiation by HDI, further supporting the selectivity of HDI-mediated modulation of mRNA expression. HDI modulated CSR, SHM, and plasma cells differentiation, and, therefore, the antibody response mainly through downregulation of *Aicda, Prdm1*, and *Xbp1*. However, the HDI-mediated downregulation of *Cxcr1*, which is preferentially expressed in $IgG1^+$ memory B cells and promotes IgG1 autoantibody production (25, 26), and *Saa3*, which can interact with Tlr4 and induce Tlr4-mediated NF-κB activation (27), suggest that downregulation of these elements can contribute to HDI-mediated modulation of antibody response. The reduction of *Cxcr3, Fut1*, and *Rhobtb1* expression was associated with an increased expression of miR-148b, miR-125a, and miR-182, which target *Cxcr3*, *Fut1*, and *Rhobtb1* mRNAs, respectively, suggesting that, in addition to *Aicda* and *Prdm1*, which are already downregulated by HDI, other genes can also be downregulated by HDI through upregulation of their targeting miRNAs. This does not, however, exclude the possibility that the HDI-mediated reduction of gene expression may at least partially result from altered expression or activation of other B cell factors.

A total of 18 HDACs, which are not functionally redundant, have been identified in human beings and mice (12). These 18 HDACs are grouped into four classes based on their function and sequence similarity (12). Classes I, II, and IV consist of 11 HDACs that require zinc as a cofactor. Class I includes HDAC1, HDAC2, HDAC3, and HDAC8, which display homology to yeast RPD3; Class IIa includes HDAC4, HDAC5, HDAC7, and HDAC9, which display homology to yeast HDA1; Class IIb includes HDAC6 and HDAC10, which contain two catalytic sites. Class IV includes only HDAC11, which displays conserved residues in

its catalytic center, shared by both class I and class II HDACs. Class III HDACs or Sirtuins (Sirt1-7) display homology to yeast Sir2, retain NAD-dependent catalytic sites and share some functions with the classical HDACs. Unlike the classes I, II, and IV HDACs, Sirtuins are not zinc dependent and cannot be inhibited by conventional HDI, such as VPA and butyrate. They may function differently from class I/II HDACs in the regulation of the antibody response. Indeed, activation of Sirt1 by resveratrol has been shown to lead to a reduced production of IgG1 and IgG2a in pristane-induced lupus mice (53) as well as antigen-specific IgE in OVA-immunized mice (54).

Our findings showed that HDI selectively downregulated the expression of those genes that are central to CSR and plasma cell differentiation processes, that is, *Aicda* encoding AID cytidine deaminase and *Prdm1* encoding Blimp-1. They did not, however, downregulate the expression of 14-3-3 adaptors, which, as we showed, are upregulated by the stimuli that induce CSR and are important for AID targeting to S regions in CSR (2, 29, 30, 32). Rab7, an effective multifunctional regulator of autophagy, which activates the canonical NF-κB pathway to induce AID expression (35), was also unchanged by HDI. In addition, DNA repair factors, such as Ung and TLS DNA polymerases, which play important roles for CSR and SHM, were also not altered by HDI. Further, epigenetic regulators HATs and HDACs, and Tet proteins, genes that are important in cell apoptosis, such as *Mcl1, Bcl2*, and *Bcl2l1,* TLRs, and genes involved in NF-κB signaling also remained unchanged in B cells induced to undergo CSR. The selective regulation of gene expression by HDI was consistent with what have been reported in other type of cells (55–58), and the level of changes in transcription was associated with the type and doses of HDI used and the time of culture (23). Although most of the available HDI do not have a high HDAC isoform specificity, SCFA HDI have been suggested to display significant selectivity for different HDACs. For example, VPA targets class I HDACs, particularly, HDAC1 and HDAC2, and, less effectively, class IIa HDACs, butyrate targets class I, mainly HDAC1, and, less effectively, other members of class I and class IIa HDACs. Like HATs, HDACs do not directly bind to DNA, rather, they interact with DNA through multi-protein complexes that include coactivators and corepressors, the role and composition of which are often cell type-specific (59). HDAC-associated proteins would specify the selectivity of HDI, which display different affinities for different HDAC/cofactor complexes. HDI with diverse chemical properties target different HDACs and HDAC/cofactor complexes, thereby regulating gene expression in a locus- and cell type-specific fashion. Thus, the different HDAC-associated proteins and the different HDAC/cofactor complexes would provide the mechanistic underpinning for the selectivity of HDI for specific B cell differentiation genes, as we have shown here.

PRDM1/Prdm1 mRNA contains a long (2,453 bp) conserved 3′ UTR, which comprises putative target sites for multiple miRNAs. In addition to miR-23b, miR-30a, and miR-125b, which, as we showed by qRT-PCR and miRNA-Seq, are upregulated by HDI, several other putative *Prdm1* targeting miRNAs, including miR-125a, miR-96, miR-351, miR-30c, miR-182, miR-23a, miR-200b, miR-200c, miR-365, let-7, miR-98, and miR-133, were also significantly increased by HDI. miR-182 has been identified as the miRNA induced at a high level in B cells stimulated to undergo CSR (60); however, deficiency of this miRNA did not significantly alter the titers of total serum IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE, and NP-binding IgG1 in mice immunized with NP-CGG (60). miR-182 is a member of the miR-183~182 cluster which includes miR-96, miR-182, and miR-183. Like miR-182, miR-96, which, based on its sequence, could target all putative miR-182 targeting sites, is also highly expressed by B cells induced to undergo CSR and plasma cell differentiation (**Figure 8**), would compensate the function of miR-182. miR-183, another member of miR-183~182 cluster, was also upregulated by HDI. This together with our finding that all members of miR-99b~let-7e~125a cluster were increased by HDI further confirm that HDI modulation of miRNA expression occurs through modulation of miRNA primary transcript. There is no conserved miRNA-targeting site identified in the 3′ UTR of *Xbp1* mRNA. The 3′ UTR of mouse *Xbp1* mRNA contains several putative target sites for miR-199, miR-299, miR-433, miR-221, and miR-490. None of these miRNAs, however, were increased by HDI, supporting the contention that the HDI-mediated reduction of *Xbp1* resulted from decreased *Prdm1* expression (16).

These findings demonstrated that HDI modulate CSR, SHM, and plasma cell differentiation, and, therefore, antibody responses by downregulating *Aicda* and *Prdm1* expression through upregulation of targeting miRNAs. By significantly extending our recent findings (16), it provides further and strong evidence that HDI, including those commonly known as "pan-HDI," can effectively modulate a restricted spectrum of miRNAs and, thereby, mRNAs in B cells induced to undergo CSR and plasma cell differentiation. This results from HDACs existing in the unique contexts of HDAC/cofactor complexes, as occurring in B lymphocytes, particularly when in an activated state. The fine specificity of the mechanisms of miRNA/mRNA regulation revealed here was emphasized by the failure of HDI to modulate a variety of other mRNAs encoding elements that participate in but do not initiate the processes of events that leads to CSR. Finally, our studies also provide mechanistic insights into epigenetic mechanisms that directly modulate B cell-intrinsic functions in the immune response, thereby offering new clues for further therapeutic approaches, as specifically targeted to B cells.

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Epigenetic regulation of monoallelic rearrangement (allelic exclusion) of antigen receptor genes

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While most genes in the mammalian genome are transcribed from both parental chromosomes in cells where they are expressed, approximately 10% of genes are expressed monoallelically, so that any given cell will express either the paternal or maternal allele, but not both. The antigen receptor genes in B and T cells are well-studied examples of a gene family, which is expressed in a monoallelic manner, in a process coined "allelic exclusion." During lymphocyte development, only one allele of each antigen receptor undergoes V(D)J rearrangement at a time, and once productive rearrangement is sensed, rearrangement of the second allele is prevented. In this mini review, we discuss the epigenetic processes, including asynchronous replication, nuclear localization, chromatin condensation, histone modifications, and DNA methylation, which appear to regulate the primary rearrangement of a single allele, while blocking the rearrangement of the second allele.

Keywords: asynchronous replication, immunoglobulin, V(D)J recombination, DNA methylation, hematopoietic development

INTRODUCTION

In diploid genomes, most genes are transcribed and expressed from both maternal and paternal alleles, giving rise to a robust expression pattern, which allows the cells to be less susceptible to the damaging consequences of mutations and varying environmental cues. There is, however, a subset of genes that are expressed from only a single allele in a given cell, seemingly sacrificing the evolutionary benefits of the diploid genome (1). This apparent disadvantage is compensated for by other benefits, such as greater cell to cell diversity, which may allow greater robustness of the organism as a whole.

Monoallelic expression can be divided into two subgroups, non-random and random monoallelically expressed (RME) genes. The imprinted genes are a well-studied example of the non-random monoallelically expressed subgroup. In this subgroup, each gene is predetermined to be expressed either exclusively from the maternal or the paternal allele in all cells in the organism (2). Most monoallelically expressed genes fall into the second category of the RME genes. Here, each cell may express either the maternal or paternal allele, and the choice of which allele of each gene is expressed varies throughout the different cells in the body. One well-studied example of RME genes is the group of X chromosome linked genes in female mammals, where in each cell most of the genes on a single X chromosome are silenced epigenetically to create a balanced level of expression relative to that in male cells (3). Olfactory receptor genes constitute an additional family of well-defined RME genes (4). In each olfactory neuron, a single olfactory receptor is expressed from a single allele, to enable varied and specific odorant sensing. Recent studies have demonstrated that monoallelic expression is widespread in various tissues and that the percent of monoallelic expression rises sharply following differentiation from the pluripotent state (5–7). The families of genes that are subject to RME are numerous and vary highly (5–8).

ALLELIC EXCLUSION OF ANTIGEN RECEPTOR GENES

Historically, one of the earliest monoallelically expressed gene families to be recognized is that of the antigen receptors (9, 10). Each B and T cell recognizes only one antigen, as a result of the expression of a single functional V(D)J rearranged protein for each subunit of the antigen receptors. Expression of additional functional rearranged subunits from the second allele could lead to multiple specificities, with deleterious results such as autoimmunity (11). The phenomenon of monoallelic rearrangement of the antigen receptors has been coined "allelic exclusion." During B and T cell development, at the proper developmental stage, each antigen receptor locus becomes accessible to the rearrangement machinery, and one of the two alleles undergoes rearrangement. If this rearrangement produces a functional antigen receptor chain, further rearrangements are prevented by a feedback inhibition mechanism. If, however, the rearrangement fails to produce a functional protein, further rearrangements on the original allele, or on the other allele are induced until a functional protein is produced (12, 13). The only antigen receptor locus that is not subject to feedback inhibition, is the TCRα locus, where rearrangements on both alleles are seen in most mature T cells (14).

Monoallelic expression may be regulated at multiple levels. Most RME genes are regulated at the transcriptional level, so that only one allele has the possibility of being transcribed. In contrast, in the case of allelic exclusion at the antigen receptor loci, both alleles (functionally and non-functionally rearranged) may be transcribed. However, only one of these alleles will give rise to

a functionally translated protein. In fact, in an engineered mice where both endogenous alleles of either the IgH (15) or Igκ (16) loci are functionally prerearranged (i.e., the Ig alleles are both in a rearranged form prior to the cell stage when rearrangement usually occurs), both alleles are transcribed and translated in mature B cells. This signifies that the mechanisms ensuring monoallelic expression at these loci are not regulating transcription, but rather the rearrangement process itself.

There is a large amount of evidence at the Igκ locus that the primary allele which undergoes rearrangement is determined prior to the developmental stage when the rearrangement itself occurs. At an early developmental stage, the allele is selected randomly, so that overall in the B cell pool both alleles are represented equally. Only later in B cell development, does this choice become stable and clonally maintained in an epigenetic manner. Lineage tracing experiments in mice where the two Igκ alleles could be differentiated by flow cytometry showed that at early stages of B cell development, namely in hematopoietic stem cells (HSCs) and multipotent progenitor (MPP) cells, the choice of which allele is selected for rearrangement is still plastic (17). Thus, mature B cells arising from a single MPP or HSC will express either one of the Igκ alleles. At the CLP stage, however, following commitment to the lymphoid lineage, but prior to B cell commitment, two subgroups are observed. The first subgroup behaves like the earlier stages of development, where both alleles are represented on the surface of different mature B cells arising from a single CLP. The second subgroup of CLPs, however, shows a committed phenotype, where all of the mature B cells that originated from a single CLP express Igκ chains from the same allele (17). This commitment is observed in all stages following the CLP stage (pro-B and pre-B cells) leading up to the rearrangement process (17). It is plausible to assume that the committed CLP subgroup is at a later developmental stage than the uncommitted CLP subgroup, though they both fall under the definition of CLP cells. This developmental transition from a plastic to committed allelic phenotype seen in hematopoietic development strikes a delicate balance between the need for diversity in the organism, which is made possible by the early, non-committed stages, and the necessity to ensure monoallelic rearrangement at the proper stage. It will be interesting to investigate the precise developmental allelic commitment of other RME genes, including additional antigen receptor loci.

Interestingly, the choice made at the CLP stage is strong enough to override the feedback inhibition, as seen in the case of mice, which have one functionally prerearranged Igκ allele, and the other allele is in the germline configuration (16). In these mice, approximately 50% of the mature B cells express only the prerearranged allele, while the rest of the cells express both the prerearranged and a newly rearranged allele, signifying that the transcription of the prerearranged allele is not sufficient to supersede the cells where the germline allele was "chosen" for rearrangement.

ASYNCHRONOUS REPLICATION

There are a number of epigenetic mechanisms that appear to regulate the selection of a single allele for rearrangement, and allow the recombination machinery to access only the chosen allele, while repressing the other allele and making it inaccessible. One of the mechanisms that mark the antigen receptor loci already at an extremely early developmental stage is the asynchronous replication timing of the DNA during S phase of the cell cycle.

Replication timing of DNA has been seen to be mostly in correlation with the expression patterns of the genes located within the region of replication (18, 19). Early replicating zones contain a large proportion of genes that are actively expressed, while repressed genes are mostly associated with late replicating zones. Approximately 10% of the regions in the genome fall into a third category of replication timing, where one of the alleles replicates early in S phase, while the replication of the second allele takes place only later (20). Genes located within asynchronously replicating regions are often expressed monoallelically. Most well-known examples of genes expressed monoallelically, such as imprinted genes (21), olfactory receptor genes (22), and genes silenced monoallelicaly on the X chromosome in females (23) are replicated in such a manner. The replication patterns are established in the early embryo, following implantation, and regions that replicate asynchronously starting from this stage remain so throughout development (24, 25). The asynchronous replication pattern remains even in cell types where the genes contained within the replication zone are not expressed (24). Thus, asynchronous replication is an early epigenetic mark of the potential of monoallelic expression. This potential is not necessarily realized in all cells.

The antigen receptor loci replicate in an asynchronous manner. In mature B cells, the rearranged IgH and Igκ alleles replicate early, while the unrearranged alleles replicate in late S phase (24). Although the regions, which replicate asynchronously, are set at implantation, the choice of which of the two alleles will replicate early remains plastic until later stages of development (17, 24). Specifically, in the hematopoietic lineage, the early replicating Igκ allele is not maintained through multiple cell divisions in HSCs and MPPs (**Figure 1**) (17). This coincides with the fact that at these stages the allele that will undergo rearrangement in pre-B cells has not yet been determined. In the CLP stage, two distinct subgroups are observed. The first subgroup behaves in a manner similar to the HSC and MPP stages, where the replication of each allele switches between early and late timing over the course of numerous cell cycles. In the second CLP subgroup, the identity of the early replicating allele remains constant through multiple cell divisions (**Figure 1**) (17). This correlates nicely with the fact that the CLP stage contains cells that are not yet committed to the rearrangement of a particular Igκ allele, as well as cells, which will faithfully rearrange only one of the two alleles. It is likely that the allelically committed CLPs represent a more mature stage of differentiation than the allelicaly plastic CLPs. In the pre-B cell stage, the early replicating allele is the one chosen for rearrangement, when the cells are induced to differentiate (**Figure 1**) (17). Thus, asynchronous replication is seen to be a clear early marker of monoallelic potential, and commitment of a specific allele to replicate early coincides with the commitment of that allele to rearrange later in B cell development. How these patterns are set up, maintained, and translated into monoallelic expression or rearrangement is still unknown and should be the subject of future research.

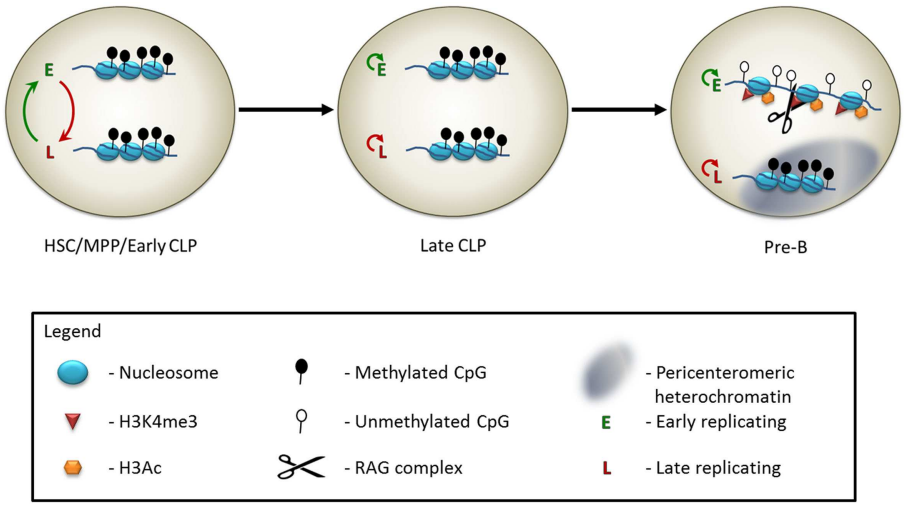

FIGURE 1 | Model of the epigenetic control of Igκ monoallelic rearrangement. In early hematopoietic progenitors, including hematopoietic stem cells (HSCs), multipotent progenitors (MPPs), and at an early stage of the common lymphoid progenitor cells (early CLPs), the two Igκ alleles appear to be identically marked – both are in a relatively inaccessible chromatin state, and are methylated on the DNA. The DNA replicates asynchronously, but the choice of which allele is early replicating switches constantly. At the late CLP stage, the alleles still appear externally similar, but the early replicating allele is now consistently maintained throughout multiple cell divisions. At the pre-B cell stage, leading up to the monoallelic rearrangement event, the late replicating allele relocates to the pericenteromeric heterochromatin, while the early replicating allele moves to a central, euchromatic region of the nucleus. The histones of the early replicating allele become marked with H3Ac and H3K4me3. The RAG protein binds to the H3K4me3 modification. Following DNA demethylation of the early replicating allele, the Igκ locus will undergo monoallelic rearrangement.

NUCLEAR LOCALIZATION AND CHROMATIN STRUCTURE

An additional level of regulation of the monoallelic choice, which comes into play closer to the actual rearrangement reaction, is that of nuclear localization and chromatin condensation. When genes are localized to the pericenteromeric heterochromatin, they are maintained in a repressive and inaccessible chromatin state, which is not optimal for the activity of the RAG machinery. At the time of recombination of the TCRα (26), TCRβ (27–29), and Igκ (30, 31) loci, it has been observed that one allele is usually located at the nuclear periphery, within a domain of pericenteromeric heterochromatin. The opposing allele is localized to more central, euchromatic regions of the nucleus, or, conversely, is looped away from the nuclear periphery. Only the allele, which is removed from the nuclear periphery, undergoes rearrangement (**Figure 1**). It is particularly noteworthy that this mechanism is present at the TCRα locus, since allelic exclusion does not occur at this locus (14), yet the rearrangement is restricted to one allele at a time. The ATM protein has been implicated as part of the mechanism that sequesters one of the alleles to the pericentromeric heterochromatin. In the absence of a functional ATM protein, the recruitment to the heterochromatin of Igκ and TCRα is impaired and many cells are observed to have RAG-mediated double strand breaks (DSBs) simultaneously on both alleles (26, 31). This function of ATM is not mediated via its canonical phosphorylation of H2AX or MDC1 as a reaction to DSBs (32). The precise mechanism of action is still not well understood.

Location and condensation are not the only ways in which two homologous antigen receptor alleles differ from each other on the chromatin level prior to rearrangement. In pre-B cells, the Igκ allele, which is destined to undergo rearrangement, is usually packaged with activating histone marks, such as H3K4me3 and H3Ac, whereas the opposing allele, which is associated with the pericenteromeric heterochromatin, is not (**Figure 1**) (17, 30). The presence of H3K4me3 at the rearrangement site is particularly noteworthy, as this acts as a docking site for the RAG2 protein (33, 34). This protein contains a PHD domain, which specifically recognizes the H3K4me3 modification that is necessary for efficient V(D)J recombination *in vivo* (35–37). Indeed, RAG2 binding at this locus is monoallelic, and occurs on the allele enriched for H3K4me3 (**Figure 1**) (17). The H3K4me3 mark is present at all of the antigen receptor loci at the developmental stage at which they undergo rearrangement (33) and the RAG complex is specifically recruited to these sites (26, 27, 34). Whether this histone mark is monoallelic at the remaining loci has not yet been examined, so it remains to be seen whether this is a widespread phenomenon.

DNA DEMETHYLATION

We will conclude this review with the discussion of the developmentally regulated removal of methylation from the DNA at the antigen receptor loci, as a final step leading to rearrangement. The antigen receptor loci are methylated in most tissues in the body following the wave of *de novo* DNA methylation, which takes place throughout the genome shortly after implantation (38, 39). It has been observed that following rearrangement in the B cell lineage, the IgH and Igκ loci are hypomethylated on the rearranged allele, while alleles, which are still in the germline configuration, remain hypermethylated (40, 41). Although it is possible that the monoallelic demethylation observed on the rearranged alleles occurs following the recombination process, there are a number

of observations, which indicate that the demethylation takes place prior to rearrangement and, in fact, enhances the rearrangement process. For one, DNA methylation has been shown to block the activity of the RAG proteins *in vitro* (42) and reduction of methylation can induce rearrangement in cell culture conditions (43). Demethylation begins on the Igκ locus at the pre-B cell stage (**Figure 1**), and will occur at this stage even in RAG$^{-/-}$ cells incapable of performing rearrangement (40). In fact, over the course of normal development, demethylation of the Igκ locus will occur in a monoallelic manner, even in the case of feedback inhibition of a prerearranged transgene, which will prevent the rearrangement of the endogenous locus (40, 44). Additionally, in a mouse where both of the endogenous Igκ alleles have been replaced with a prerearranged functional Igκ gene, one of the alleles is unmethylated in mature B cells, while the second remains fully methylated, despite the fact that both alleles are expressed at similar levels (16). Rearrangement intermediates of the Igκ locus (where the DSBs created by the RAG machinery are not yet resolved) are found among the unmethylated, but not methylated, fraction of wild type pre-B cells DNA (44). Moreover, the methylated chromatin fraction from RAG$^{-/-}$ IgH^{+} pre-B cells is not a good substrate for exogenous RAG cleavage, while the unmethylated fraction is (44). Taken together, it is clear that the monoallelic DNA demethylation is a strong mechanism hardwired into B cell development, which is independent of the rearrangement process, but which licenses monoallelic recombination.

A number of *cis* regulatory elements at the Igκ locus contribute to the demethylation process in pre-B cells. These elements include the three κ enhancers (iEκ, 3'Eκ, and Ed) (40, 45–48), as well as the recently characterized Dm element, which lies upstream of iEκ (49). Over the past few years, a number of mechanisms have been suggested, which can lead to demethylation of DNA sequences. Demethylation may occur either in an active manner, which transpires independently of DNA replication, or in a passive manner, where the methylation is not regenerated following replication and is thus diluted over the course of multiple cell divisions. The proteins from the Tet family, which catalyze the oxidation of 5-methyl cytosine (5mC) residues into 5-hydroxymethyl cytosine (5hmC) (50), have been suggested as mediators of DNA demethylation in a number of tissues, in both an active and passive manner. Once the 5hmC intermediate has replaced the 5mC, it can be either be actively excised from the genome and substituted with an unmodified cytosine (51, 52), or, alternatively, may be passively lost over the course of DNA replication (53) [since DNMT1, does not recognize 5hmC as a substrate for maintenance of DNA methylation (54)]. A different strategy of passive demethylation, which has been reported, is the sequestration of DNMT1 from the DNA by *cis* acting non-coding RNA (55). This allows the DNA to become unmethylated by a passive mechanism during cell division. Whether any of the above described mechanisms is utilized by the cell for demethylation of the Igκ locus has yet to be determined.

CONCLUSION

Altogether, we see that the cell uses multiple layers of epigenetic regulation, starting from the early post-implantation embryo, to ensure that the antigen receptor genes undergo rearrangement on one allele at a time, and thus allow for the clonal monoallelic expression of the antigen receptors on B and T cells, giving rise to the great diversity and specificity of the system. In the Igκ locus, asynchronous replication is the first epigenetic mark to be fixed upon a specific allele. This is apparently followed by histone modifications, which begin to appear on the chosen allele in the pro-B cell stage, before the alleles move to separate nuclear compartments in pre-B cells (17, 30). In the pre-B cell stage, one allele becomes more strongly marked with active histone modifications, whereas the other allele is located adjacent to the heterochromatin. The final epigenetic change, which precedes rearrangement, is the removal of DNA methylation from the allele that is then cleaved by the recombination machinery. It is not yet clear what the comparative contribution of each epigenetic event is toward the regulation of the monoallelic rearrangement. There is still a large gap in our understanding of how exactly these patterns are established, maintained, and translated into the monoallelic rearrangement phenotype. Future research will improve our understanding of this, and perhaps other monoallelically expressed systems. It will be exciting to see what more can be learned about this fascinating system.

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Clonal progression during the T cell-dependent B cell antibody response depends on the immunoglobulin D_H gene segment repertoire

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The diversity of the third complementarity determining region of the IgH chain is constrained by natural selection of immunoglobulin diversity (D_H) sequence. To test the functional significance of this constraint in the context of thymus-dependent (TD) immune responses, we immunized BALB/c mice with WT or altered D_H sequence with 2-phenyloxazolone-coupled chicken serum albumin (phOx-CSA). We chose this antigen because studies of the humoral immune response to the hapten phOx were instrumental in the development of the current theoretical framework on which our understanding of the forces driving TD responses is based. To allow direct comparison, we used the classic approach of generating monoclonal Ab (mAb) from various stages of the immune response to phOx to assess the effect of changing the sequence of the D_H on clonal expansion, class switching, and affinity maturation, which are hallmarks of TD responses. Compared to WT, TD-induced humoral IgM as well as IgG antibody production in the D-altered ΔD-DμFS and ΔD-iD strains were significantly reduced. An increased prevalence of IgM-producing hybridomas from late primary, secondary, and tertiary memory responses suggested either impaired class switch recombination (CSR) or impaired clonal expansion of class switched B cells with phOx reactivity. Neither of the D-altered strains demonstrated the restriction in the V_H/V_L repertoire, the elimination of V_H1 family-encoded antibodies, the focusing of the distribution of CDR-H3 lengths, or the selection for the normally dominant Ox1 clonotype, which all are hallmarks of the anti-phOx response in WT mice. These changes in clonal selection and expansion, as well as CSR indicate that the genetic constitution of the D_H locus, which has been selected by evolution, can strongly influence the functional outcome of a TD humoral response.

Keywords: rodent, B cells, antibodies, class switch recombination, repertoire development

INTRODUCTION

In immunoglobulins, juxtaposition of the three complementary determining regions (CDRs) of the L chain and the three of the H chain creates the site at which antigen binds (1, 2). While CDRs 1 and 2 are entirely of germline origin and CDR-L3 is largely so, CDR-H3 is the direct product of VDJ rearrangement and N nucleotide addition (3). This makes CDR-H3 the focus for pre-immune Ig diversity. In combination, this diversity and its physical location at the center of the antigen binding site tends to endow CDR-H3 with the ability to define the antigen binding specificity and affinity of the antibody.

Analyses of anti-hapten immune responses have been crucial for the dissection of the roles played by T cells in initiating and regulating humoral immune maturation. Immune maturation in the classic humoral immune response of BALB/c mice to the hapten 2-phenyloxazolone (phOx) (4) focuses on the clonal expansion and somatic hypermutation of Ig bearing the dominant Ox1 Id (Id_{Ox1}). While this Id is marked by the use of a combination of V_HOx1 and $V_κ$Ox1 variable genes, the presence of a short DRG peptide sequence in CDR-H3 is typically determinative (4, 5).

To test the role of natural selection of D gene segment sequence on humoral immune function, we previously created a panel of BALB/c-derived D-altered mutant mouse strains (6–8). ΔD-DμFS and ΔD-iD B cells produce two alternative, polyclonal Ig repertoires with a normal and intact set of V_H, J_H, and C_H exons that are fully capable of undergoing somatic hypermutation and class switching (6, 8). The only change that has been made is the simplification of D_H locus to contain only one D of alternative sequence. After VDJ rearrangement, even the loxP sites are deleted, leaving only the imprint of the three to seven amino acids encoded by the D_H. The CDR-H3s that contain identifiable D_H sequence create an antigen binding site repertoire that differs greatly in the pattern of amino acid use from WT. However,

Abbreviations: CSA, chicken serum albumin; Id_{Ox1}, Ox1 idiotype; phOx, 2-phenyloxazolone.

CDR-H3 sequences that lack identifiable D_H sequence and are created by V, J, and N sequence alone appear indistinguishable from similar sequences created in wild-type (WT) mice (Figures S1 and S2 in Supplementary Material).

The DRG peptide sequence characteristic of the dominant Ox1 Id is an example of a CDR-H3 that can be easily created either with or without D gene segment sequence. The nine nucleotides used to encode DRG can include three to five nucleotides from 5 of the 13 D_H gene segments. However, the DRG sequence can also be created by simply introducing five N nucleotides between V_HOx1 and J_H3. Our panel of D-altered mice thus provided us with the means to test the extent to which loss of the naturally selected D-dependent CDR-H3 repertoire would influence the development of a classic T dependent response to a defined hapten even when the loss of D sequence could be easily mitigated by N addition alone.

To allow direct comparisons to previous studies, we used the classic approach of generating monoclonal Ab (mAb) from various stages of the immune response to phOx. We found that changing conserved elements of the sequence of the diversity gene segment locus led to the failure to select for the use of V_HOx1/V_κOx1 gene combination, the failure to yield the normal focusing of CDR-H3 sequence, and thus the loss of Id_{Ox1} dominance. Further, we observed an enhanced and persistent production of hybridomas secreting low affinity IgM indicating a profound failure to develop a fully mature, class switched IgG response. Together, these findings suggest that TD B cell responses can be heavily influenced by the effects of natural selection of D_H content on CDR-H3 repertoire diversity.

MATERIALS AND METHODS

ANIMALS

Wild-type female BALB/c (H-2^d) mice (Harlan-Winkelmann; Borchen, Germany) and BALB/c D-altered homozygous ΔD-DμFS (7) and ΔD-iD (6) mice were reared under clean conventional conditions the University of Kiel animal house. Immunizations and experimental procedures were approved by the "*Ministry of Agriculture, Environment and Rural Areas*" of the local government of Schleswig-Holstein, permission no. V 312-72241.121-3 (35-3/06).

ANTIGENS, IMMUNIZATIONS, ANTIBODY TITRATIONS, AND PRODUCTION OF MONOCLONAL ANTIBODIES

BALB/c WT and D-altered ΔD-DμFS and ΔD-iD mice were immunized with the TD hapten–protein complex phOx-coupled chicken serum albumin (phOx-CSA) (molar ratio ~11) (9, 10). At 3–4 months of age, animals of all three strains received 80 μg of phOx-CSA adsorbed to $Al(OH)_3$ as a primary intraperitoneal immunization. Venous blood was serially obtained by tail vein phlebotomy and serum antibody concentrations were determined after primary or secondary immunization. PhOx-binding IgM and IgG titers were determined with ELISA using class-specific secondary antisera. Spleen cells of phOx-CSA-immunized WT or D-altered mice were fused with the non-secretor Ag8.653 myeloma cells by means of the conventional PEG-mediated hybridization technique. Fusions were performed 7 or 14 days after primary immunization and 3 days after each secondary (memory) immunization. Resulting mAb reactive with phOx-BSA but not with BSA alone were selected for further study.

DETERMINATION OF RELATIVE AFFINITIES OF ANTI-phOx ANTIBODIES

The relative affinities of mAb antibodies to phOx were determined with a hapten-inhibition test as previously described (9). These affinities were assessed in comparison to that of two prototypic Ox1-idiotypic mAb: H11.5 (μ,κ) for IgM and NQ2/16.2 (γ,κ) for IgG Ab. Briefly, the binding of comparable amounts of anti-phOx mAb to surface-bound phOx-BSA was inhibited with graded concentrations of soluble phOx-caproic acid. Concentration values giving 50% inhibition were taken as relative affinity measures. An affinity factor was generated as the quotient of the relative affinity of H11.5 (μ,κ) for IgM and NQ2/16.2 (γ,κ) for IgG mAb divided by that of a particular mAb. MAb with higher affinity than the controls exhibited an affinity factor greater than 1, and those with a lower affinity exhibited an affinity factor of less than 1. Independent confirmation of these measures of affinity was obtained by fluorescence quenching (10).

SEQUENCING OF ANTIBODY V REGION GENES

Sequence analysis of the variable regions of mAb was performed as previously described (11) with some modifications. Briefly, total RNA of anti-phOx Ab secreting hybrid cell lines was isolated with TRIZOL® (GIBCO-BRL, Eggenstein, Germany) and transcribed into cDNA with SuperScript™ II RNAse H reverse transcriptase (GIBCO-BRL, Eggenstein, Germany) using pd(N)6 random and $pd(T)_{12–18}$ primers. The V_H and V_L mRNA sequences were first amplified by PCR using 10 primer sets for each of the V_H-regions, and 7 primer sets for each of the V_L-regions and two forward primers specific for the 3′-end for the first domain of the V_H and V_L constant regions, respectively. A second semi-nested amplification at 3′-end was then performed with relevant primers coupled to M13 oligonucleotides. This amplimer product was then used for sequencing (MWG Biotech; Ebersberg, Germany). V region sequences were analyzed with the integrative database VBASE2 (http://www.vbase2.org/).

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison post test, using Graphpad Prism for Windows (version 4.02, GraphPad Software Inc., San Diego, USA) was used for statistical comparisons of individual IgM and IgG end-point titers.

ACCESSION NUMBERS

GenBank accession numbers for all antibodies are indicated in the supplementary table legends of each group of antibodies.

RESULTS

HUMORAL IMMUNE RESPONSE AND ANALYSIS OF 2-PHENYL-5-OXAZOLONE-SPECIFIC MONOCLONAL ANTIBODIES

The humoral Ab response to phOx in BALB/c WT mice was compared to that of homozygous D-altered ΔD-DμFS and ΔD-iD mutant mice. The D_H locus in these strains was first simplified by deleting 12 of the 13 D_H gene segments by means of cre/loxP gene-targeting (12); and then modifying the sequence

of the remaining D_H. In the ΔD-DμFS strain, the WT sequence of the DFL16.1 D_H segment was modified by introducing two frame-shift mutations that flipped the normal preference for reading frame 1 (RF1), which encodes neutral tyrosine and glycine, to RF2, which encodes hydrophobic valine. In the ΔD-iD strain, the center of the DFL16.1 segment was replaced with the DSP2.2 D_H gene segment in inverted form. This leads to CDR-H3 enriched for charged arginine, asparagine, and histidine in place of tyrosine and glycine (6, 8).

In both mutant strains, primary immunization with the TD Ag phOx-CSA-induced significantly lower IgM and IgG anti-phOx Ab titers than WT (**Figures 1A,B**). Following secondary (memory) immunization, the IgG response remained suppressed while IgM production was meager and indistinguishable between all three strains.

Study of the molecular events during immune maturation of the TD anti-phOx response classically focused on analysis of mAb (9, 13, 14) secreted by hybridomas produced by fusion of permanently growing myeloma cells with immune splenocytes obtained after sequential primary and memory immunization (9, 13, 14). In order to directly compare the effect of altering D_H locus content in the same classic context, we generated anti-phOx mAb-secreting hybridomas from immune splenocytes harvested after primary, secondary, and tertiary immunizations of D-altered ΔD-DμFS and ΔD-iD mutant mice.

The characteristic parameters of these mAb, including isotype, relative affinity, usage of V_H/V_L genes and D_H and J_H gene segments, amino acid sequence, and reading frame usage in CDR-H3, and correspondence with the normally dominating Id_{Ox1} are summarized Tables S1 and S2 in Supplementary Material. A comparison of these mAb with those of previously published mAb (10) from WT mice revealed crucial differences induced by the change in D_H sequence content. Unlike WT mice, where the majority of hybridomas (>80%) isolated during late primary responses and

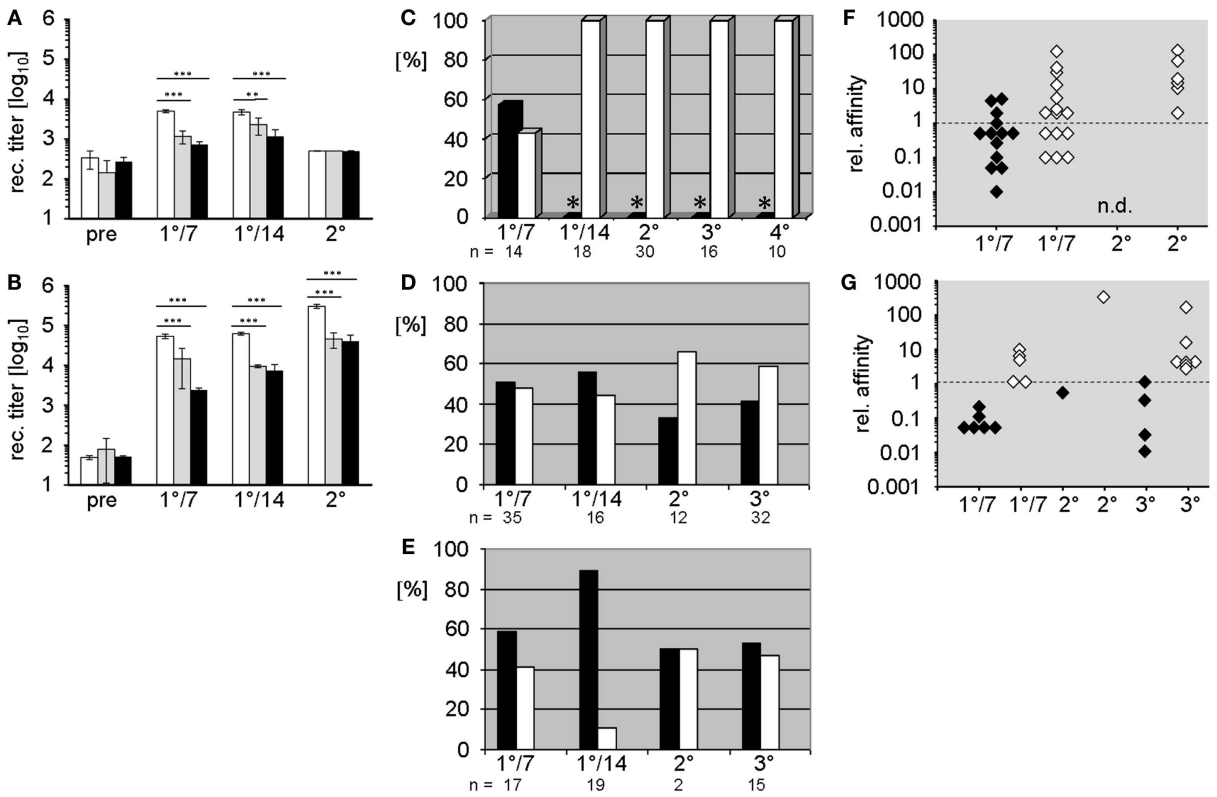

FIGURE 1 | Comparison of humoral Ab responses to the hapten 2-phenyloxazolone in BALB/c wild-type and D-altered ΔD-DμFS and ΔD-iD mutant mice. **(A,B)** IgM **(A)** and IgG **(B)** anti-phOx Ab titers of primary and secondary thymus-dependent immune responses after immunization with phOx-CSA in BALB/c wild-type mice (white bars), ΔD-DμFS mice (gray bars), and ΔD-iD mice (black bars). The bars show the mean + SD of five mice per group. Pre – pre-immune sera; 1°/7 – primary day 7 and 1°/14 – primary day 14 responses. Secondary immunization followed 14 weeks after primary and Ab titers were determined after two more weeks. Significance is indicated at $**p < 0.01$; $***p < 0.001$. **(C–E)** Monoclonal anti-phOx mAb were prepared from **(C)** BALB/c wild-type, **(D)** ΔD-DμFS, and **(E)** ΔD-iD mice on day 7 (1°/7) and day 14 (1°/14) after primary immunization with phOx-CSA and 3 days after secondary (2°), tertiary (3°), and from wild-type mice also after quaternary (4°) immunizations. For ΔD-DμFS and ΔD-iD mice, percentages of IgM and IgG mAb are indicated as black and white bars, respectively. However, because extremely low numbers of IgM mAb have been isolated from the late primary and memory responses of BALB/c wild-type mice (*), only IgG mAb have been produced. **(F,G)** The relative affinities of IgM (black diamonds) and IgG (white diamonds) anti-phOx mAb from different stages of the immune response of **(F)** ΔD-DμFS and **(G)** ΔD-iD mutant mice are compared either to the IgM Id_{Ox1} mAb H11.5 (μ,κ) or to the IgG Id_{Ox1}-prototypic mAb NQ2/16.2 (γ,κ). Affinity factors of >1 indicate higher while affinity factors of <1 indicate lower affinities than H11.5 and NQ2/16.2, respectively.

on day 3 of secondary and tertiary responses produce IgG, and thus reflect T cell aided CSR (15); only half of the hybridomas obtained from the D-altered ΔD-DμFS and ΔD-iD mice produced IgG. This included both the hybridomas generated after the late primary response as well as hybridomas harvested from splenocytes 3 days after memory immunizations (**Figures 1D,E**). Thus, the normal pattern of favoring production of IgG secreting B cells failed to occur in mice lacking a WT D_H locus, and therefore, a WT CDR-H3 repertoire. A full depiction of these antibodies is provided in Tables S1 and S2 in Supplementary Material.

To assess the quality of these mAb, we compared their relative affinities to those of prototypic IgM and IgG Id_{Ox1} mAb. Early primary IgM mAb of both mutant strains, as well as those from secondary and tertiary responses, were mostly of rather low affinity (**Figures 1F,G**). However, three IgM mAb from ΔD-DμFS mice exhibited higher affinities than the IgM Id_{Ox1} mAb H11.5 (**Figure 1F**), even though none of them included the Id_{Ox1} V gene combination of V_HOx1/$V_κ$Ox1, which is designated V_H171/$V_κ$072 in the integrative database VBASE2.

Early primary IgG mAb generally exhibited higher affinities than the IgM mAb, with some IgG mAb displaying even higher affinities than the IgG Id_{Ox1} mAb NQ16.2. However, none of the mAb expressed the Id_{Ox1} V_HOx1/$V_κ$Ox1 gene combination. IgG of higher affinity were found among secondary and tertiary response mAb. However, immune maturation did not progress in the steady way that characterizes the classic response of WT mice (see below).

In accordance with historical studies, in WT mice the anti-phOx response exhibited a drastic CSR-associated reduction in the variability of the V_H and V_L repertoires (10) (**Figures 2A,D**), especially in the class switched secondary response. No such reduction in V_H or V_L variability was observed in the D-altered ΔD-DμFS and ΔD-iD mice (**Figures 2B,E** and **C,F**). The classic secondary phOx response in WT mice is associated with a shift away from use of the V_H1 family (10). However, in the D-altered mice hybridomas secreting V_H1 family-encoded IgM and IgG mAb continued to be isolated at all phases of immune maturation (Tables S1 and S2 in Supplementary Material).

In WT mice, the TD response to phOx-CSA is dominated by V_H171/$V_κ$072-encoded Id_{Ox1} Ab (4) whose idiotypic determinant

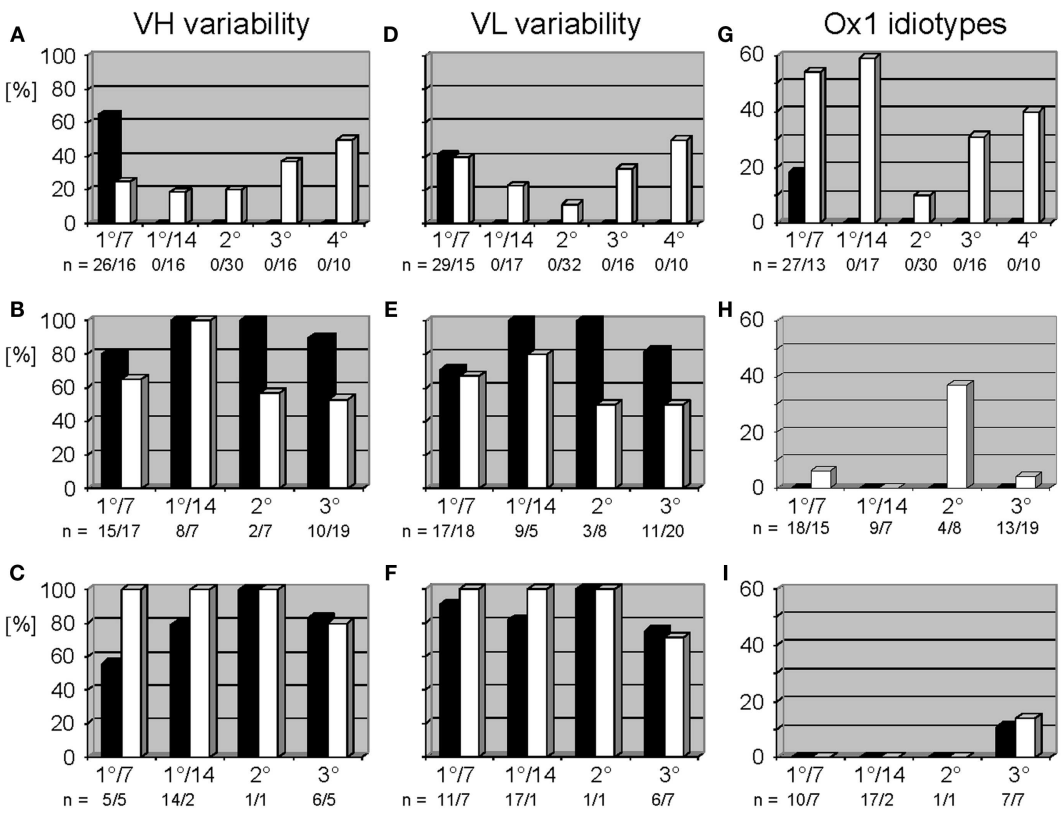

FIGURE 2 | Variable gene usage of mAb of the thymus-dependent anti-phOx immune response in BALB/c wild-type and ΔD-DμFS and ΔD-iD D_H mutant mice. Mice were immunized with the TD Ag phOx-CSA and mAb were prepared on day 7 (1°/7) and day 14 (1°/14°) after primary immunization and 3 days after secondary (2°), tertiary (3°), and quaternary (4°) immunizations, respectively. The characteristic attributes and utilized variable genes of these Ab are depicted in Tables S1 and S2 in Supplementary Material. Variability is calculated as the number of genes expressed by a group of antibodies divided by the number of monoclonal antibodies of this group and multiplied by 100. In BALB/c mice, IgM mAb (black bars) were only prepared early after primary immunization; see also legend to **Figure 1**. Data for IgG (white bars) are taken from two previous publications (9, 10). V_H and V_L variability is indicated for anti-phOx mAb of BALB/c wild-type mice **(A,D)**, ΔD-DμFS mice **(B,E)**, and ΔD-iD mice **(C,F)**. **(G–I)** Expression of Ox1-idiotypic gene combination VH171/Vκ072 by monoclonal anti-phOx Ab from the TD response in BALB/c wild-type mice **(G)**, ΔD-DμFS mice **(H)**, and ΔD-iD mice **(I)**. Data of BALB/c wild-type mice are taken from previous publications (5, 9, 10).

is located in CDR-H3 (5). A compilation of previous data (9, 10) shows that mAb with the gene combination $V_H171/V_\kappa072$ (the "genetic" Id_{Ox1}) first dominate after CSR among IgG mAb (**Figure 2G**). This gene combination is counter-selected in secondary response mAb, but increases again in tertiary and quaternary responses. However, in both of the D-altered strains the Id_{Ox1} gene combination $V_H171/V_\kappa072$ only represented a minority of clones. Among ΔD-DμFS mAb, the $V_H171/V_\kappa072$ gene combination was not identified among the IgM hybridomas. It was detected in IgG mAb drawn from the primary response, but at a low level. A plurality of the secondary IgG response used this combination, but this was again lost in the tertiary response (**Figure 2H**). In ΔD-iD mice, anti-phOx antibodies with the $V_H171/V_\kappa072$ gene combination were not observed in either the primary or secondary responses. It was found among tertiary IgM and IgG, but again at a low level (**Figure 2I**).

ANALYSIS OF CDR-H3 OF MONOCLONAL ANTI-2-PHENYL-5-OXAZOLONE ANTIBODIES

In WT mice, CSR and affinity maturation are strongly associated with a focusing of CDR-H3 content. For example, whereas the CDR-H3 of anti-phOx IgM hybridomas varied between 15 and 51 nucleotides, 70–100% of primary and memory IgG mAb exhibited a clearly dominating length of 21 nucleotides (10). This CSR-associated restriction of CDR-H3 lengths was not observed in D-altered ΔD-DμFS and ΔD-iD mice (**Figure 3**). CDR-H3 lengths with 21 and 36 nucleotides were slightly increased among ΔD-DμFS mAb (**Figure 3A**) and to an even lesser degree among ΔD-iD mAb (**Figure 3B**). Otherwise, IgM as well as IgG mAb from both strains of mice demonstrated a wide distribution of lengths.

The distribution of charged, neutral, and hydrophobic amino acids dictates the average hydropathicity of the CDR-H3 loop. During immune maturation among WT mice, highly hydrophobic amino acids found among IgM anti-phOx CDR-H3 are gradually supplanted by arginine, aspartic acid, glycine, and tryptophan (Figures S3A–H in Supplementary Material). In contrast, from the primary to tertiary responses in both ΔD-DμFS mice (**Figure 4**) as well as ΔD-iD mice (data not shown), hydrophobic amino acids were found to a similar extent in the CDR-H3 of both IgM and IgG anti-phOx antibodies. The content of hydrophobic amino acids in the CDR-H3 loops was also reflected in the corresponding hydrophobicity values. In anti-phOx mAb from WT mice, about 25% of IgM mAb exhibited CDR-H3 with positive average hydropathicity values, whereas only 6% of IgG antibodies (with the exception of secondary IgG) belonged to this category (Figures S4A–H in Supplementary Material). In contrast, IgM as well as IgG mAb produced by ΔD-DμFS B cells expressed similar proportions of CDR-H3 loops with positive average hydropathicity values (**Figure 5**). [The numbers of mAbs generated from the ΔD-iD mice were insufficient to allow a firm conclusion on whether average hydrophobicity was maintained or altered in this strain (data not shown).]

Since idiotypic determinants defining Id_{Ox1} antibodies are located in CDR-H3, we analyzed antibodies bearing this idiotype to assess, which D_H gene segments could be used to generate the typical Id_{Ox1} CDR-H3 sequence C_ARDRGAY. In solely phOx-CSA-induced mAb from WT mice (10), this sequence could be generated from the five D_H gene segments DSP2.2, DSP2.9, DSP2.11, DST4, and DQ52 (**Table 1**). In contrast, after a primary immunization with the TI-2 Ag phOx-Ficoll and a subsequent TD immunization (primary to quaternary) with phOx-CSA, CDR-H3 sequences from anti-phOx mAb were drawn from a larger set of eight D_H segments (DSP2.2, DSP2.3, DSP2.5, DSP2.6, DSP2.9, DSP2.10) plus two sequences from inverted DFL16.1 and DFL 16.2, respectively (9). Moreover, in half of the mAb, the D_H segment could not be identified due to extensive nibbling (9). This raised the issue of whether ΔD-DμFS mutant mice bearing a frameshifted DFL16.1 D_H segment or ΔD-iD mice with an inverted DSP2.2 gene sequence would be able to generate a typical Id_{Ox1} CDR-H3 sequence in association with the VH171 gene.

C_ARDRGAY-related sequences were identified (**Table 2A**). The majority of these CDR-H3s lacked identifiable D_H sequences and instead was largely the product of N and P nucleotide addition. Among the mAbs drawn from the ΔD-DμFS mice, two contained CDR-H3 amino acids coded by the frameshifted DFL16.1 gene segment. Five additional ΔD-DμFS mAb (FS1°14/05, FS3°/08, FS3°/09, FS3°/22, FS3°/18) also exhibited CDR-H3 sequences already found in Id_{Ox1} antibodies from WT mice, but these mAbs used N and P nucleotides to recreate the sequence. Among the mAbs drawn from the ΔD-iD mice, one early primary mAb, iD1°7/06, exhibited the correct Id_{Ox1} sequence in CDR-H3. These findings made it clear that both D-altered strains were able to generate Id_{Ox1}-typical or related CDR-H3 sequences in spite of the alteration in D_H sequence content. Thus, differences in the CDR-H3 repertoire in these D-altered strains cannot be simply explained by an inability to generate the classic Id_{Ox1} sequence.

Short CDR-H3 with 6–8 amino acids (i.e., in the range of CDR-H3 of Id_{Ox1} antibodies) were also found in association with V_H genes other than V_H171. These alternative V_H belonged to non-V_H2 families (**Table 2B**). Two of the V_H171-encoded antibodies exhibited longer CDR-H3. Thus, the great majority of anti-phOx mAbs produced in these D-altered mice used H chains that differed greatly from the characteristic Id_{Ox1} sequence, even though classic Id_{Ox1}-like CDR-H3s could be generated by both D-altered mice.

It has been argued that diversity at CDR-H3 is sufficient for the creation of Ag-specificity and that a particular specificity can be correlated with identical or very similar amino acids sequences in CDR-H3 (16). Our anti-phOx mAb from the two D-limited mouse strains offered the opportunity to check this hypothesis in another experimental system. A survey of all anti-phOx antibodies from ΔD-DμFS and ΔD-iD mice is depicted in Tables S3A,B in Supplementary Material, respectively. From the 83 mAb of ΔD-DμFS mice, 58 (70%) made use of varying lengths of the genomic D_H sequence, 2 (2.4%) used inverted sequences, and in 23 (28%) the genomic D_H sequence was not detectable. From the 44 mAb of ΔD-iD mice, 23 (52%) used varying lengths of the D_H segment, 5 (11%) used variable lengths of the inverted sequence, and in 16 mAb (36%), no portion of the D_H segment could be identified. This large variability of CDR-H3-coding sequences led to an equal variability of 0–13 amino acid sequences in CDR-H3 loops. The average hydropathicity values of CDR-H3 loops varied from strongly hydrophobic to strongly hydrophilic (**Table 3**). Similar findings were obtained in WT mice (data not shown). Thus, since

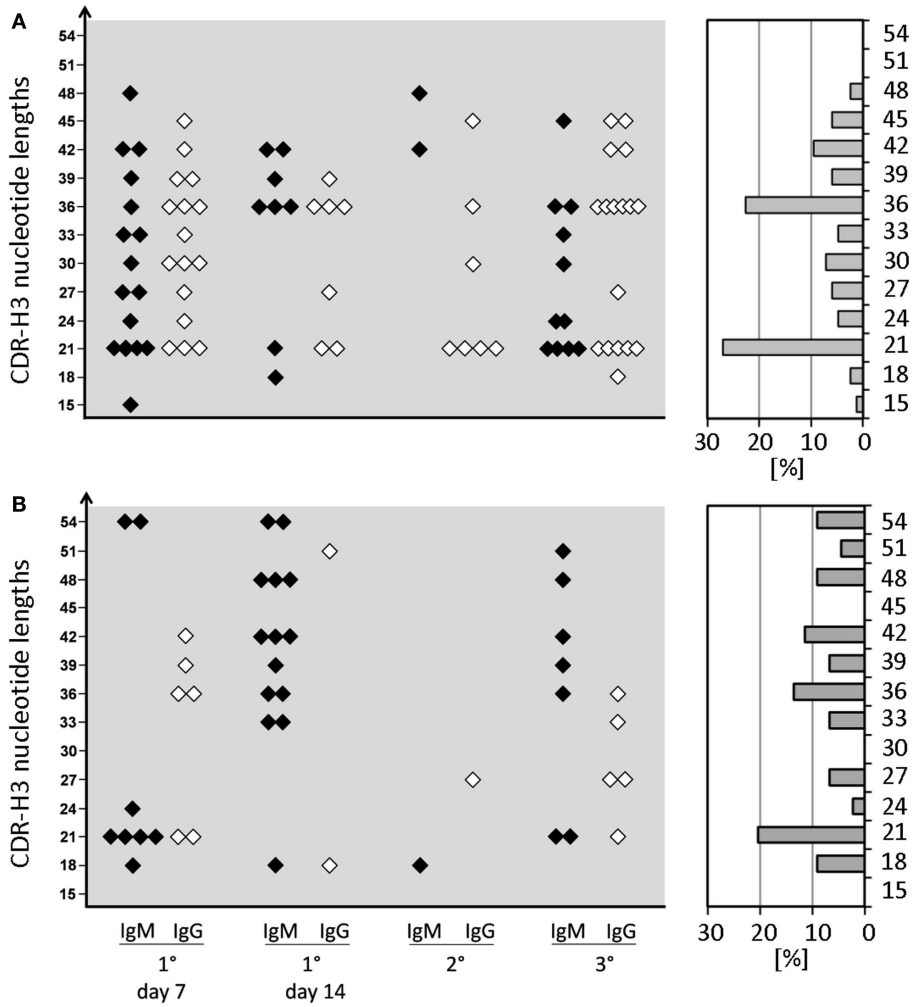

FIGURE 3 | CDR-H3 nucleotide lengths of IgM and IgG mAb of the anti-phOx immune response of D-altered ΔD-DμFS and ΔD-iD mutant mice. Monoclonal Ab were prepared from mice that were immunized various times with the TD Ag phOx-CSA (see previous figures). The characteristic attributes of these Ab are depicted in Tables S1 and S2 in Supplementary Material. The CDR-H3 nucleotide lengths of IgM (black symbols) and IgG (white symbols) monoclonal anti-phOx Ab from different stages of the immune response of ΔD-DμFS mice are shown in **(A)** and those of ΔD-iD mice in **(B)**. The percental contribution of each nucleotide length is indicated by gray bars at the right hand side.

common sequences or sequence motifs in CDR-H3 could not be detected, we conclude that Ag-specificity in this model, immune response is not limited by either this parameter or by other, readily apparent physicochemical properties of the CDR-H3 interval.

DISCUSSION

In WT BALB/c, the preference for Id_{Ox1} anti-phOx Ab reflects both the failure of B cells producing phOx-binding IgM antibodies that use V_H1-family genes to contribute to the IgG Ab repertoire and a focusing of CDR-H3 content (10). While the increase of Ab encoded by non-Id_{Ox1} V_H/V_L gene combinations from later stages of the response may reflect enhanced K_a (17), the initial dominance of the Id_{Ox1} has been attributed to its superior K_D for the hapten phOx (14). This view has been challenged by the finding that the early primary IgM response contains a considerable number of anti-phOx Ab that demonstrate similar or even higher affinities than Id_{Ox1} Ab (10). Because these high affinity IgM progenitors do not have counterparts among IgG Ab (10), these findings have raised questions about a strict attribution of higher affinity for Ag as the primary force behind the clonal selection and CSR exhibited by B cells that express Ig bearing the Id_{Ox1} idiotype.

Attribution of the preference for the Id_{Ox1} idiotype as reflective of higher Ag affinity carries with it the presumption that the choice of specific Ab reflects selection from a random repertoire of antigen binding sites. However, although VDJ rearrangement and N addition yields a tremendously diverse CDR-H3 repertoire, inspection of the actual composition of the antigen binding site repertoire reveals a major bias in CDR-H3 amino acid content. Tyrosine and glycine are preferred, whereas charged and hydrophobic amino acids are under-represented. This non-random pattern of amino acid usage reflects natural selection for a bias in D gene segment sequence by reading frame that is coupled to a bias in

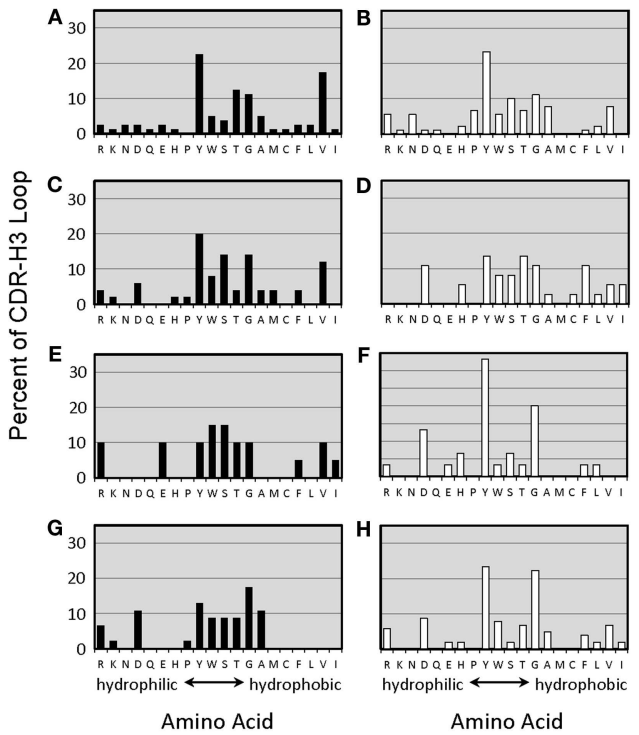

FIGURE 4 | Amino acids of CDR-H3 loops of monoclonal anti-phOx Ab of D-altered ΔD-Dμ*FS* mice. Amino acids are indicated at the *x*-axis in the one-letter code. Hybridomas secreting anti-phOx antibodies (IgM black bars, IgG white bars) were generated after primary immunization on day 7 **(A)** IgM ($n = 16$ mAb), **(B)** IgG ($n = 14$ mAb), and on day 14 **(C)** IgM ($n = 8$ mAb), **(D)** IgG ($n = 7$ mAb), 3 days after secondary immunization **(E)** IgM ($n = 2$ mAb), **(F)** IgG ($n = 7$ mAb), 3 days after tertiary immunization, **(G)** IgM ($n = 11$ mAb), and **(H)** IgG ($n = 17$ mAb). See also Figure S3 in Supplementary Material.

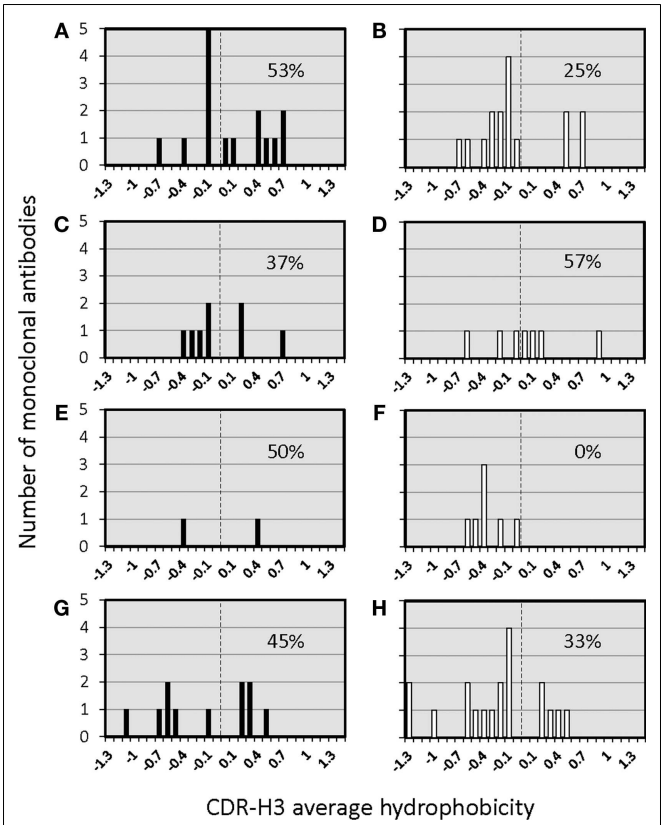

FIGURE 5 | Distribution of average hydropathicity values of CDR-H3 loops of monoclonal anti-phOx Ab of D-altered ΔD-Dμ*FS* mutant mice. The average hydropathicity values of CDR-H3 loops were calculated with the normalized Kyte–Doolittle hydrophobicity scale (8). Anti-phOx antibody-secreting hybridomas (IgM black bars, IgG white bars) were generated after primary immunization on day 7 **(A)** IgM ($n = 16$ mAb), **(B)** IgG ($n = 14$ mAb), and on day 14 **(C)** IgM ($n = 8$ mAb), **(D)** IgG ($n = 7$ mAb), 3 days after secondary immunization **(E)** IgM ($n = 2$ mAb), **(F)** IgG ($n = 7$ mAb), 3 days after tertiary immunization, **(G)** IgM ($n = 11$ mAb), and **(H)** IgG ($n = 17$ mAb). See also Figure S3 in Supplementary Material.

reading frame choice (8). Together, these conserved biases yield a greatly restricted repertoire with some types of antigen binding sites represented more frequently than random chance would allow, and others grossly under-represented.

To test the functional significance of constraints introduced by natural selection of the germline antibody repertoire on B cell development and antibody production, we previously altered the D_H locus in BALB/c mice to force use of alternative D_H sequence (8). Violation of naturally selected germline constraints on CDR-H3 content led to impairments in total serum IgG levels, in tetanus-specific IgG antibody production after tetanus toxoid vaccination, and to the ability to protect against re-infection with influenza virus of a different serotype, termed heterosubtypic immunity. All of these facets of antibody production and protection are T cell dependent.

In the present work, we have gone beyond global measurements of total immunoglobulin production or protection against infection with a pathogen to assess the effects on T dependent antibody production at the individual B cell sequence level in the classic phOx system, whose study yielded many of the paradigms still in common thought to this day.

Here, we report that changes in naturally selected D_H sequence have not only yielded a decrease in serum immunoglobulin levels directed against yet another T cell dependent antigen; they have resulted in major changes at the molecular level to the nature of the antibody produced even though the simple addition of only five nucleotides of N addition can yield the classic I Id_{Ox1} irrespective of the sequence of the D. In particular, we observe enhanced and persistent production of hybridomas secreting low affinity IgM after secondary and tertiary challenge, as well as a failure to develop a fully mature, class switched IgG response equivalent to that produced by WT mice.

Classically, anti-phOx hybridomas obtained on day 3 of memory responses predominantly produce IgG mAb (13, 15). Therefore, we anticipated that the D-altered mice would also primarily produce IgG mAb. The consistently high rescue of IgM-producing hybridomas from memory responses was thus unexpected.

Development of a fully mature IgG response in WT mice is marked by clonal expansion of B cells using the $V_HOx1/V_\kappa Ox1$

Table 1 | D_H segment usage of IGVH171-encoded monoclonal anti-2-phenyloxazolone antibodies from BALB/c wild-type mice immunized with two different immunization schemes.

	FL16.1	FL16.2	SP2.2	SP2.3	SP2.5	SP2.6	SP2.9	SP2.10	SP2.11	ST4	Q52	Not found
Scheme 1 ($n = 22$)	–	–	1[a]	–	–	–	6	–	6	2/3[b]	4/3	–
Scheme 2 ($n = 39$)	–/1	–/1	2	2	1	1/1	4/2	4	–	–	–	20[c]

Scheme 1 – usage of D_H segments of monoclonal antibodies prepared from mice immunized with the thymus-dependent antigen 2-phenyloxazolone-(phOx)-coupled chicken serum albumin (phOx-CSA) (3).

Scheme 2 – here, monoclonal antibodies are combined, which were generated in the course of the TD response to phOx-CSA of mice previously immunized with the TI-2 antigen phOx-Ficoll (12). Both groups comprise antibodies from the early primary to hyper-immune responses.

[a]*Numbers without or before a slash indicate the amount of antibodies using the respective D_H gene segment.*

[b]*Numbers behind the slash indicate usage of inverse D_H gene segments.*

[c]*Assignment of a D_H gene segment was not possible.*

gene combination coupled with a focusing of CDR-H3 sequence with preference given to CDR-H3s encoded a short, DRG containing peptide sequence. In the D-altered mice, V_H and V_L variability persisted (**Figure 2**), V_H1 family-encoded mAb continued to be produced, and no evidence of focusing of CDR-H3 sequence and structure was observed (**Figures 2** and **3**). This failure to clonally select for the various elements of the Id_{Ox1} idiotype occurred in spite of the fact that both mutant strains were able to generate an anti-phOx antibodies containing both the Id_{Ox1} V_H171 H chain/Vκ072 L chain combination in conjunction with a CDR-H3 sequence similar or near identical to the classic idiotype (Table S3a in Supplementary Material).

Together, these findings suggest that the impairment in antibody production that we had observed in previous studies as a result of violation of evolutionarily conserved D gene sequence is accompanied by impairment of hallmarks of affinity maturation, such as clonal expansion and class switching. This occurs even when the classically preferred anti-phOx sequence can not only in theory be generated in the absence of D specific sequence, but is present in practice and thus available for clonal expansion and class switching.

The mechanism(s) that have led to the failure of antigen driven T cell dependent clonal expansion to produce the expected outcome are unclear. Two possible mechanisms involve B cells alone. First, the failure of the Id_{Ox1} idiotype to dominate could reflect the reduced likelihood of creating the Id_{Ox1} CDR-H3 sequence that might result from the loss of the D_H gene segments that normally contribute to its generation. However, comparable Id_{Ox1} CDR-H3 sequence was detected in the D-altered mice. Moreover, in WT mice the Id_{Ox1}^+ Ab begins as a minority of the early primary IgM response. It only dominates with the help of T cells, since it occurs only after class switching (**Figure 2**). Thus, attributing the change in outcome to an absence or diminution in the initial prevalence of Id_{Ox1} CDR-H3 is not compelling.

Second, the immunological imprinting that normally occurs during ontogeny might be altered as a result of the global change in the CDR-H3 repertoire (18, 19). The importance of controlling the B cell repertoire is underscored by the observation that neonatal injection or maternally derived anti-idiotypic antibodies may induce a drastic distortion of the adult B cell repertoire (20, 21) and maternal anti-idiotypes can even induce a long-lasting transgenerational suppression of IgE responsiveness (22). However, these types of early imprinted responses typically involve B cells expressing specificities directed against antigens encountered early in life. PhOx, however, is a foreign and manufactured antigen.

Alternatively, since the response that we have studied is T dependent, the mechanisms that have led to failure of clonal expansion and class switch recombination (CSR) could also reflect a D_H sequence dependent effect on interactions between T cells and B cells. There are several possible mechanisms by which this could occur. First, in the D-altered mice, we have previously observed changes in the distribution of B cell subset numbers and in the repertoire expressed by these B cells (8). It has been suggested that TD Ag-activated IgG^+ and IgM^+ memory B cells form a whole spectrum of memory B cell populations (23), although this view is not undissented (24). Most memory B cells appear to differentiate as a result of germinal center reactions. However, they can also be generated in a GC-independent manner in the follicles or even outside follicles (25). Many GC-independent follicular memory B cells are of the IgM^+-only type (25–27), contain few or no somatic mutations and have not undergone affinity maturation (28–30). These same attributes are found in our memory IgM anti-phOx mAb (**Figure 1**). It is thus possible that the memory IgM mAb in the D-altred mice derive from a GC-independent pathway.

A second possibility is that the change in the repertoire of D sequence-associated antigen binding sites alters the antigen presentation properties of B cells for the phOx bearing antigen. D-alteration shifts the distribution of antigen binding sites, including enrichment for sites that are normally rare and depletion of sites that are normally common. The production of novel immunoglobulins with high affinity for the hapten could lead to changes in the peptides derived from the carrier protein that are presented to T cells by B cells in their capacity as antigen presenting cells (31). A global altered pattern of epitope recognition by responding T cells could inhibit T cell helper driven clonal expansion and affinity maturation.

A third possible T dependent mechanism could reflect the role of CDR-H3 as a potential T cell epitope, and thus contribute to distortions in T cell–B cell interactions (32–35). For example, the

Table 2 | CDR-H3 amino acid sequences of (A) V_H171- and (B) non-V_H171-encoded monoclonal anti-phOx antibodies from D-altered ΔD-DμFS and ΔD-iD mutant mice[a].

ΔD-DμFS mice					ΔD-iD mice				
mAb[b]	**Is[c]**	**Fam[d]**	**V_H[e]**	**CDR-H3[f]**	**mAb[b]**	**Is[c]**	**Fam[d]**	**V_H[e]**	**CDR-H3[f]**
(A)									
1°7/09	μ	2	171	C_AR***LTQT***FAY	1°7/04	μ	2	171	C_AR***DRG***DY
1°7/24[g]	γ	2	171	C_AR***DPG***AY	1°7/05	μ	2	171	C_SR***DRG***DY
1°14/11	γ	2	171	C_AR***DFGK***D	1°7/06	μ	2	171	C_AR***DRG***AY
2°/05[g]	γ	2	171	C_AR***DSG***DY	1°7/12	γ	2	171	C_AR***DRG***DY
2°/06[g]	γ	2	171	C_AR***DGG***AY	1°7/13	γ	2	171	C_AR***SYRNHSRT***AY
2°/07[g]	γ	2	171	C_AR**DY*GI***Y	3°/02	μ	2	171	C_AR***DGG***DY
2°/08	γ	2	171	C_AR***DGG***DY	3°/03[g]	μ	2	171	C_AR***DGGI***S
3°/05	μ	2	171	C_AR***DSG***DY	3°/04	μ	2	171	C_AR***AGRSYG***WYFDV
3°/19[g]	γ	2	171	C_AR***DGG***TY	3°/11[g]	γ	2	171	C_AR***DGG***AF
3°/20	γ	2	171	C_AR***DEGV***N					
(B)									
1°7/03	μ	1	627	C_ARGYFDV	1°7/03	μ	1	286	C_AR***WGN***DY
1°7/04	μ	1	627	C_AR***A***NFDY	1°7/08	μ	6	114	C_TR***RG***TH
1°7/07	μ	1	396	C_***AIR***DY	1°7/11	γ	11	183	C_AR***NWG***DY
1°7/12	μ	3	128	C_ARRYFDV	1°14/01	μ	1	706	C_AR***R***DAY
1°7/25	γ	3	128	C_ARRYFDV	1°14/18	γ	1	528	C_AR***R***FAY
1°7/29	γ	5	139	C_AR***SPG***DY	2°/01	μ	6	114	C_TR***RG***DY
1°7/32	γ	14	125	C_***VPV***AWFAY					
1°14/03	μ	1	495	C_A***RWE***AY					
1°14/05	μ	1	073	C_AR***DGG***AY[h]					
3°/04	μ	1	286	C_AR***RDG***AY					
3°/08	μ	5	139	C_AR**DYG**DY[h]					
3°/09	μ	5	139	C_AR**DYG**DY[h]					
3°/10	μ	6	494	C_T***GG***PWFAY					
3°/18	γ	1	175	C_AR***DWGD***Y[h]					
3°/22	γ	5	139	C_AR**DYG**AY[h]					
3°/23	γ	6	114	C_T***TRG***DY					
3°/29	γ	14	125	C_AS**DYG*L***Y					

[a]The complete characteristics of these antibodies are compiled in Tables S1 and S2 in Supplementary Material.

[b]The designation of antibodies of ΔD-DμFS and ΔD-iD mutant mice indicates their generation on day 7 (1°7) or day 14 (1°14) after primary or on day 3 after secondary (2°) or tertiary (3°) immunization and is followed by a sequential number.

[c]Isotype of antibodies.

[d]Indicates the V_H gene family.

[e]V_H gene numbers according to the integrative database VBASE2.

[f]The CDR-H3 amino acid sequences are given in the one-letter code. Amino acids in bold are derived from the respective D_H gene segments while those in italics and underlined are generated by N region insertions and P nucleotides.

[g]V_H171-encoded antibodies in combination with the Id_{Ox1} V_L gene Vκ072 are indicated by a gray background.

[h]These CDR-H3 amino acid sequences have already been observed in Id_{Ox1} antibodies from BALB/c WT mice.

response to the TI-2 antigen α (1–3) dextran can be influenced by CDR-H3-specific T cells, which inhibit CSR of the dominant J558 IgM idiotype (36). And, BCR-specific T cells have been shown to be capable of interrupting an ongoing GC reaction, favoring the differentiation of short-lived extrafollicular plasmablasts (37). This interaction could help explain the enhanced yield of IgM hybridomas that we observed during memory responses in the D-altered mice, as well as explaining the reduced humoral IgM titers at later times of these responses (**Figure 1**). We would note that none of these potential mechanisms are mutually exclusive. Thus, one or more could be contributing to the failure of this TD response. Studies to clarify the mechanisms by which this has occurred are ongoing in our laboratory.

In this manuscript, we report a test of the hypothesis that conservation of the sequence signature of H chain diversity gene segments, which reflects the effect of natural selection, can influence the outcome of a T dependent response to antigen at the Ab molecular level. We found that subverting the effects of natural selection on the B cell CDR-H3 repertoire led to an alteration of the pattern of T cell-dependent CSR-associated clonal progression

Table 3 | Exemplary representation of the spectrum of CDR-H3 lengths and average hydropathicity values of CDR-H3 loops of selected anti-phOx antibodies from D-altered ΔD-DμFS and ΔD-iD mice.

mAb[a]	Is[b]	CDR-H3[c]																HD[d]
					CDR-H3 Loop[e]													
FS1°7/07	μ	C	A	I	-									R	D	Y	W	---
FS1°14/03	μ	C	A	R	W									E	A	Y	W	-0.14
FS3°/23	γ	C	T	T	R									G	D	Y	W	-1.3
FS1°7/31	γ	C	V	P	V	A	W							F	A	Y	W	+0.74
iD1°14/07	μ	C	A	R	S	I	V	I	I	V	E			A	K	L	W	+1.03
FS1°14/16	γ	C	A	S	F	F	I	T	T	V	V			R	A	Y	W	+0.99
FS1°7/32	γ	C	A	L	Y	Y	Y	G	S	G	T	W		F	S	Y	W	-0.13
FS1°7/11	μ	C	A	L	F	T	T	V	V	S	Y	V	L	F	D	Y	W	+0.75
FS2°/09	γ	C	A	R	H	E	F	Y	Y	Y	G	S	Y	L	R	A	Y	W	-0.06
FS1°7/16	μ	C	A	G	V	H	N	Y	Y	G	S	Y	G	W	Y	F	D	V	W	-0.14
iD1°14/05	μ	C	A	R	K	D	R	N	H	S	R	R	D	W	Y	F	D	V	W	-0.86
iD1°7/07	μ	C	A	R	Q	G	R	N	H	S	R	S	Y	P	Y	W	Y	F	D	V	W	-0.54

[a]*The complete characteristics of these antibodies are compiled in Tables S1 and S2 in Supplementary Material. The designation of antibodies of ΔD-DμFS (FS) and ΔD-iD (iD) mutant mice indicates their generation on day 7 (1°7) or day 14 (1°14) after primary or on day 3 after secondary (2°) or tertiary (3°) immunization and is followed by a sequential number.*

[b]*Isotype of antibodies.*

[c]*The CDR-H3 amino acid sequences are given in the one-letter code.*

[d]*Average hydropathicity of CDR-H3 loops.*

[e]*Amino acids of the CDR-H3 loops are accented by a gray background.*

in these mutant mice (no dominant idiotypes, no elimination of V_H1 family-encoded antibodies, and no selection of homogeneous CDR-H3). Our finding that the sequence of the D_H controls this specific TD response suggests that a full understanding of protective and non-protective responses to self or exogenous antigens, including vaccines, pathogens, and self antigens, will likely require clarification of the role of natural constraints on the antigen binding site repertoire. Vaccination strategies may need to be modified in order to take into account the constraints on humoral responses imposed by evolution.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00385/abstract

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Epigenetic codes programing class switch recombination

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Class switch recombination imparts B cells with a fitness-associated adaptive advantage during a humoral immune response by using a precision-tailored DNA excision and ligation process to swap the default constant region gene of the antibody with a new one that has unique effector functions. This secondary diversification of the antibody repertoire is a hallmark of the adaptability of B cells when confronted with environmental and pathogenic challenges. Given that the nucleotide sequence of genes during class switching remains unchanged (genetic constraints), it is logical and necessary therefore, to integrate the adaptability of B cells to an epigenetic state, which is dynamic and can be heritably modulated before, after, or even during an antibody-dependent immune response. Epigenetic regulation encompasses heritable changes that affect function (phenotype) without altering the sequence information embedded in a gene, and include histone, DNA and RNA modifications. Here, we review current literature on how B cells use an epigenetic code language as a means to ensure antibody plasticity in light of pathogenic insults.

Keywords: AID, DNA repair, DNA deamination, recombination, non-coding RNAs

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Introduction

Genes are the basic molecular unit of heredity in all organisms since it is the gene (genotype) and not the trait (phenotype; except for imprinted genes) that is inherited. A gene usually refers to a particular sequence of nucleotides that has an annotated function. However, phenotypic manifestations can be governed by factors beyond (epi) genes (genetics), which too are hereditary. Epigenetic alterations are carried out by a repertoire of modifiers (writers and erasers) and readers, which can act on either proteins (histones) or nucleic acid (DNA/RNA) (1–3). Epigenetic regulation also includes non-coding RNAs (micro-RNA and long non-coding RNA), which can directly or indirectly (via recruitment of proteins) affect gene expression (4–6). This complex layer of gene regulation is a testament to the pliability that the system needs and possesses. The immune system exemplifies one such complex system that is geared to adapt to the environment, and B cells that make antibodies also need to reshape their antibody repertoire during antigenic challenges. Thus, it is not surprising that B cells overcome genetic constraints and integrate environmental cues into a complex network of gene regulation, which is both flexible and heritable. Dynamic epigenetic alterations engineered with spatiotemporal precision that expand the genetic code beyond A, G, C, and T, would be ideal for B cells in their quest to diversify the antibody repertoire during infection by an ever-evolving array of pathogens.

Class switch recombination (CSR) is one such secondary antibody diversification that occurs in peripheral lymphoid organs when B cells encounter antigen, and is dependent on cytokine/chemokine cues generated by T cells and stromal cells (7). The switching of the antibody isotype from IgM (or IgD)

to IgG, IgE, or IgA is necessary to impart distinct effector functions (8, 9). At the molecular level, CSR is a deletional-recombination reaction occurring between repetitive DNA elements, called switch (S) regions that precede each constant region (C_H) segment. Cytokine stimulation and/or antigen binding to B cells stimulate "germline" transcription through the S regions and promote accessibility of the DNA deaminase AID (activation-induced cytidine deaminase), whose activity leads to the generation of DNA double-strand breaks (DSBs) at S regions. End-joining of DSBs between donor (usually Sμ) and acceptor S (Sγ, Sε, or Sα) regions replaces Cμ for a different C_H gene segment downstream of the rearranged variable region segment to complete CSR (**Figure 1**). The drivers of germline transcription, AID expression, its target specificity and factors in end-joining have been extensively studied and reviewed elsewhere in Ref. (8, 10–13).

The role of histone modifications, DNA methylation, and non-coding RNAs during CSR has been recognized and appreciated significantly in the last decade (4, 14, 15). Given that B cells encounter an inflammatory milieu during an immune response and since the same cues can also program epigenetic changes, it suffices to say that there must be an underlying intricate chromatin network landscape, which is shaped to govern CSR. In this review, we will focus on recent advances that buttress the existence of epigenetic codes that orchestrate CSR.

Epigenetic Control of Switch (S) Region Locus Accessibility and Antibody Response

S regions are highly repetitive G-rich sequences that precede each constant region gene. Transcription through these regions is necessary for CSR. However, constitutive (donor S region) and inducible (acceptor S region) transcription is associated either with a poised and dynamic epigenetic state, respectively (4). Donor S region (Sμ) has a transcriptionally active state even in naïve B cells, suggesting that it is poised for participation in CSR. Histone modifications like H3K4me3 and H3K9ac are present at Sμ in naïve B cells and increases upon stimulation (16–19). These are marks associated with an open chromatin conformation that would favor accessibility of the CSR machinery including tethering AID to donor S region.

On the other hand, the acceptor S regions (Sγ1, Sγ3, Sγ2a, Sε, and Sα) are inaccessible to the CSR machinery at the basal state. When the B cell faces an antigenic challenge that provides CSR triggers including T cell help (CD40 ligation), pattern-recognition receptor ligation and the cytokine milieu (IL4, TGFβ, retinoic acid, BAFF, and IFNγ), the cognate S acceptor region accumulate histone modifications that are permissive to transcription and accessibility of only the particular S acceptor participating in CSR. The alterations include removal of repressive chromatin marks (H3K9me3 and H3K27me3) and also induction of H3K9ac and H3K4me3 (**Figure 2**) (17, 18). Knockdown of histone chaperone FACT complex components (SSRP1 and SPT16), Spt6, and methyl transferases (Ash2 and Wdr5) in CH12F3 cells have revealed the pertinence of the histone marks in regulating locus accessibility and S region cleavage, and thus CSR to IgA, a function which extends beyond germline transcript induction (20–22). If the same holds true in primary B cells and is it generalizable for CSR to other antibody isotypes remains to be determined. Additionally, H3K9me3 mark found at donor S region can recruit HP1γ-KAP1 complex, which facilitates AID tethering (17). The enzyme Suv39h1, which is probably the histone methyl transferase involved in the deposition of this histone mark, plays a positive role in inducing CSR to IgA, since deletion of the gene impairs IgA CSR in primary B cells without affecting GLT (23). It is not specifically clear as to why there is isotype specificity if H3K9me3 is a donor S region mark.

A word of caution in the interpretation of all these results is warranted since most of the studies show strong correlative but

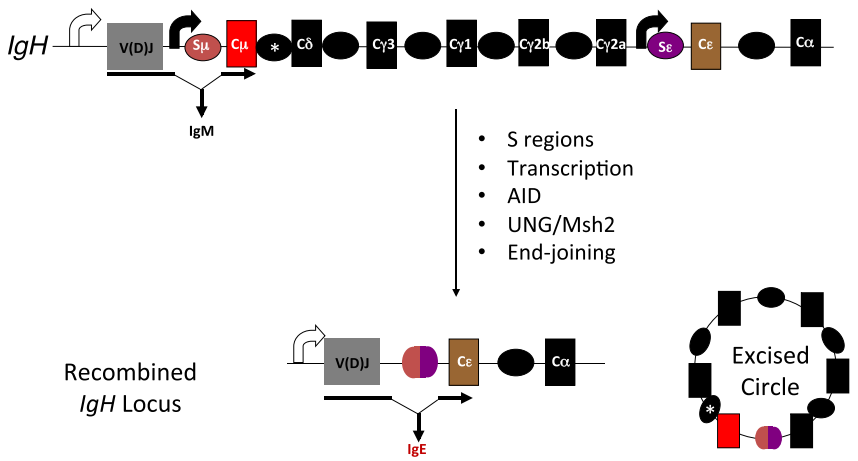

FIGURE 1 | Overview of CSR. Each of the CH genes is preceded by transcribed, repetitive DNA elements called switch (S) regions. AID mediated DNA deamination, followed by processing of deaminated cytidines by components of the base excision and mismatch repair machineries (UNG and Msh2, respectively) lead to the generation of DNA double-strand breaks. End-joining of double-strand breaks between donor and acceptor S regions completes CSR. Scheme for CSR to IgE is shown; block curved arrows indicate germline transcription from promoters upstream of Sμ and Sε; *represents a putative, but uncharacterized switch region upstream of Cδ.

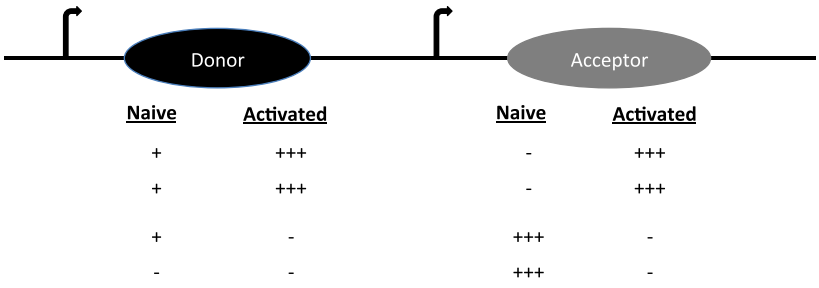

FIGURE 2 | The histone modifications on donor and acceptor S regions in naïve and activated B cells are shown.

not direct causative evidence; genetic models targeting the histone modifying enzymes would be required to unequivocally support the claims. Even the use of genetic or pharmacologic approach to manipulate histone modifications might not provide conclusive proof of their specific role in locus accessibility due to functional promiscuity/essentiality of the enzymes. Future studies are necessary to uncover the complex relationships that probably exist between multiple factors influenced by a single histone modification, and how an ensemble of spatiotemporal histone modifications orchestrates CSR.

The function of mixed-lineage leukemia (MLL)-like H3K4 methyl transferase complex in CSR was tested by deletion of a key component PTIP conditionally in B cells (24, 25). These studies revealed that compromised H3K4me and acetylation and global chromatin architectural changes associated with the loss of PTIP leads to decreased S region accessibility, germline transcription, and thus compromised CSR to multiple isotypes (24, 25).

The function of histone acetylation/deacetylation in B cells was uncovered by conditionally inactivating MOZ (H3K9 acetyl transferase) and HDAC-1 and -2 genes in mature B cells (26, 27). Stage-specific MOZ deletion suggested that it is required for optimal GC response (proper affinity maturation and memory formation) (26, 28). HDAC-1/2 were absolutely necessary for the proliferative burst that B cells undergo during stimulation for CSR, and double-deficient B cells fail to divide and die by apoptosis when challenged with LPS + IL4 (29). In contrast, pharmacological intervention with HDAC inhibitors, butyrate and valproic acid, *in vivo* during T-dependent/independent antibody response and *ex vivo*, did not affect B cell proliferation and viability (30). However, they did block CSR and plasma cell differentiation by upregulating cognate micro-RNAs that dampen AID and Blimp1, critical for CSR and plasma cell differentiation, respectively. Interestingly, the therapeutic potential of HDAC inhibitors in treating antibody-mediated lupus was highlighted by the finding that they ameliorated disease and prolonged survival in a mouse model of lupus (30). Discrepancies between genetic and pharmacological approaches can be due to milder effect of inhibitors or more broad effects on multiple HDACs beside HDAC-1/2.

Finally, the function of polycomb repressive complex (PRC)-2 component, Ezh2 (histone methyl transferase), which was shown to be necessary for B cell development (31), during GC response was tested by conditional inactivation of Ezh2 using Cγ1-cre (32, 33). Two independent studies provided compelling evidence that the germinal center (GC) formation and consequent cell-fate decisions including CSR, SHM, plasma cell differentiation and memory response are critically shaped by Ezh2 (32, 33). H3K27me3 and H3K4me3 ChIP-Seq in Ezh2-sufficient versus -deficient GC B cells revealed that Ezh2 negatively regulates terminal differentiation by epigenetically (via PRC2) regulating Blimp1, Xbp1, and Irf4, and thus promotes long-lasting immunity by sustaining antibody diversification and memory B cell differentiation (26, 33). Gain of function mutant Ezh2 alleles (found in lymphoma patients) cause GC hyperplasia in mice and cooperate with Bcl2 to accelerate and sustain malignant transformation of GC B cells (32). Combinational therapeutic targeting of Ezh2 for specific B cell malignancies (GC-DLBCL) will definitely be enticing (32, 33).

Epigenetic Control of AID Expression

AID is the key enzyme that is essential for CSR. It is probably one of the only proteins unique to stimulated mature B cells, and AID fate-mapping studies corroborate that physiologically functional levels of expression are largely restricted to B cells (34). It instigates DNA lesions in the form of deaminated deoxycytidine (dC), i.e., deoxyuridine (dU) at donor and acceptor S regions, which is subsequently processed by the general base excision repair, mismatch repair machineries, and DNA end-processing enzymes, such as, Mre11 and CtIP to generate DNA DSBs (10). Since it can induce DSBs, it is a potential mutator, and thus, AID expression is stringently controlled at the transcriptional and post-translational levels (8, 12). However, herein we review epigenetic factors modulating AID expression.

At the epigenetic level, AID expression is controlled by DNA methylation–demethylation and micro-RNAs. The AID locus, especially the promoter sites for cognate stimuli-induced transcriptional factors are hypermethylated in naïve B cells and the mark is reversibly modulated during different stages of B cell-fate program (35). Activated B cells or GC (GL7+Fas+) B cells acquire a permissive epigenetic landscape of hypomethylation that allows robust AID expression by STAT6, NF-κβ and Hox-C4 transcription factors (4). Besides, phylogenetic footprinting, histone acetylation, and DNase1 hypersensitivity site (DHS)-mapping revealed that the AID locus is dynamically shaped during an ongoing immune response (36). Histone H3 acetylation is increased at AID regulatory regions upon *in vitro* stimulation of naïve B cells and in GC B cells. In activated B cells, a conserved non-coding

sequence 7kb downstream of the AID locus maps to a DHS and regulates AID expression positively, via the binding of a yet to be identified protein (36). However, AID is turned off epigenetically upon terminal differentiation, probably as a means to preserve antigen specificity of the antibody secreting B cells (36).

Post-transcriptional regulation of AID by micro-RNAs 155, 181b, 93, and 361, provides an additional layer of safeguard against a potent genome mutator (12). miR155, is the best studied one, which suppresses *aicda* expression by binding to a canonical site on the 3′-UTR of *aicda*. Although miR155 has functions way beyond suppressing AID in B cells undergoing CSR, as evidenced by compromised CSR in absence of miR155, yet its specific effect on regulating AID is significant. Mutation of the miR155-binding site on *aicda* 3′-UTR leads to increased AID levels that potentiate cMyc-IgH translocations (37, 38). Besides, miR155 and AID levels are inversely correlated in Burkitt's lymphoma, and an IL10/miR155 axis can potentially modulate AID expression during chronic inflammation and lymphomagenesis (39).

Epigenetic Control of AID Targeting

An enzyme like AID is a dual-edged sword; on one hand, it is mandatory for optimal humoral immunity but on the other, a threat to genomic integrity. Therefore, a normal B cell must delegate adequate layers of safeguard in addition to regulating AID expression, which would primarily target AID to the physiological targets. Genetic factors controlling AID targeting and function have been reviewed elsewhere in Ref. (12, 13, 40). Herein, we will focus on epigenetic guides of this potent mutator.

Histone Modifications

One way to limit the risk of collateral damage would be to sequester AID at hotspot target motifs. S regions are GC-rich and possess stretches of 5′-AGCT-3′, which are AID hotspots (13). These regions, when transcribed form stable R-loop structures that provide single-stranded DNA substrates for AID (10). An intriguing finding is that histone modifications, such as, H3S10 phosphorylation induced in CSR-activated B cells have also been linked to R-loop formation (41). Stable R-loops formed during CSR stimulation of B cells at S regions also accumulate H3K9AcS10ph modification. The classical adaptor protein 14-3-3, which has unique specificity for 5′-AGCT-3′ repeats and H3K9AcS10ph modification, also directly binds AID (42, 43). Thus, it is well poised to recruit AID to recombining S regions during CSR, thereby serving as transducers of the epigenetic code (4). It remains to be seen, however, if genome-wide occupancy of 14-3-3, H3K9AcS10ph, and AID overlap, or if 14-3-3 only functions during physiological AID targeting. Another study focused on the chromatin-bound AID-interactome to reveal that the RNA polymerase-associated factor (PAF) complex member LEO1 is required for efficient targeting of AID to Sμ in CH12F3A cells (44). It will be interesting to test if the function of LEO1 is also pertinent in B cells undergoing *ex vivo* CSR and more importantly in GC B cells. AID was also shown to bind the KAP1-HP1γ complex, the latter of which recognizes H3K9me3 (17). AID targeting was dependent on KAP1 and also on its association with HP1, since genetic manipulation studies clearly revealed that AID occupancy at Sμ was dampened due to loss of KAP1 alone or its interaction with HP1 (17).

Super-Enhancers and Regulatory Clusters

Enhancers are classically defined as a class of DNA elements that function in promoting transcription of gene from a distance, and irrespective of their orientation with respect to the target gene (45). Advance is sequencing techniques like DHS mapping (Dnase-seq), ChIP-seq and 3C-5C, Hi-Seq in the last decades has enabled genome-wide characterization of enhancers. Key features include presence of Dnase1 hypersensitive sites, multiple transcription factor binding sites, histone modifications like H3K4me1 and H3K27Ac, and looping to contact promoter elements far apart in the genome (46). Essentially now, the presence of a chromatin profile as alluded to above is considered as a hallmark of enhancers, although to date these are strongly correlative yet not always functionally causative. A new class of regulatory DNA elements has been defined recently, termed super-enhancers or stretch-enhancers (47, 48). These are cell-type specific enhancers that play a key role in establishing lineage or cell identity (47, 48). They are defined as clusters of large regulatory domains that have remarkable enrichment for transcription factor and coactivator (Mediator) binding along with a characteristic chromatin landscape (nucleosome occupancy and histone modifications) (46). Since AID expression and CSR are unique to B cells, it would make sense to regulate this cell-type specific expression and targeting by integrating stimulation cues to topologically associated domains i.e., super-enhancers. Recent work from several laboratories has greatly advanced our understanding of AID targeting/mistargeting (49–51).

Using genome-wide sequencing approaches including GRO-seq, DNase-seq, and ChIP-seq, AID targets were found to be mostly unique in different cell-types (B cells, MEFs) although they shared common features of being transcriptionally active regions. However, transcription alone was not sufficient to explain the distinct set of hotspot in MEF versus B cells (49). Analysis of genes transcribed in both cell-types but only targeted in one, led to identification of a shared set of epigenetic attributes including H3K27Ac and H3K36me3, which typify enhancers (49). Deep sequencing techniques allowed uncovering a remarkable overlap of AID target sites to regions of the genome that constitute super-enhancers (50, 51). The AID off-targets like Cd83 in CSR-activated B cells map to regions enriched in chromatin marks, typical of super-enhancers, and lie within sites of convergent transcription (sense transcription from promoter of genes and anti-sense transcription from super-enhancer) (50, 51). A majority of AID-instigated lesions (irrespective of cell-type) occurred at transcription start sites, which were connected over long distances to multiple topologically active "regulatory clusters" (51). A central theme that came out from these elegant studies is that the nuclear microenvironment, which can vary from one cell type to another (even between *ex vivo* CSR-activated B cells and GC B cells), greatly influences AID target selection, yet the targets do share the following commonalities: (a) highly transcribed super-enhancers, (b) topologically interconnected clusters, and (c) sites of convergent transcription (50–52) (**Figure 3**). How these findings fit the available models of stalled Pol-II (Spt5), 14-3-3, RNA

exosome, PTBP2-mediated AID targeting remains to be explored (8). Additionally, these strong correlative evidences should now be tested for causation using the CRISPR-Cas9 system by abrogating transcription or knocking out the eRNA transcripts, to query if it affects the mutational landscape in AID-expressing cells. Another open question is whether convergent transcription regulates physiological Ig locus targeting of AID during CSR. These exciting avenues remain to be explored and would be at the forefront of research of AID biology in the coming years.

Non-Coding RNA

AID, being a member of the APOBEC-family of enzymes, has long been known to be associated with RNA and RNA metabolism-associated factors, such as, RNA Pol-II, Spt5, RNA exosome, PTBP2, and CTNNBL1 (8). Since AID activity has been strongly linked to transcription, R-loop formation and anti-sense RNA processing, a recent study delved into the details of how non-coding RNA biology can impact AID targeting (14). Using a mouse model of conditional inactivation of an essential component of the RNA exosome (Exosc3), it was revealed that the RNA exosome shapes the non-coding transcriptome in B cells in a way that allows AID to access sites of anti-sense and divergent transcription (14). This was true for many of the well-characterized off-target sites of AID in B cells including Cd79b, Cd83, Pim1, IL4ra, and cMyc. However, the proposed model of divergently transcribed loci generating RNA exosome substrates facilitating single-stranded DNA access to AID is in contradiction with the convergent transcription model (14, 50), and future work is necessary to address the discrepancies.

Another elegant finding of Exosc3 and 10 conditional deletions in mature B cells was that it unraveled a novel role of this cellular RNA degradation factory in regulating enhancer (e) and super-enhancer (se) RNAs, which as discussed before might have a remarkable impact on AID mistargeting (14, 50, 51, 53). The genome-wide mapping of changes in non-coding RNA transcriptome in RNA exosome-deficient B cells undergoing CSR led to the identification of a distal divergent eRNA-transcribing element (lncRNA-CSR) (53). Ablation of transcription from this element profoundly impacted looping-dependent long-range DNA interactions with IgH 3′ regulatory region super-enhancer and compromised CSR. Additionally, the RNA exosome appears to promote genomic integrity in activated B cells by chewing up genome-destabilizing R-loop structures that emanate from active enhancers and also by regulating chromatin silencing (53). Questions come to mind as to how the CSR-promoting R-loop structures at S regions are preserved temporally, and how the RNA exosome function in facilitating template strand deamination by AID is in unison with its more global functions in genomic integrity (54). Nonetheless, these compelling studies have definitely united super-enhancer and ncRNA biology into B cell research, especially CSR, and will propel the field forward in coming years.

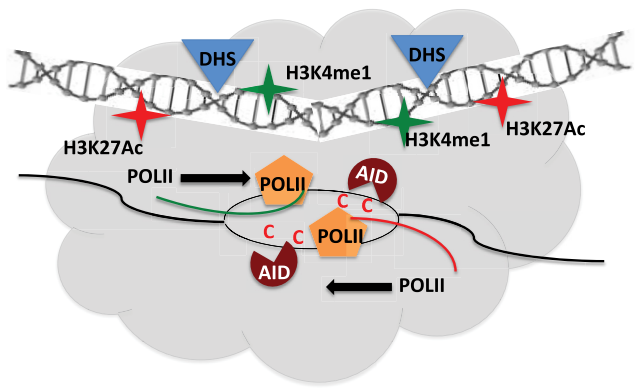

FIGURE 3 | Regulatory clusters containing super-enhancers modulate AID mistargeting. Convergent transcription between promoters of target gene and super-enhancers from topologically associated domains (TAD) allow access of AID to single-stranded DNA and promote off-target activity. DHS – Dnase1 hypersensitive sites.

Co-transcriptional processes and factors including RNA pol-II stalling, RNA exosomes, and even eRNA/seRNA appear to reinforce the transcription and R-loop dependent AID targeting model (12, 14, 16, 50, 51, 53, 54). It can be concluded that the requirement for transcription (convergent/divergent) for AID targeting/mistargeting has been testified. However, an intriguing finding predating the discovery of AID was that processing of switch transcripts by splicing was also necessary for CSR (55, 56). The role of transcript *per se* was elusive and post-transcriptional/splicing mechanisms regulating CSR have been understudied. To address if the sterile switch transcripts have a role in CSR, a mouse model of debranching enzyme (DBR1) haploinsufficiency and a knockdown approach in CH12F3A cells was used (15). These experiments revealed that if the generation of the transcript, post lariat debranching is abrogated, without affecting transcription or splicing, still, CSR is substantially impaired. The defect in CSR in DBR knockdown cells could be fully rescued by providing the switch RNA *in trans*, buttressing specificity of the phenotype (15). The defect in CSR was attributable to a failure to recruit AID to S regions, suggesting that the RNA might act *in trans* to guide AID back to the DNA locus due to sequence complementarity. This was indeed true, because provision of Sα transcripts *in trans* (in DBR1 knockdown CH12F3A cells) although could rescue AID recruitment to Sα but failed to target AID to Sμ, and thus failed to rescue CSR.

The structural basis of AID binding to RNA was due to the ability of the S region RNA to fold into G-quadruplex structures, which could be recognized by a recognition motif in AID that has a conserved G133 residue (15). Interestingly, this residue was also mutated (G133V) in human patients with hyper IgM syndrome (57). Indeed, G133V AID could not be recruited to S regions in B cells and failed completely to rescue CSR in AID-deficient cells, despite being catalytically active, expressed at similar levels and having similar subcellular localization as WT AID (15). The model proposed based on these findings is that following transcription of the S regions, the spliced out lariat is debranched by DBR1, the debranched RNA assumes G-quadruplex structures, which facilitates AID binding and allows sequence- and structure-specific recruitment of AID to DNA during CSR (**Figure 4**). This physiological targeting mechanism can have implications for off-target AID activity as well because primary transcripts of many off-target AID genes (compared to non-target ones) have the potential to form G-quadruplex structures (15).

This opens up the field with many questions: how does the hand-off of AID-RNA complex to the DNA take place, and how the co-transcriptional mechanisms of AID targeting are complemented by this post-splicing mechanism to ensure a coordinated system enabling efficient CSR.

Epigenetic Control of DNA Repair

The culmination of AID activity and lesion processing at recombining S regions during CSR is the generation of DNA DSBs, which constitute one of the most toxic lesions in a cell. Every cell in the body has evolved highly efficient and elaborate system dedicated to repair DSBs in order to promote survival and prevent translocations. However, a B cell faces a daunting challenge; not only does it have to repair the breaks to limit translocation potential, but it also has to time the process with such exquisite precision that it promotes long-range recombination. The non-homologous end-joining repair pathway is the most well-characterized one that is used during CSR (10). However, other pathways, such as, homologous recombination and alternative end-joining also play a role albeit to a lesser extent (8). The function of major players (genetic) in the AID-induced DNA damage response/repair phase that allow recombination including ATM, H2AX, Ligase IV, 53BP1, and Rif1 have been studied in-depth and reviewed elsewhere in Ref. (8, 10, 11, 13).

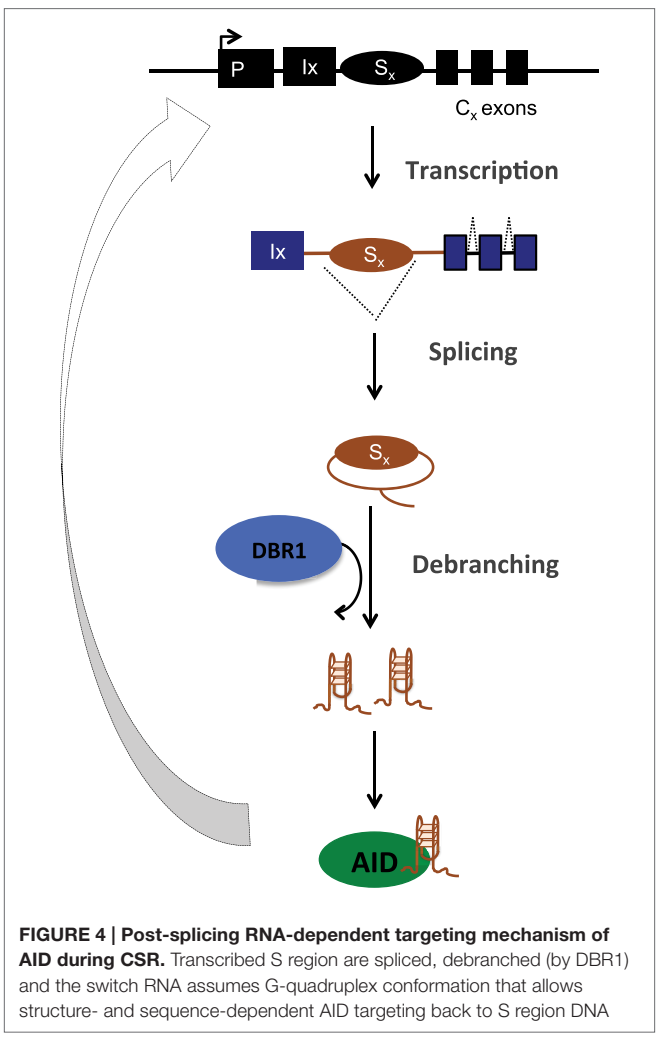

FIGURE 4 | Post-splicing RNA-dependent targeting mechanism of AID during CSR. Transcribed S region are spliced, debranched (by DBR1) and the switch RNA assumes G-quadruplex conformation that allows structure- and sequence-dependent AID targeting back to S region DNA

DNA insults have to be dealt within the context of a highly organized chromatin microenvironment and it is rational to assume that DNA DSB repair would be modulated significantly by epigenetic factors (58–60). Histones and many other DNA repair proteins undergo different post-translational modifications (PTMs) including phosphorylation, ubiquitylation, acetylation, methylation, sumoylation, and PARylation during an active repair response (61). These modifications can serve multiple purposes including serving as scaffold to recruit essential DNA repair factors, many of which harbor complementary domains to recognize the PTM. The histone variant H2AX gets phosphorylated at Ser 139 (γH2AX) in response to DNA damage by PI3K-like family of kinases (ATM, ATR, and DNA-PKcs), a classical DNA DSB marker. H2AX deficiency compromises CSR possibly due to defect in long-range chromatin remodeling and synapsis. BRCT domain containing proteins like MDC1 and 53BP1 also play an important role in the cascade of DNA repair events following DSB induction (62). MDC1, via its BRCT domain recognizes γH2AX and allows recruitment of E3 ubiquitin ligases RNF8 and RNF168, both of which are required for efficient CSR. The CSR defects are milder as compared to that in 53BP1-deficient B cells (63–65). 53BP1 functions mainly to prevent resection of DNA ends by recruiting Rif1 (66), and allows for timely persistence of breaks to be joined *in trans* (to acceptor S regions) rather than in cis (intra-switch recombination). 53BP1 recruitment to DNA is dependent on H4K20me2 mark, which is recognized via its tudor domain. Mutation in the tudor domain leads to compromised CSR, implying that reading the chromatin mark is important in potentiating its function (67). 53BP1 has been recently shown to recognize DNA damage-induced H2AK15 ubiquitylation (68), and it remains to be seen if this function also regulates CSR. The histone methyl transferase MMSET (WHSC1, implicated in Wolf-Hirschornn Syndrome) also functions during CSR, since knockdown of MMSET impairs 53BP1 recruitment to DNA damage site and compromises CSR (69). Lastly, the bromo-domain reader, Brd4, was also shown to function during CSR by providing an ideal chromatin platform during DNA repair phase (70). Brd4 inhibitor JQ1 perturbed CSR by affecting 53BP1 accumulation and end-joining pathway choice (70). JQ1 also compromised *in vivo* GC response during T-dependent Ag challenge, and this was suggested to be due to failure of NF-kappa B signaling and Bcl6 upregulation (71). However JQ1 can have non-specific effects, and genetic or domain-specific mutational approaches to test Brd4 function must be employed in the future to establish its role in CSR.

The methyl cytosine dioxygenase family of enzymes (TETs), has been in focus for their ability to demethylate specific regions of the genome (indirectly, via conversion of methylated cytidines to hydroxymethylated cytidines followed by engagement of base-excison repair) derepress genes (2). These TET enzymes have been found to be mutated or shut-off in many tumors, suggesting that they might have epigenetic tumor suppressive functions. Indeed, deletion of TET1 in mice was associated with increased propensity of development of B cell lymphomas (72). TET1 was required for maintenance of normal 5hmC levels and distribution across

the genome, which allowed adequate expression of essential DNA repair genes (e.g., *lig1*, *ogg1*, *rad50*, and *rad51*). Absence of TET1 led to increased γH2AX foci and DNA damage sensitivity due to lack of repair and potentiated lymphomagenesis (pre-malignant B lymphomas at pro-B cell stage) (72). Outstanding questions about the role of other TET enzymes (TET2 and TET3), and even AID as a demethylase in B cell physiology and pathology remain to be explored (73, 74).

Lastly, chromatin remodeling complexes too have been implicated in reorganizing the chromatin during CSR. Defects in INO80 nucleosome remodeler in humans and in CH12F3A cells compromises CSR, likely due to improper loading of the cohesion complex and synaptic complex formation (75). Indeed, defects in cohesion loading proteins do cause abrogation of CSR (76, 77). These findings need to be introspected further because INO80 and cohesion complex have global cellular functions that extend beyond CSR.

Summary and Perspective

Taken together, a bevy of epigenetic factors including "histone codes", ncRNA, micro-RNAs, and super-enhancers communicate and coordinate to provide a dynamic chromatin landscape, which is geared for optimal diversification of the antibody repertoire.

Advances in sequencing-based techniques and data from the ENCODE project have greatly propelled research in the last decade (78). B cell biology, especially CSR has been investigated at depths like never before, and this has revealed the complexities that underlie this mechanistically counterintuitive process, which the B cell has to accommodate as a cost for co-evolution with pathogens. The smooth collisions of the chromatin and enhancer landscapes that facilitate CSR have been unraveled, however a multitude of questions lie ahead.

New modifications of histones continue to be identified. For example, crotonylation (79), which is thought to be similar to acetylation in being catalyzed by p300 in presence of intracellular crotonyl-CoA (79, 80), and even its erasers have been identified (81). Given that histone acetylation has functions in CSR, it remains to be seen if and how crotonylation can modulate CSR. DNA methylation has been extensively studied over the years and its role in locus accessibility and gene expression (epigenomic) is well characterized. But recently there has been identification of RNA methylation (N^6-methyl-adenosine) as an epitranscriptomic modification (1). Transcriptome-wide m^6A-mapping has provided great insight into the prevalence and relevance of this RNA modification, and how it impacts gene expression (82). Identification of m^6A "readers", "writers" and "erasers" has also propelled epitranscriptomic research (82). Given that CSR is impacted significantly by an integral non-coding RNA (eRNA, seRNA, lncRNA, and micro-RNA) component, and that RNA methylation is more prominent in non-coding RNAs, it will be unsurprising if this modification impacts CSR (83–85). Additionally, a very recent identification of N^6-methyl-deoxyadenosine modification in the DNA of lower eukaryotes as a new "epigenetic" mark also opens up the question whether the writers and erasers of this mark will impact CSR in higher eukaryotes (86–88).

Recent upsurge in research on the impact of microbiota on a multitude of processes in the immune system during health and disease has uncovered the significance of this symbiotic association over centuries of evolution (89). Given that microbes have been shown to produce metabolites that functionally impact the epigenetic status of the host (90), it is tempting to speculate that even antibody responses *in vivo* will be epigenetically shaped by commensal-derived metabolites.

Most research reviewed herein has focused on positive regulation of CSR. But, one aspect that has to be borne in mind is that *in vivo*, CSR is only one of the multiple cell-fates that a clonally expanding population of B cells undergoes. So, there has to be coordinated allocation to other cell-fates including plasma cell differentiation and memory B cell differentiation, possibly by negative modulation of CSR. There has to be active genetic and epigenetic modules that skew cell-fate decisions toward one versus another path. It is rational to presume that such fine-tuning would be necessary in order to achieve an overall humoral response encompassing all aspects of adaptive immunity (adaptability, specificity, and memory). Future research is warranted to understand molecular mechanisms of negative regulation of the individual modules of cell-fate decision programs in B cells during a GC response.

A programed DNA damage process, such as, CSR is rare and unique to B cells, the fundamental understanding of which will propel basic (understanding DNA repair, recombination, and immunity) and translational science (ontogeny of lymphomas and its therapies). Epigenetics undoubtedly has enormous influence on physiology (adaptive immunity) and pathology (autoimmunity and lymphomagenesis) (4). Thus, exciting times lie ahead for research in CSR due to the establishment of the solid framework of the epigenetic landscape governing CSR.

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Epigenetic function of activation-induced cytidine deaminase and its link to lymphomagenesis

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Activation-induced cytidine deaminase (AID) is essential for somatic hypermutation and class switch recombination of immunoglobulin (Ig) genes during B cell maturation and immune response. Expression of AID is tightly regulated due to its mutagenic and recombinogenic potential, which is known to target not only Ig genes, but also non-Ig genes, contributing to lymphomagenesis. In recent years, a new epigenetic function of AID and its link to DNA demethylation came to light in several developmental systems. In this review, we summarize existing evidence linking deamination of unmodified and modified cytidine by AID to base-excision repair and mismatch repair machinery resulting in passive or active removal of DNA methylation mark, with the focus on B cell biology. We also discuss potential contribution of AID-dependent DNA hypomethylation to lymphomagenesis.

Keywords: activation-induced cytidine deaminase, DNA methylation, epigenetics, B cells, lymphomagenesis

DYNAMIC NATURE OF METHYLOME DURING B CELL DEVELOPMENT

Epigenetic mechanisms of regulation including histone modifications, non-coding RNA-mediated gene regulation, chromatin remodeling, and DNA methylation (1) play an important role in B cell differentiation and the antibody response (2). DNA methylation is essential during X-chromosome inactivation, imprinting, and tissue differentiation (3, 4). This epigenetic modification refers to the addition of a methyl group at the C5 position of cytosine (C), mostly when C is bound to guanine (G) creating a CpG site in mammalian organisms (5, 6), with less common non-CpG methylation detected in embryonic stem cells (ESCs) and brain tissue (7, 8). The addition of the methyl group is catalyzed by the family of DNA methyltransferases (DNMTs) (9), whereas DNA demethylation can be a passive or an active process. Passive demethylation occurs when methylC (mC) mark is not faithfully reproduced either during replication or due to DNA damage, while active demethylation requires the action of enzymes and can be replication-independent (10, 11). However, the molecules involved in the 5-methylC (5mC) active demethylation are still not clearly defined (see below). Several studies have investigated the DNA methylation landscape in B cells and the role of the DNA methylome in B cell development. Ji et al. demonstrated in a mouse model using Comprehensive High-throughput Array-based Relative Methylation analysis (CHARM) (12) that lymphoid commitment requires more DNA methylation than myeloid lineage with myeloid skewing of lineages in DNMT1 hypomorphic animals. Loss of methylation predominates during progression of Multipotent Progenitors (MPPs) to Common Lymphoid Progenitors (CLPs) (13, 14). During transition from naïve B cells (NBs) to germinal center B cells (GCBs), occurring in the secondary lymphoid organs after T cell-mediated activation, there is marked demethylation of the genome (15, 16). Memory and plasma cells display DNA methylation patterning very similar to GCBs, although the three subpopulations of B cells differ substantially at the transcriptional level (16). The use of the DNA demethylating agent Decitabine results in complete abrogation of the GCs, while preserving the primary follicles (15), underscoring an important biological role of methylation in B cell development. The fact that entrance of NBs into the GC reaction is characterized by marked upregulation of DNMT1 and simultaneous hypomethylation of many genomic loci suggests yet unknown mechanisms of passive or active demethylation after B cell activation. In this review, we would like to explore the potential role of activation-induced cytidine deaminase (AID) in modifying the methylome of mature B cells. We will present and analyze large amount of conflicting evidence that accumulated up to date linking AID with epigenetic modifications during development and B cell differentiation.

Abbreviations: BCR, B cell receptor; BER, base-excision repair; BL, Burkitt lymphoma; CSR, class switch recombination; DLBCL, diffuse large B cell lymphoma; DNMTs, DNA methyltransferases; DSBs, double strand DNA breaks; EMT, epithelial–mesenchymal transition; ESC, embryonic stem cells; FL, follicular lymphoma; GCBs, germinal center B cells; Ig, immunoglobulin; iPSCs, induced pluripotent stem cells; MMR, mismatch repair; NBs, naïve B cells; PGCs, primordial germ cells; SHM, somatic hypermutation; TLR, toll-like receptor.

FUNCTION OF AID IN B CELL DEVELOPMENT

NBs enter the GC reaction and undergo marked changes in transcriptional program, including dramatic upregulation of the enzyme AID (or AICDA), a member of the APOBEC family of cytidine deaminases that is required for both somatic hypermutation (SHM) and class switch recombination (CSR) (17–19). SHM results in the introduction of somatic mutations in the rearranged V(D)J region of the Ig genes (IgV) in order to generate antibodies with high affinity antigen binding sites (20, 21). Additionally, GCBs undergo a process of CSR of the constant

region of the immunoglobulin (Ig) heavy chain (C_H), generating isotypes with different immunological functions but the same antigen-specificity (22–24). Both SHM and CSR are initiated by the deaminase activity of AID, which is able to convert deoxycytosines (dC) into deoxyuracils (dU) in a single-stranded DNA, producing dU:dG mismatches that are removed by base-excision repair (BER) and mismatch repair (MMR) pathways (19, 25, 26). AID can also deaminate 5mC to thymine (T), although with less efficiency (27). AID is expressed in B cells in a stage specific manner during transition of NB through the GCs (28). NBs integrate signals through B cell receptor (BCR) and Toll-like receptors (TLRs), along with stimulation via surface receptors, such as CD40 and cytokine receptors, to initiate the NF-κB-mediated AID transcription (29). In the secondary lymphoid organs, AID is expressed in a subpopulation of dark and outer zone GCBs and in large extrafollicular B cells, which have evidence of CD40 and BCR stimulation (28), and is downregulated after differentiation of GCBs to memory and/or plasma cells (30, 31). In addition, viral infection of B cells can induce expression of AID (31–34). Ig genes are the main targets of AID, with a mutation rate of 10^{-4} to 10^{-3}/bp per generation (35, 36). Nonetheless, non-Ig genes such as *BCL6*, *CD79A*, *CD79B*, or *CD95* are also susceptible to AID-mediated mutations (37–40). Moreover, Liu et al. reported that around 25% of the highly expressed genes in GCBs, including *Bcl6*, *Cd83*, *Pim1*, *Pax5*, and *Myc* among others, experienced AID-mediated deamination and low-level of SHM (41). The authors demonstrated that these non-Ig genes were protected from mutations in normal B cells due to the activity of high-fidelity BER factors. Besides, they observed that there was correlation between target regions of AID and sites of chromosomal translocations and deletions present in human lymphomas (41). They proposed that malfunction of repair machinery could lead to AID-mediated mutations and chromosomal instability, and finally to tumorigenesis. In line with these results, Yamane et al. performed chromatin immunoprecipitation sequencing (CHIP-seq) for AID in *ex vivo* activated B cells and proposed that AID was recruited genome-wide by stalled PolII polymerases, inducing low-level of hypermutation in those AID-targeted genes (42). Importantly, these findings provided insight into the role of AID in lymphomagenesis.

EVIDENCE LINKING AID TO DNA DEMETHYLATION

There is a body of accumulating evidence linking AID to genome-wide epigenetic changes, and specifically to DNA demethylation (43). A significant discovery has been made in recent years that implicated AID in DNA demethylation in three paradigms: epigenetic reprogramming in heterokaryons using mouse ESCs, demethylation in zebrafish, and global demethylation in mouse primordial germ cells (PGCs) (44–46). The capacity of AID to deaminate 5mC in a single-stranded DNA was established *in vitro* (27), although the efficiency of 5mC deamination by AID was 5–10 times lower compared to unmethylated-C (47). The same report by Morgan et al. (27) demonstrated that AID mRNA was highly expressed in oocytes and ovaries, and moderately expressed in pluripotent cells (embryonic germ cells, ESCs, and PGCs), which can undergo epigenetic reprograming. Those results indicated that expression of AID was not restricted to activated B cells in the GCs of lymphoid organs and prompted the authors to suggest that the upregulation of AID (and other members of the APOBEC family) in pluripotent tissues could play a role in the epigenetic reprogramming during development. Rai et al. followed that hypothesis and presented the first evidence of the epigenetic role of AID (45). They proposed an active DNA demethylation mechanism in zebrafish embryos in which 5mC was converted to T through the cytosine deaminase activity of AID and the subsequent G:T mismatch was repaired by the thymine glycosylase methyl-CpG binding domain protein 4 (MBD4). The injection of methylated DNA (M-DNA) at the single-cell stage induced genome-wide demethylation in zebrafish embryos, allowing the analysis of epigenetic changes in both the injected M-DNA and the bulk genome. After knockdown of AID/APOBEC enzymes using anti-sense morpholino-modified oligonucleotides there was a reduction in demethylation. On the contrary, overexpression of AID (or APOBEC2A/B) and MBD4 induced DNA demethylation of M-DNA and the embryo genome (45).

Thereafter, the production of interspecies heterokaryons (mouse ESCs fused to primary human fibroblasts using polyethylene glycol) allowed the identification of AID-mediated demethylation as a key process for nuclear reprogramming toward pluripotency (44). Transfection experiments with small interference RNAs (siRNAs) for mouse and human AID mRNA 24 h before fusion inhibited expression of the ESC-specific genes *OCT4* and *NANOG* and reduced the CpG demethylation in the promoter of those genes. The transient overexpression of the human AID protein before siRNA transfection returned *NANOG* promoter demethylation and gene expression to normal levels and partially rescued *OCT4* demethylation and gene expression.

Primordial germ cells also undergo a process of epigenetic reprogramming, including DNA demethylation, which is pivotal for the acquisition of pluripotency during the germ line development (48, 49). Popp et al. demonstrated, on a genome-wide scale, that the genome from fully reprogramed PGCs at E13.5 was extensively hypomethylated and that the absence of AID increased DNA methylation levels, mainly in introns and repetitive elements and also in exons, but not in the promoter regions (46). Importantly, the epigenetic effect of AID on the genome of PGCs was confirmed by two independent techniques: bisulfite next generation sequencing (BS-Seq) and Sequenom MassARRAY. This finding of DNA hypermethylation in cells from *Aicda*$^{-/-}$ mice was restricted to PGCs since the genome-wide methylation levels remained unaffected in the fetus, the placenta, and the sperm. A biological process required during embryogenesis is the epithelial–mesenchymal transition (EMT), in which epithelial cells acquire a mesenchymal phenotype characterized by increased migratory capacity and invasiveness and production of extracellular components (50). In addition, EMT is a driving force for tumor metastasis (51). AID is upregulated in epithelial cells during inflammation *in vivo* (52–54) and by inflammatory factors *in vitro* (55, 56) and was shown to be required for the EMT in both normal mammary epithelial cells and breast cancer cell lines (55). Knockdown of AID in epithelial cell lines blocked the upregulation of *SNAI2*, *ZEB1*, and *ZEB2*, which are master regulator genes for EMT. Those genes inhibited by AID-deficiency presented high levels of methylation in the CpG islands associated with the promoters and were

re-expressed in the presence of the demethylating agent 5-aza-2′deoxycitidine, indicating that AID regulated the transcription of EMT-associated genes through DNA demethylation (55).

The *in vitro* generation of induced pluripotent stem cells (iPSCs) by Yamanaka's group from adult somatic cells through the addition of four transcription factors: OCT4, KLF4, SOX2, and c-MYC (57) opened new possibilities for regenerative medicine and autologous therapies. AID was identified as a critical factor for the initiation of mouse embryonic fibroblasts (MEFs) reprogramming to iPSCs (58). Inhibition of AID expression with shRNAs during the first 72 h after reprogramming induction reduced the number of iPSCs colonies. In addition, rescue experiments with a construct containing a catalytically deficient version of AID (E58Q) demonstrated that the deaminase activity of AID was required for the induction of iPSCs (58). However, experiments performed by different groups with *Aicda*$^{-/-}$ mice challenged this conclusion because AID-deficient fibroblasts were able to generate iPSCs, although with different kinetics depending on the concentration of virus supernatant, the transfection plasmids, and the culture conditions, including the number of cell passages (59–61). Interestingly, Kumar et al. observed that cells from *Aicda*$^{-/-}$ mice generated iPSCs, but failed to stabilize the expression of pluripotency genes after 3 weeks of culture (59), a phenomenon that correlated with genome hypermethylation in reprogramming cells, especially near RGYW motifs. Additionally, genes that were upregulated later during reprogramming (*Rex1*, *Gdf3*, *Dnmt3l*, *Dnmt1*, *Apobec1*, *Cbx7*, and *Zfp96*) presented higher levels of methylation, both by RRBS and Sequenom MassArray, and were not expressed in the absence of AID (59). The authors proposed that AID was essential for the late phase of reprogramming, which is characterized by the genome-wide erasure of DNA methylation to stabilize a pluripotent phenotype (62).

Although AID is highly expressed in the GCBs and is responsible for the generation of high affinity antibodies through the induction of SHM and CSR (20, 43), to date only few studies have addressed the epigenetic role of AID in activated B cells (15, 63). An important link between SHM in B cells and DNA hypomethylation has been made by several studies (19, 64, 65). Endonuclease sensitivity sites within the loci containing CpG nucleotides and located close to J-C intronic enhancers are methylated in somatic cells, but are demethylated in B cells (66–68). Jolly et al. studied DNA methylation and SHM in *Jκ5* region, due to the high density of CpGs and high level of SHMs in those loci. They demonstrated that the locus was heavily methylated in mouse tail DNA, whereas it was totally demethylated in the GCBs from Peyer's patches (64). Studying the Igκ transgene and analyzing the *Jκ5* locus they were able to demonstrate that B cells contained a mixture of loci with different state of methylation. Most importantly, the degree of demethylation correlated with the burden of SHM: only demethylated loci contained detectable mutations. This finding led the authors to conclude that transcription and demethylation are requited for SHM, and thus for AID targeting. Our current view is that it is equally possible that demethylation is a consequence of the deaminase activity of AID, which introduces mutations that are subsequently repaired. Hypomethylation along with double strand DNA breaks (DSBs) are likely to be an unwanted consequence of SHM. It is not clear how efficient are DNMTs (DNMT1 and DNMT3b are expressed in the GCBs (15)] in remethylating aberrantly hypomethylated loci. There does not seem to be a detectable loss of 5mC in the GCBs based on high-performance liquid chromatography (HPLC) measurements in isolated B cell fractions. Nevertheless, the methylation profiling indicates that there is a locus-specific loss of methylation in GCBs (15). DNMT1 localizes to the sites of DSBs and phosphoH2AX foci, and its absence results in increased number of DSBs, making it a likely candidate to remethylate AID-dependent demethylated loci (69). Global post-replicative remethylation of DNA depends on association of DNMT1 with PCNA and replication machinery. In addition, DNMT1 has been shown to associate with various transcription factors and remethylate various genes during not only S phase, but also G1 and G2 phases of the cell cycle (70, 71). Hervouet et al. also demonstrated that some genes remained hemi-methylated when leaving S phase and showed a delay in remethylation later in the cell cycle (70). How cell cycle and proliferative rate of GCBs affect global and locus-specific demethylation is an interesting question. It is conceivable that faster proliferation with shorter cell cycle may result in accumulation of hypomethylation. The extent of such passive demethylation is likely to be limited due to the protective effect of Hayflick limit of cell divisions in normal cells (72, 73). In cancer, on the other hand, this stochastic accumulation of hypomethylation may contribute to detectable levels of genomic hypomethylation. However, our data in diffuse large B cell lymphoma (DLBCL) cell lines does not reveal different degrees of genome methylation in cell lines with different duration of the cell cycle (unpublished data).

Using microarray-based DNA methylation profiling of NBs and GCBs (15), we demonstrated that hypomethylated loci within GCBs were significantly associated with RGYW-like AID-recognition motif (74) and CHIP-seq experiments identified AID-binding sites (42), providing another line of indirect evidence for the link between AID and DNA hypomethylation in B cells. On the other hand, Fritz et al. performed *ex vivo* experiments using *Aicda*$^{-/-}$ mice and reported that the absence of AID did not affect the methylome of activated B cells generated from splenic CD43^{-} B cells stimulated with lipopolysaccharide (LPS), interleukin-(IL)-4, and anti-CD40. However, the authors did not exclude that AID could function as a DNA demethylase *in vivo* (63). Indeed, this *ex vivo* system of mouse B cell activation is not equivalent to *in vivo* GC reaction. For instance, the mutation rate in activated B cells is one order of magnitude lower than in GCBs (41, 75, 76). In addition, Hogenbirk et al. profiled purified GCB from immunized mice using MethylCap-Seq and failed to detect any difference in DNA methylation between wt and *Aicda*$^{-/-}$ mice (77). Nevertheless, it would be necessary to apply higher resolution genome-wide techniques to definitely answer this question. A useful tool to prove the demethylase function of AID in B cells would be a mouse model of GC-specific overexpression of AID, since to date the only available conditional AID-transgenic mice overexpress AID under the CD19 promoter, a molecule expressed in B cells from the early stages of differentiation (78). Therefore, better tools and approaches are necessary to elucidate the role of AID in the DNA demethylation process that occurs in B cells during their transit through the GC reaction (15).

PROPOSED MECHANISMS OF ACTIVE DEMETHYLATION

In order to understand the link between deaminase activity of AID and DNA demethylation, we will review the current state of knowledge of the mechanisms involved in active DNA demethylation. Flowering plants possess a set of DNA glycosylases (DEMETER, ROS1, DML2, and DML3) capable of recognizing and removing 5mC in a discrete number of loci, such as imprinted genes and silenced transgenes (79–81). Due to the lack of homologs of 5mC DNA glycosylases in vertebrates, other mechanisms of DNA demethylation have been proposed in mammals. It is well established that the BER machinery, which restores nucleotide lesions originated after base deamination, alkylation, or oxidation (82), is involved in the process of active DNA demethylation (83, 84). The most frequent lesion in DNA is uracil, which is removed by members of the UDG family: UNG, thymine DNA glycosylase (TDG), single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1), and MBD4 (85). The first model for 5mC demethylation was described in zebrafish embryos and proposed that deamination of 5mC by AID generated a T:G mismatch that was excised by MBD4, with the cooperation of GADD45, and finally repaired to restore C (45). The formation of a complex containing AID, GADD45, and a DNA glycosylase was also observed in an independent study using HEK293 cells, although the authors identified TDG as the glycosylase of the BER machinery involved in demethylation (86). The discovery of the base 5-hydroxymethyl-2′-deoxycytidine (5hmC) (87) as a result of the 5mC oxidation by proteins of the (ten-eleven translocation) TET family (88) enabled the identification of other possible mechanisms of active DNA demethylation (89). One report proposed that the conversion of 5mC to 5hmC by TET1 initiated an oxidative-deamination process mediated by the coordinated action of AID/APOBEC proteins and BER pathway, which led to DNA demethylation (90). 5hmC deamination by AID generated 5hmU, which was excised and repaired by the BER machinery. The specific 5hmU DNA glycosylases were not identified, but subsequent experiments with $TDG^{-/-}$ mice suggested that TDG was the DNA glycosylase responsible for the excision of 5hmU (or T) after deamination by AID of 5hmC (or C) (86). This model of active DNA demethylation involved the deamination of 5hmC to 5hmU by AID/APOBEC proteins, a step that was questioned later on due to the voluminous size of the hydroxymethyl group (91). The biochemical analysis of the AID enzymatic activity using a ssDNA oligonucleotide deamination assay (ODA) (27) indicated that AID could deaminate C and 5mC, but was unable to remove the side chain at C5 position from 5hmC due to the size of the hydroxyl group (91). In line with these results, Nabel et al. demonstrated that the deamination activity of AID diminished with the increasing size of the substituent at five position of C due to steric hindrance (47). The rate of 5mC deamination was only 10% relative to unmethylated-C, and no activity was detected on 5hmC *in vitro*. Therefore, if *in vitro* findings by Nabel et al. also apply *in vivo*, the role of AID in direct demethylation of 5mC and 5hmC may be limited. As an alternative, Petersen-Mahrt's group has recently proposed that methylated-Cs do not have to be directly targeted by AID to be demethylated (92). Using an *in vitro* resolution (IVR) assay consisting of a methylated plasmid containing GAL4 DNA-binding sites and a GAL4-AID fusion protein combined with *xenopus laevis* egg extracts-containing molecules of the BER machinery, this study provides evidence for the activation of different DNA repair pathways after AID-mediated deamination. The short-patch-BER machinery restores only one base, whereas the processive DNA polymerase pathways (long-patch-BER or MMR) incorporate multiple nucleotides during the repair process (82, 93, 94). In that last scenario, the deamination of C (or 5mC) to U (or T) by AID would promote the activation of a processive DNA polymerase, which would introduce from 2 to 12 nt in the case of the LP-BER (93), or up to 2 kb of ssDNA if the MMR pathway is triggered (95). As a result, there would be extensive demethylation along the section of DNA around the initial lesion and 5hmC demethylation would occur independently of targeted AID deamination (92). However, the physiological relevance of this model still needs to be confirmed. All these proposed mechanisms of AID-mediated demethylation rely on the lesion-induced activation of the DNA repair pathways. In all scenarios, the deamination activity of AID introduces a modified base that is targeted by the repair machinery. SHM results from recruitment of the error-prone short-patch BER-especially in Ig genes, leading to a single nucleotide substitution, and a loss of a "methylatable" C. The active and direct loss of methylation is possible if AID deaminates 5mC to T. On the other hand, if LP-BER or MMR are involved in repair, the outcome is more marked demethylation that extends beyond the original single nucleotide lesion (96). It remains unclear how AID is recruited to its DNA targets. SHM is linked to transcription: AID requires ssDNA to initiate the deamination of IgV regions (97). Regarding AID partners, it has been demonstrated that stalled Pol II, Spt5, and RPA are necessary for recruitment of AID to Ig and non-Ig targets (98, 99). Duke et al. delineated that a combination of E-box with YY1 and C/EBP-β binding sites targets AID in B cells (100). However, it is not known if demethylation and SHM targets are always the same. Hypothetically, the demethylation targets may exceed the numbers of SHM targets due to successful repair, which would leave the AID target site as mutation-free but demethylated. Also, hypomethylated areas are located in introns, intergenic regions and repeat elements rather than promoters, suggesting a different targeting mechanism (46). In contrast, other studies have proposed that active DNA demethylation does not involve AID/APOBECs-mediated deamination, but it occurs through the different intermediates generated after the successive oxidation of 5hmC by TET proteins: 5-formyl-C (5fC) and 5-carboxyl-C (5caC), which are excised by TDG and replaced by unmodified cytosine (101, 102). Finally, it has been demonstrated recently that TETs are able to oxidize T to 5hmU in mouse ESCs (103). This finding unveils another possible mechanism of demethylation consisting of AID-mediated deamination of mC to T followed by conversion of T to 5hmU by TET proteins. Based on the previously mentioned results, it seems reasonable to conclude that both AID-dependent and TET-dependent mechanisms participate in the active demethylation of the genome (**Figure 1**). To what extent they are interconnected or which one is preferentially activated depending on the cell type needs to be further investigated.

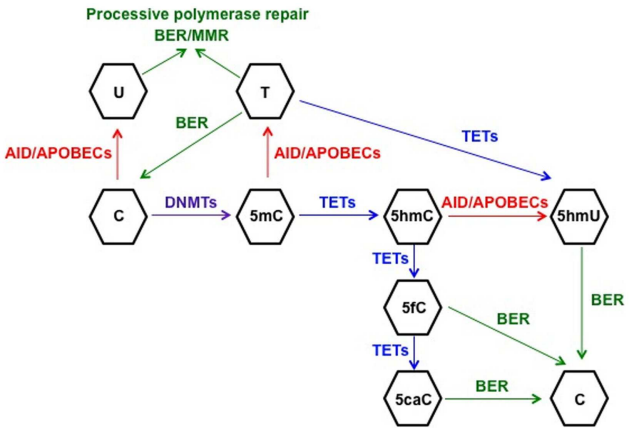

FIGURE 1 | Schematic representation of the proposed mechanisms for active DNA demethylation. DNMTs catalyze the methylation of cytosine (C) to 5-methyl-C (5mC), which can be deaminated to thymine (T) by AID. The generated mismatch can be repaired by the short-patch BER machinery, restoring C, or by one of the processive repair pathways (long-patch BER or MMR), leading to demethylation of a fragment of DNA. This machinery involving processive DNA polymerases can also repair the uracil:guanine (dU:dG) mismatches generated after C deamination to U. On the other hand, TET-mediated hydroxylation of 5mC generates 5-hydroxymethyl-C (5hmC), which can be deaminated by AID/APOBECs to 5-hydroxymethyl-U (5hmU) and replaced by C through BER. 5hmC can be further oxidized by TETs proteins to 5-formyl-C (5fC) and 5-carboxyl-C (5caC), leading to activation of BER to restore C. Finally, another potential mechanism of active DNA demethylation would involve 5mC deamination to T and TET-mediated oxidation of T to 5hmU, which would be replaced by C through BER, although this model has not been proven as yet.

DNA HYPOMETHYLATION AND CANCER

The question of AID activity, its possible link to DNA hypomethylation and predisposition to cancer is a fundamental one. At the moment, only a tenuous link exists between AID-induced SHM and DNA hypomethylation in B cells and B cell lymphomas. There is a large body of evidence though linking hypomethylation to cancer (70, 104, 105). Many subtypes of lymphomas reveal genome-wide hypomethylation. Wahlfors et al. made an early observation that chronic lymphocytic leukemia (CLL) genome undergoes global loss of methylation using digestion of genomic DNA with isoschizomer enzymes *Hpa*II and *Msp*I, followed by validation using HPLC (104). More recently, these findings were confirmed by whole methylome sequencing studies, which revealed that aberrant hypomethylation was centered in repetitive sequences, like ALU and LINES and were particularly pronounced in CLL with TP53 mutations (106). A subset of DLBCLs also revealed loss of DNA methylation, as demonstrated by Chambwe et al. (107). Methylome interrogation in the follicular lymphoma (FL) cell line RL and in $CD19^+$ B cells using 454 sequencing technology (108) revealed hypermethylation in the promoters, but hypomethylation in intra- and intergenic areas of the genome.

DNA methylation patterns in all tissues and cell types are a result of two main forces: deterministic patterning and stochastic changes [reviewed in Ref. (109)]. Deterministic changes in DNA methylation reflect the tissue-specific forces, which are dependent on transcription factors and epigenetic factors that reflect the tissue type and determine cellular identity. On the other hand, the stochastic changes reflect the cell-to-cell variability, with individual cells at the same tissue and differentiation stage displaying epigenetic heterogeneity. There are several possible sources of stochastic variability: aging, ambient mutagens, oxidative damage, errors, and off-target activity of epigenetic enzymatic factors (110, 111). Several mechanisms have been proposed to contribute to DNA hypomethylation in normal development and disease, which to different degrees can explain deterministic and stochastic DNA hypomethylation. One explanation is a possible reduction of intracellular concentration of DNA methyl donor S-adenosylmethionine (AdoMet) or an increased concentration of the product of the reaction and its inhibitor S-Adenosylhomocysteine (AdoHcy) (112). Methyl-deficient diet has been shown to lead to AdoMet deficiency in a mouse model (113), but has not been proven to contribute to carcinogenesis in humans. Another logical explanation for DNA hypomethylation would be the decreased activity of DNMTs or increased activity of demethylases. Published observations in tumors and normal development suggest that hypomethylation does not correlate with decreased expression of DNMTs or their mutations (114–116). On the other hand, active demethylation can take place either by the action of yet unidentified demethylases or through DNA repair process. Enzymatic demethylation is not favored because of the stability of the chemical bond. Ramchandani et al. reported the biochemical purification of a DNA demethylase from tumor cells (117). The same group demonstrated that the rate limiting step in the reaction is initiation of demethylation, which is sequence specific and progresses in a processive manner sliding along the DNA and demethylating CpGs in cis position (118). MBD2 was demonstrated to possess demethylase activity in biochemical *in vitro* experiments and in solid cancers, but its function as demethylase remains controversial (119, 120). Another possibility is modification of DNA nucleotides by deaminases resulting in eventual excision and repair of that nucleotide and loss of methylation. AID is the key candidate for this mechanism of demethylation, as discussed before. AID is known to contribute to chromosomal instability and induce chromosomal translocations by inducing DSBs (see below). B lymphocytes from IgκAID-transgenic mice showed increased number of chromosomal translocations (6% of cells) and increased frequency of DNA breaks (8% of cells) (121). The contribution of hypomethylation to AID-dependent genomic instability has not been addressed and warrants further investigation.

The biological effect of DNA hypomethylation is its contribution to reactivation of transposable elements, activation of oncogenes, and increased chromosomal fragility, as demonstrated elegantly by Gaudet et al. in experiments with hypomorphic DNMT1 mice (122, 123). Mice with low DNMT1 expression at 10% of wild type level demonstrated marked reduction in genome-wide DNA methylation and revealed significant increase in genomic instability and activation of proto-oncogenes, like *c-Myc* (124). The maintenance of methylation in mammalian cells is accomplished by DNMTs, particularly DNMT1 and its complex with PCNA and UHFR1. Disruption of this complex results in global

hypomethylation and serves as a biomarker of poor prognosis in GBM tumors (125).

ROLE OF AID IN LYMPHOMAGENESIS

AID is expressed in half of Burkitt lymphomas (BLs), 30% DLBCLs and 25% FLs, and is absent in B cell precursor lymphoblastic leukemia and mantle cell lymphomas (126, 127). Due to its DNA mutator capacity, AID activity represents a potential risk for genomic instability. It is clear that AID can introduce point mutations in Ig and non-Ig genes and also induce chromosome translocations involving oncogenes, which contribute to the development of B cell neoplasms (128). Pasqualucci et al. found that proto-oncogenes such as *PIM1*, *MYC*, *RHOH*/*TTF*, and *PAX5* were aberrantly mutated in more than 50% of the DLBCLs cases analyzed, probably due to failure of the SHM pathway (129). In addition, chromosomal translocations are frequent in lymphomas and myelomas, including DLBCLs, BLs, multiple myeloma, and mouse plasmacytoma (130, 131). Specifically, translocations between *c-MYC* and the Ig C_H genes (*c-MYC/IgH*) are a hallmark of BLs and were demonstrated to be AID-dependent, since AID-deficiency eliminated the presence of canonical *cMyc* translocations in BCL6 or Bcl-xL transgenic mice (132, 133), although the frequency of DSBs was lower in *c-Myc* than in *IgH* (76). Moreover, mutations in the anti-apoptotic protein *BCL2* are frequent in DLBCLs and are enriched in the AID-binding motif WRCY (134).

Multiple mouse models have been established to study lymphomagenesis *in vivo*, and more specifically to clarify the role of AID in the induction and development of B cell neoplasms. Experiments with IμHABCL6 transgenic mice, which develop lymphomas due to the deregulated expression of the transcriptional repressor BCL6 under the control of the IgH Iμ promoter (135), demonstrated that AID had a role in lymphomagenesis (136). AID-deficiency in IμHABCL6 background prevented the formation of GC-derived lymphomas. This effect was not observed when crossing *Aicda*$^{-/-}$ mice with other lymphoma-prone mouse models such as λMYC mice, which develop pre-GC-derived lymphomas, or λMYC/IμHABCL6 mice, characterized by the formation of post-GC-derived malignancies. The same report showed that *ex vivo* stimulated B cells from IμHABCL6 mice presented a high number of *c-Myc/IgH* translocations, which were not present in activated IμHABCL6/*Aicda*$^{-/-}$ cells. The authors proposed that AID-mediated translocations contributed to lymphoma formation (136). Another study reported that AID activity determined the frequency of lymphocytes with *c-Myc/IgH* translocations, influencing the incidence of B cell tumor development in a mouse model of plasmacytoma (137). An independent group demonstrated the link between AID-mediated chromosome translocations and mature B cell lymphomas using another transgenic mouse strain, which expressed AID under the control regulatory elements of the light chain Igκ (121). In this experimental design, deregulated AID expression generated DSBs in the genome of B cells, inducing *c-Myc/IgH* chromosome translocations. Nonetheless, AID overexpression was not sufficient to drive B cell lymphomagenesis, requiring the concomitant loss of the tumor suppressor p53 in that model (121). The generation of a transgenic mouse strain with sporadic *c-Myc* activation in GCBs (Vk*MYC), which led to a multiple myeloma phenotype, indicated that AID-mediated SHM was required for the aberrant *c-Myc* expression and the subsequent plasma cells expansion (138). Constitutive expression of AID using a transgenic mouse model, which expresses AID under the control of the ubiquitous CAG promoter, led to T cell lymphomas containing no translocations but abundant point mutations in *c-Myc* and the *TCR* genes (139). Bone marrow transplantation experiments with AID-transduced cells also resulted in the development of T-lymphomas, with frequent point mutations in *Notch1*, *PTEN*, and *c-Myc*. Noteworthy, some of the mice presented B-lymphomas after transplantation. *Pax5* and *Ebf1* were mutated in these B cell-derived malignancies and no chromosome translocations were found (140). The aforementioned mouse models proposed different mechanisms of AID-mediated genomic instability, focused on the analysis of somatic mutations or translocations involving proto-oncogenes generated as a consequence of the deaminase activity of AID. Intriguingly, the role of AID activity in the epigenetic stability of the genome and its implication in lymphomagenesis remain largely unexplored. De et al. (141) analyzed the DNA methylation patterning in three subtypes of primary lymphomas: FL, GCB–DLBCL, and ABC–DLBCL (142), which differs in their level of aggressiveness (FL < GCB–DLBCL < ABC–DLBCL). This study demonstrated that normal GCBs presented higher level of epigenetic heterogeneity than NBs, and also that inter-sample and intra-sample methylation heterogeneity increased with lymphoma aggressiveness, correlating with adverse outcome (141). Trying to discover the cause for the abnormal DNA methylation patterns in B cell lymphomas, the authors found that the promoters of the targets of *BCL6* and *EZH2* showed an aberrant hypermethylated status compared to normal B cells. On the contrary, AID target genes presented an abnormal promoter hypomethylation. In addition, the expression level of AID was significantly correlated with genome-wide aberrant hypomethylation (141). This result, along with the established role of AID in active DNA demethylation during normal development and also with the significantly higher hypomethylation in GCBs compared to NBs in regions enriched for the putative AID-binding site RGYW, suggests an epigenetic role for AID during GC transit of normal B cells and in GC-derived lymphomagenesis. Such a role needs to be formally proven and will have great implications on our understanding of B cell biology.

AUTHOR CONTRIBUTIONS

Pilar M. Dominguez and Rita Shaknovich conceptualized and wrote the manuscript.

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Distinct transcriptomic features are associated with transitional and mature B-cell populations in the mouse spleen

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Splenic transitional B-cells (T1 and T2) are selected to avoid self-reactivity and to safeguard against autoimmunity, then differentiate into mature follicular (FO-I and FO-II) and marginal zone (MZ) subsets. Transcriptomic analysis by RNA-seq of the five B-cell subsets revealed T1 cell signature genes included RAG suggesting a potential for receptor revision. T1 to T2 B-cell differentiation was marked by a switch from Myb to Myc, increased expression of the PI3K adapter DAP10 and MHC class II. FO-II may be an intermediate in FO-I differentiation and may also become MZ B-cells as suggested by principle component analysis. MZ B-cells possessed the most distinct transcriptome including down-regulation of CD45 phosphatase-associated protein (CD45-AP/PTPRC-AP), as well as upregulation of IL-9R and innate molecules TLR3, TLR7, and bactericidal Perforin-2 (MPEG1). Among the endosomal TLRs, stimulation via TLR3 further enhanced Perforin-2 expression exclusively in MZ B-cells. Using gene-deleted and overexpressing transgenic mice we show that IL-9/IL-9R interaction resulted in rapid activation of STAT1, 3, and 5, primarily in MZ B-cells. Importantly, CD45-AP mutant mice had reduced transitional and increased mature MZ and FO B-cells, suggesting that it prevents premature entry of transitional B-cells to the mature B-cell pool or their survival and proliferation. Together, these findings suggest, developmental plasticity among splenic B-cell subsets, potential for receptor revision in peripheral tolerance whereas enhanced metabolism coincides with T2 to mature B-cell differentiation. Further, unique core transcriptional signatures in MZ B-cells may control their innate features.

Keywords: transcriptome by RNA-seq technique, splenic transitional B-cells, follicular 1 and 2 B-cells, marginal zone B-cells, DAP10 PI3K pathway, IL-9/IL-9R, Myb Myc, Toll-like receptors 3 and 7

INTRODUCTION

The role of B-lymphocytes is to produce antigen specific antibodies to neutralize pathogens. B-cells develop in the bone marrow (BM) where most autoreactive clones are triaged by the central tolerance mechanisms of clonal deletion, anergy, or receptor editing (1). Surviving $sIgM^+$ immature or transitional 1 (T1) B-cells migrate to the spleen, where they are again tested for autoreactivity. Innocuous clones are allowed to develop into transitional 2 (T2) cells (2). Observations that T1 cells are extremely sensitive to BCR-induced apoptosis *in vitro* suggest that the T1-stage serves as a peripheral tolerance checkpoint (3–7). Dysregulation of peripheral checkpoint can lead to autoimmune pathologies such as SLE, RA, and MS (8–10).

The immature T2 cell stage is believed to serve as the branching point for selection into functionally distinct mature B-cell subsets comprised of follicular I and II (FO-I and FO-II), B1, and marginal zone (MZ) B-cell compartments [reviewed in Ref. (11)]. FO-I cells specialize in T cell-dependent (TD) immune responses whereas MZ B-cells specialize in rapid T cell-independent (TI) antibody responses and possess innate-like properties (11–13). The function of the FO-II subset is unknown (14). A comprehensive analysis to identify transcriptional changes associated with peripheral tolerance at the transitional stages and functional specialization of mature B-cell subsets may provide a framework for hypothesis-driven experiments to identify key processes responsible for B-cell biological properties.

The Immunological Genome consortia (ImmGen) has provided a rich resource for gene expression data sets to the immunological community including all known mouse B-cell subsets using microarray. Analyses of these gene expression data sets have

produced gene-network models laying the foundation for experimentally testable hypotheses for various hematopoietic lineage cell developmental relationships and acquisition of functional specialization. However, such analysis has not been reported for the B lineage. Here, we report bioinformatics analysis performed on data obtained with next generation sequencing (NGS) on highly purified B-cell subsets that are either not available from ImmGen (FoB-II) or were phenotypically defined differently than the current study. Our splenic B-cell populations were enriched using a combination of schemes and to achieve maximum cell homogeneity defined as; $T1^{21/23DN}$ ($B220^+$, $AA4.1^+$, $CD23^-$, $CD21^-$, $CD24^{hi}$), $T2^{CD21int}$ ($B220^+$, $AA4.1^+$, $CD23^+$, $CD21^{int}$, $CD24^{hi}$), FO-I ($B220^+$, IgM^{lo}, $CD21^{int}$, IgD^+, $CD23^+$, $CD24^{lo}$, $CD9^-$), FO-II ($B220^+$, IgM^{hi}, $CD21^{int}$, IgD^+, $CD23^+$, $CD24^{lo}$, $CD9^-$), and MZ^{CD9+} ($B220^+$, IgM^{hi}, $CD21^{hi}$, IgD^-, $CD23^-$, $CD24^{int}$, $CD9^+$).

We identified many novel stage-specific transcripts not identified by ImmGen data sets and associated processes. Our comparative analysis of transcriptomes in specific B-cell subsets has advanced our understanding of the transcriptional networks associated with peripheral B-cell development and selection as well as functional specialization acquired by mature B-cell subsets. We highlight transcripts contributing to innate MZ B-cell function (TLR3 and Perforin-2) and demonstrate a previously unknown function for IL-9R and CD45-AP in B-cells.

MATERIALS AND METHODS

MICE

C57BL/6 mice were purchased from The Jackson Laboratory and maintained at University of Miami animal facility. *CD45-AP*$^{-/-}$ (C57BL/6), IL-$9R^{-/-}$, and IL-9 transgenic (Tg5) mice have been previously described (15–17). Unless indicated otherwise, mice used in these studies were aged between 6 and 10 weeks. These studies were approved by the Institutional Animal Care and Use Committee.

FLOW CYTOMETRIC ANALYSIS AND CELL SORTING

$CD93^+$ enriched immature and $CD93^+CD43^+$ depleted mature splenocytes were incubated with fluorochrome labeled antibodies to sort $T1^{21/23DN}$, $T2^{CD21int}$, FO-I, FO-II, and MZ^{CD9+} on a BD FACS Aria II yielding 95–99% purity as described in Figure S1 in Supplementary Material. Briefly, B-cell enrichment was performed using Mouse B Lymphocyte Enrichment set-DM (BD Biosciences). Transitional B-cells were sorted from pooled splenocytes after two rounds of AA4.1 positive selection. Sorting from $DAPI^-$, $B220^+$ cells produced two relatively homogeneous transitional subsets. $T1^{21/23DN}$ were $AA4.1^+$, $CD23^-$, $CD21^-$, $CD24^{hi}$, whereas $T2^{CD21int}$ were $AA4.1^+$, $CD23^+$, $CD21^{int}$, $CD24^{hi}$. For mature B-cell sorting, pooled splenocytes were enriched for B-cells and simultaneously depleted of $AA4.1^+$ (transitional) cells. Three purified mature B-cell populations were sorted from the $DAPI^-$, $B220^+$ gate. FO-I were IgM^{lo}, $CD21^{int}$, IgD^+, $CD23^+$, $CD24^{lo}$, and $CD9^-$. FO-II were IgM^{hi}, $CD21^{int}$, IgD^+, $CD23^+$, $CD24^{lo}$, and $CD9^-$. MZ^{CD9+} were IgM^{hi}, $CD21^{hi}$, IgD^-, $CD23^-$, $CD24^{int}$, and $CD9^+$.

Flow cytometric analysis of splenocytes from CD45-AP-deficient mice and IL-$9R^{-/-}$/IL-9 transgenic were performed on a LSRII Flow cytometer and LSR Fortessa, respectively (BD Bioscience). For intracellular staining, cells were stimulated 15 min with IL-9 for 15 min. Cells were then fixed 10 min in 2% PFA, 90% methanol permeabilized (30 min), washed extensively, blocked and then incubated 1 h with anti-pSTAT, and surface marker antibodies. FCM data were analyzed using FlowJo software (TreeStar). Antibodies used are listed as follows; CD93 Biotin Clone AA4.1, CD23 PE/Biotin Clone B3B4, CD21 FITC/PE Clone 7G6, Streptavidin PE Cy7, B220 V500 Clone RA3-6B2, CD19 Clone 1D3, CD9 Biotin Clone KCM8, pSTAT3 (pY705) Alexa Fluor 647 Clone 4/pSTAT3, pSTAT1 (pY701) Alexa Fluor 647 Clone 4a, pSTAT5 (pY694) Alexa Fluor 647 Clone 47/Stat5 (pY694) (BD Bioscience), CD93 APC Clone AA4.1, CD24 PerCP Cy5.5 Clone M1/69, IgD PacBlue Clone 11-26 (eBioscience), B220 Alexa 700 Clone RA3-6B2, Streptavidin PerCP (Biolegend), IgM 649 Fab Fragment (Jackson ImmunoResearch), CD9 FITC Clone MZ3 (Santa Cruz Biotechnology), DAPI (Invitrogen). B220 Alexa 700, CD9 Biotin, Streptavidin PerCP, and pSTAT antibodies were used for IL-9R/IL-9 studies. CD21 Clone 7G6 is used for both sorting and IL-9R/IL-9 studies.

RNA ISOLATION FOR NEXT GENERATION SEQUENCING AND ANALYSIS

RNA was prepared from the sorted cells using Qiagen RLT buffer. PolyA RNA was selected and library constructed using Illumina RNA sample preparation reagents following manufacturer's recommendations (Illumina). RNA integrity was assessed using a Bioanalyzer 2100 (Agilent) as well as Nanodrop 8000 Spectrophotometer (Thermo Scientific). Four of the five samples were sequenced on the Illumina GAIIx using Cluster Generation Kit v4 and Sequencing Kit v4, generating 74base single-end reads. The fifth sample (MZ^{CD9+}) was sequenced on the Illumina HiSeq2000 using the reagents provided in the Illumina TruSeq PE Cluster Kit v3 and the TruSeq SBS Kit – HS (200 cycle) kit, generating 99base paired-end reads. Read2 was not used in this study and read1 was trimmed to match the 74base reads generated by the GAIIx. Quality of the RNA-seq data was reviewed using FastQC software version 0.10.1[1] . Average phred-like quality scores were >30 in all samples if calculated per-base and over 36 if calculated per sequence. This quality was considered sufficient and no reads were filtered out. All samples passed testing on basic parameters in FastQC (data not shown), except sequence duplication levels (Table S1 in Supplementary Material). Elevated duplication levels may arise in RNA-seq due to "over-sequencing" of high abundant transcripts as well as bias caused by non-random hexamer priming (18). We did not remove any duplicates because there is no consensus so far how it affects expression level estimations. Table S1 in Supplementary Material shows basic statistics on the samples.

Quantification of transcriptome was done in two steps as described in TopHat protocol (19). In the first step, TopHat was used to map the reads to the reference genome (UCSC build mm10 GRCm38 from September 2012) with default settings and novel splice discovery disabled. In the second step, Cufdiff was employed to calculate FPKM values using reference transcriptome along with BAM files from the first step for each sample. Data were analyzed in automated fashion on the cluster hosted by the

[1]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

High-Performance Computing core in the Center for Computational Science, University of Miami. All transcript expression below FPKM 1 was set to 1. Quantitative FPKM values were log2 transformed and converted to Z-scores. miRNA, rRNA, and hemoglobin transcripts were removed from the analysis as these transcripts likely represented artifacts (20). Table S1 in Supplementary Material contains FPKM, log2 transformed FPKM, *z*-scores, and fold-change (FC) relative to $T2^{CD21int}$.

RNA species compositions (biotypes) were analyzed and visualized using NOIseq package in Bioconductor[2]. Biotypes from Ensemble annotation were used (Figure S1 in Supplementary Material). Data analysis was performed using R software[3]. Scatterplot was generated using "pairs" R function. Principle component analysis (PCA) was performed with "prcomp" R function. *Z*-scores for gene expression were visualized as heatmaps using "heatmap.2" function from "gplots" R library. Venn diagrams were generated using "VennDiagram" library in R. Functional annotation clustering was done using DAVID bioinformatics online software (21, 22). Each term is ranked based on enrichment score along with corresponding *P* value. Prioritization of clusters was based on enrichment score using highest stringency settings. GeneGo software (MetaCore, Thomson Reuters) was used to predict transcription factor (TF) regulation during development. All differentially expressed (DE) genes (FC > 2) between two subsets (or signature genes) were used as input.

REAL-TIME PCR

RNA for quantitative Real-Time PCR (qRT-PCR) was isolated using RNeasy Minikit and reverse-transcribed utilizing Quantitect Reverse Transcription kit (Qiagen). qRT-PCR was performed with TaqMan Fast Universal PCR Master Mix in Step One Real-Time PCR System (Applied Biosystems). TaqMan primer/probes (Applied Biosystems) are as follow: Gfi1 Mm00515855_m1, Tlr3 Mm01207404_m1, Tlr7 Mm00446590_m1, Tlr9 Mm00446193_m1, Rag1 Mm01270936_m1, Rag2 Mm01270938_m1, IL-9R Mm0043413_m1, Tnfrsf13c/BAFF-R Mm00840578_g1, Ptprc-ap/CD45-AP Mm01236556_m1, Mpeg1/Perforin-2 Mm01222137_g1, Hcst/Dap10 Mm01270936_m1, Bmf Mm00506773_m1, IKKε Mm00444862_m1, Tnfrsf13b/TACI Mm00840182_m1, and Gapdh Mm99999915_g1.

WESTERN BLOT

T1, T2, and Mature B-cell (FO) subsets were sorted and western-blotted as previously described in Ref. (7). Briefly, 20 μg/lane of total cellular extracts from FACS cells were analyzed by immunoblotting with antibodies specific for the indicated anti-apoptotic proteins. Anti-p38 was used as a loading control. Antibody information is as follows: Mcl-1 (Rockland Immunochemicals); Bcl-2, Bcl-xL, p38, Pim-2 (Santa Cruz Biotechnology); A1 (R&D Systems); and c-IAP2 (Cell Signaling Technology).

B-CELL ISOLATION FOR *IN VITRO* STIMULATION

Splenic B-cells enriched by CD43 negative selection or CD45/B220 positive selection beads (BD Bioscience) were cultured (7) and stimulated with Poly(I:C) (#tlrl-pic), CL097 (#tlrl-c97), or CpG (tlrl-1826) from Invivogen.

STATISTICAL ANALYSIS

Biological data were analyzed using Student's *t*-test. All data are represented as mean ± SEM. Values of $^*P \leq 0.05$ were considered statistically significant. Analysis performed using Prism software (GraphPad).

RESULTS

TRANSCRIPTOME ANALYSIS REVEALS PREVIOUSLY UNKNOWN RELATIONSHIPS AMONG SPLENIC B-CELL SUBSETS

We FACS-purified five splenic B-cell subsets using a combination of schemes previously described for mouse and human B-cells (2, 4, 6, 14, 23–25). This purification scheme was used to maximize homogeneity for RNA-Seq analysis (Figure S1 in Supplementary Material). Hereafter, B-cell subsets will be referred to as $T1^{21/23DN}$, $T2^{CD21int}$, FO-I, FO-II, and MZ^{CD9+} to denote distinction from other sorting schemes. As shown in **Figures 1A,B**, the NGS data corroborate well with cell-surface marker expression used in sorting thereby confirming both phenotype and purity. qPCR further verified the accuracy of our NGS data (**Figures 2–9**).

Transcriptome analysis yielded 10,851 unique transcripts among our subsets. Approximately 19% (2057) of transcripts were up-regulated and 16.5% (1789) were down-regulated (FC > 2) in at least one subset relative to $T2^{CD21int}$. This subset was used for comparison because of its central position between $T1^{21/23DN}$ and mature subsets. MZ^{CD9+} displayed the highest number of DE genes, whereas FO-II had the lowest (**Figure 1C**). There were significant differences in the number of DE genes detected in our data-set compared to ImmGen (Table S1 in Supplementary Material). For example, ImmGen identified seven genes higher in T1 versus T2 and 23 genes higher in T2 versus T1 (FC > 2 cut-off). Our RNA-Seq data identified 375 genes higher in $T1^{21/23DN}$ versus $T2^{CD21int}$ and 326 genes higher in $T2^{CD21int}$ versus $T1^{21/23DN}$ (also FC > 2 cut-off). This clearly demonstrates the utility and advantage of our sorting scheme as well as use of RNA-Seq technology.

Available information suggests that T1 differentiate into T2, which give rise to mature B-cells. To determine B-cell subset relationships, we used the number of DE genes (FC > 2) to construct a distance graph, which suggested three additional differentiation paths (dashed lines): (1) $T1^{21/23DN}$ to MZ^{CD9+}, (2) FO-II to FO-I, and (3) FO-II to MZ^{CD9+} (**Figure 1D**). Scatterplot and PCA analysis illustrates that among our subsets, MZ^{CD9+} was most closely related to FO-II. Surprisingly, $T1^{21/23DN}$ and $T2^{CD21int}$ were almost equally related to MZ^{CD9+} cells (**Figures 1E,F**). These three potential MZ^{CD9+} precursors shared significant overlap in DE genes relative to MZ^{CD9+} (**Figure 1G**). This analysis also shows that FO-II are in the middle of a follicular differentiation pathway between $T2^{CD21int}$ and FO-I (**Figures 1E,F**). Consistently, there was a high degree of overlap in DE genes between $T2^{CD21int}$ and FO-II or FO-I (**Figure 1H**).

DISTINCT TRANSCRIPTION PROGRAMS DEFINE TRANSITIONAL AND MATURE B-CELL SUBSETS

To systematically identify transcription regulators (TR) (TF plus regulatory genes) that control co-expression of B-cell

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[3]http://www.r-project.org/

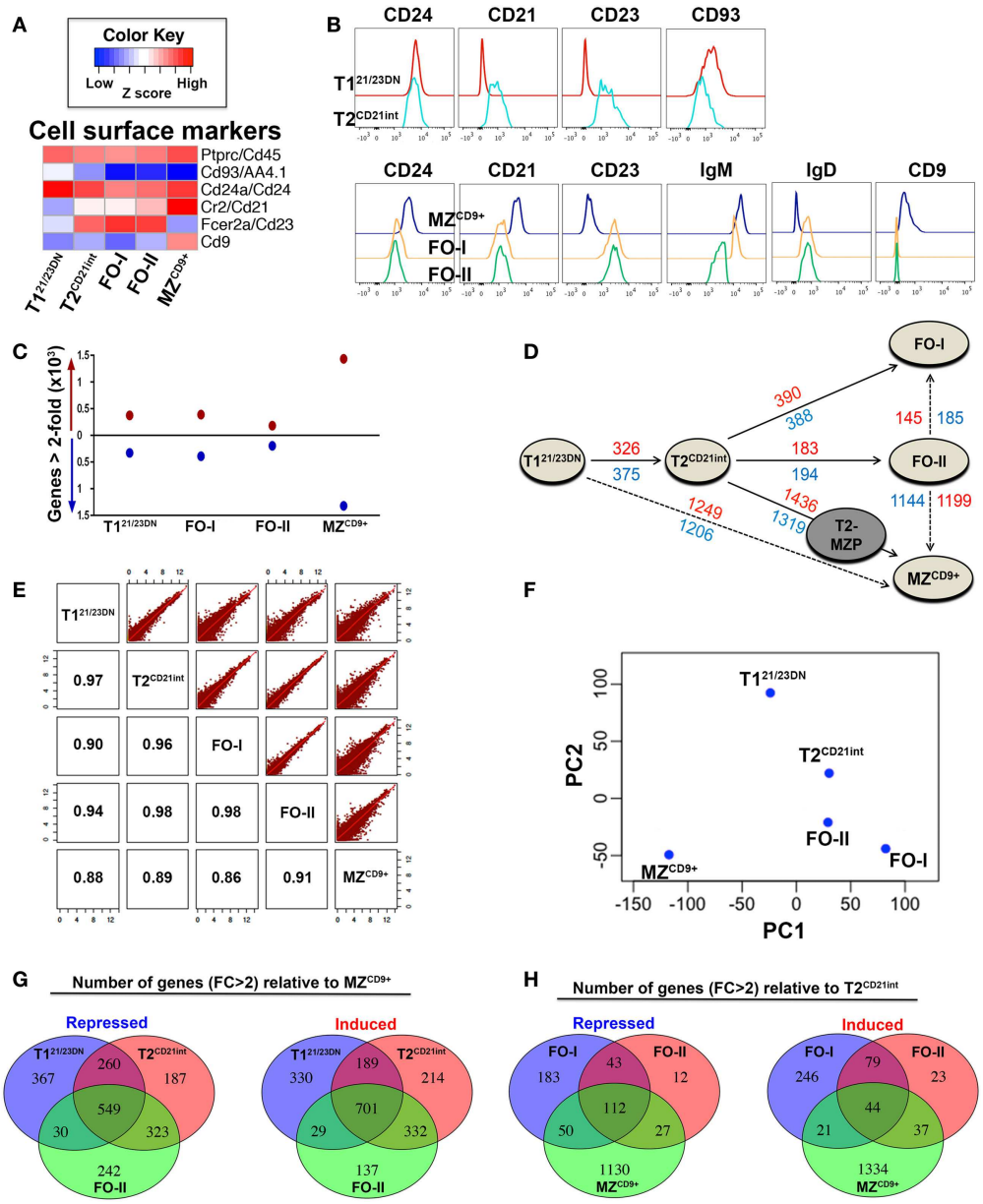

FIGURE 1 | Global gene expression profiles define hierarchical relationships among splenic B-cell subsets. **(A)** NGS results of gene expression profiles of each B-cell subset corroborate well with the cell-surface marker expression shown in **(B)**. Heatmap displaying expression of mRNAs (median log-transformed relative fold-change) encoding cell-surface markers that define $T1^{21/23DN}$, $T2^{CD21int}$, FO-I, FO-II, and MZ^{CD9+} B-cell subsets and were used for cell sorting by FACS (see Figure S1 in Supplementary Material for details). Immunoglubulin sequences were excluded from NGS data analysis. Red color indicates high *z*-score (high expression) while blue indicates a low *z*-score (low expression). **(B)** Histograms depict cell-surface marker expression that define transitional (top panels) and mature (bottom panels) splenic B-cell subsets as in **(A)**. **(C)** Graphic depiction of the number of genes differentially expressed (DE) relative to $T2^{CD21int}$ (FC > 2). Red circles (above horizontal line at 0) represent the number of up-regulated genes while the blue circles (below horizontal line at 0) represent the number of down-regulated genes. $T1^{21/23DN}$, FO-I, FO-II, and MZ^{CD9+} had 6.46, 7.17, 3.74, and 25.39% DE genes (includes up- and down-regulated), respectively. **(D)**, Distance plot indicating the number of up-regulated genes (red) and down-regulated genes (blue) in various maturation steps (FC > 2). Solid arrows indicate known paths of maturation whereas dotted arrows indicate alternate maturation paths predicted by NGS data analysis in this study. Although we did not sort T2-MZP (gray), it is included for completeness. It is believed to be an intermediate between T2 to MZ B-cell differentiation. **(E)** Scatterplot analysis of log-transformed NGS data showing similarity in global gene expression between all B-cell subsets. Numbers inside intersecting boxes indicate the percentage of similarity. Graphical representation of this percentage is displayed on upper panels where each red dot corresponds to a single gene. **(F)** Principle component analysis (PCA) using two principle components (PC1 and PC2) where PC1 explained the majority of gene expression differences followed by PC2. Similarity between B-cell subsets can be ascertained by the physical difference between circles on the plot. Both scatterplot and PCA analysis do not involve fold-change cut-offs and involve all subset cross comparisons. **(G)** The number of repressed or induced genes overlapping during differentiation from either $T1^{21/23DN}$, $T2^{CD21int}$, or FO-II to MZ^{CD9+} cells. **(H)** The number of repressed or induced genes overlapping in differentiation from $T2^{CD21int}$ to either FO-I, FO-II, or MZ^{CD9+} cells.

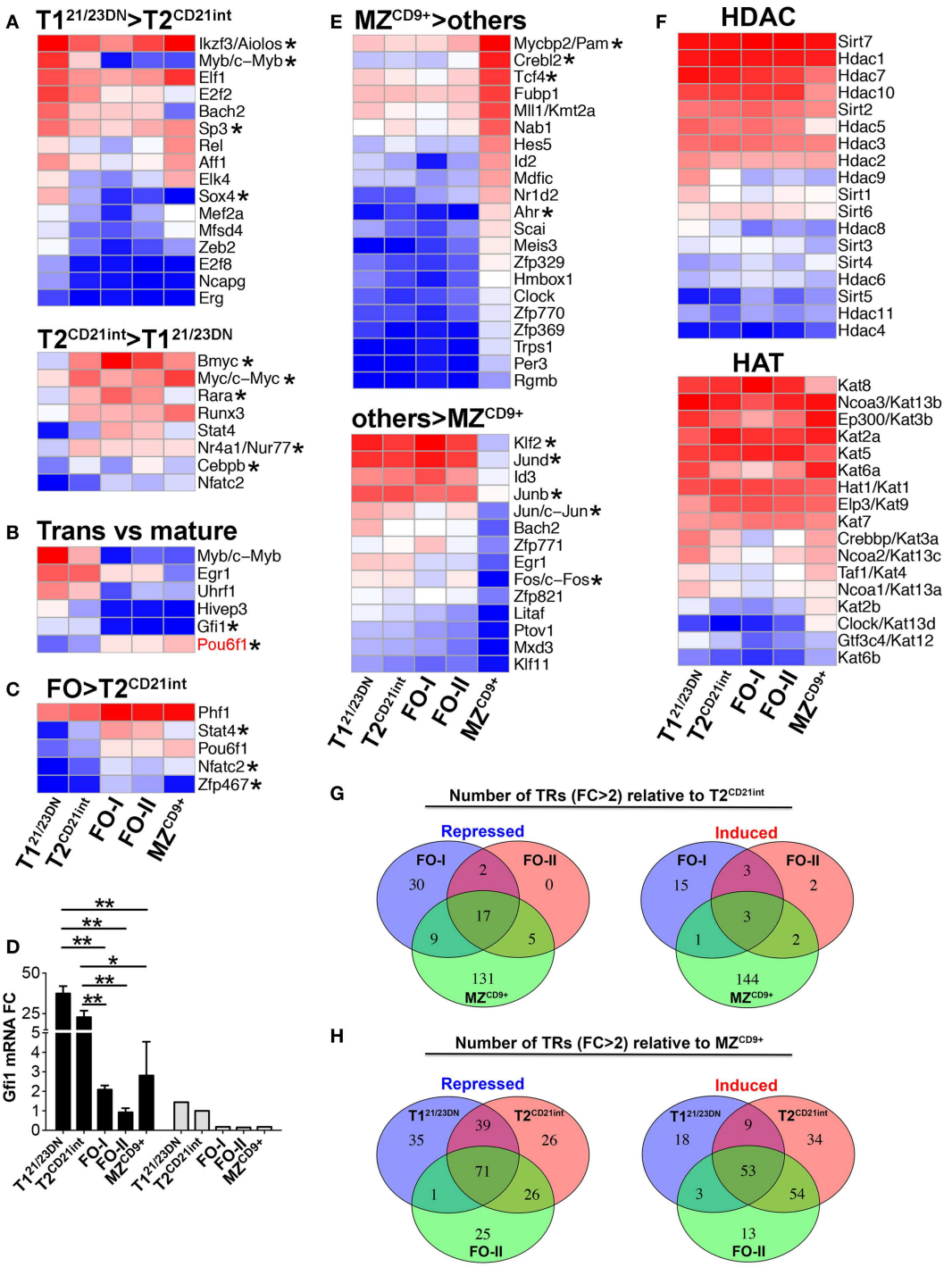

FIGURE 2 | Expression of distinct transcription regulators in splenic B-cell subsets. Heatmap display of NGS analysis of DE transcription regulators (including TFs) using *z*-scores comparing: **(A)** $T1^{21/23DN}$ to $T2^{CD21int}$ (FC > 3 and select FC > 2); **(B)** transitional cells ($T1^{21/23DN}$ and $T2^{CD21int}$) to all mature B-cells (FC > 3); **(C)** FO (FO-I and FO-II) to $T2^{CD21int}$ (FC > 2); and **(E)** MZ^{CD9+} to all other subsets (FC > 3), up-regulated (top heatmap) and down-regulated (bottom heatmap). Pou6f1, the only gene over threefold higher in mature relative to transitional cells, is shown in red **(B)**. Asterisks denote important genes discussed in the text. Many of the TFs shown are predicted to regulate genes that are DE in the B-cell subsets (refer to Table S3 in Supplementary Material). **(D)** Gfi1 is down-regulated during transitional to mature B-cell differentiation. qRT-PCR (black bars) validation of NGS results (gray bars). qRT-PCR data are representative of three independent experiments with FACS-sorted B-cell subsets, where each experiment utilized pooled splenocytes from 2 to 5 mice. $*P \leq 0.05$; $**P \leq 0.01$. **(F)** Heatmap display of transcripts encoding histone deacetylase (HDAC, top) and histone acetylase (HAT, bottom) enzymes. **(G)** The number of repressed or induced TRs that overlap during differentiation from $T2^{CD21int}$ to either FO-I, FO-II, or MZ^{CD9+} cells. **(H)** The number of repressed or induced genes that overlap during differentiation from either $T1^{21/23DN}$, $T2^{CD21int}$, or FO-II to MZ^{CD9+} cells.

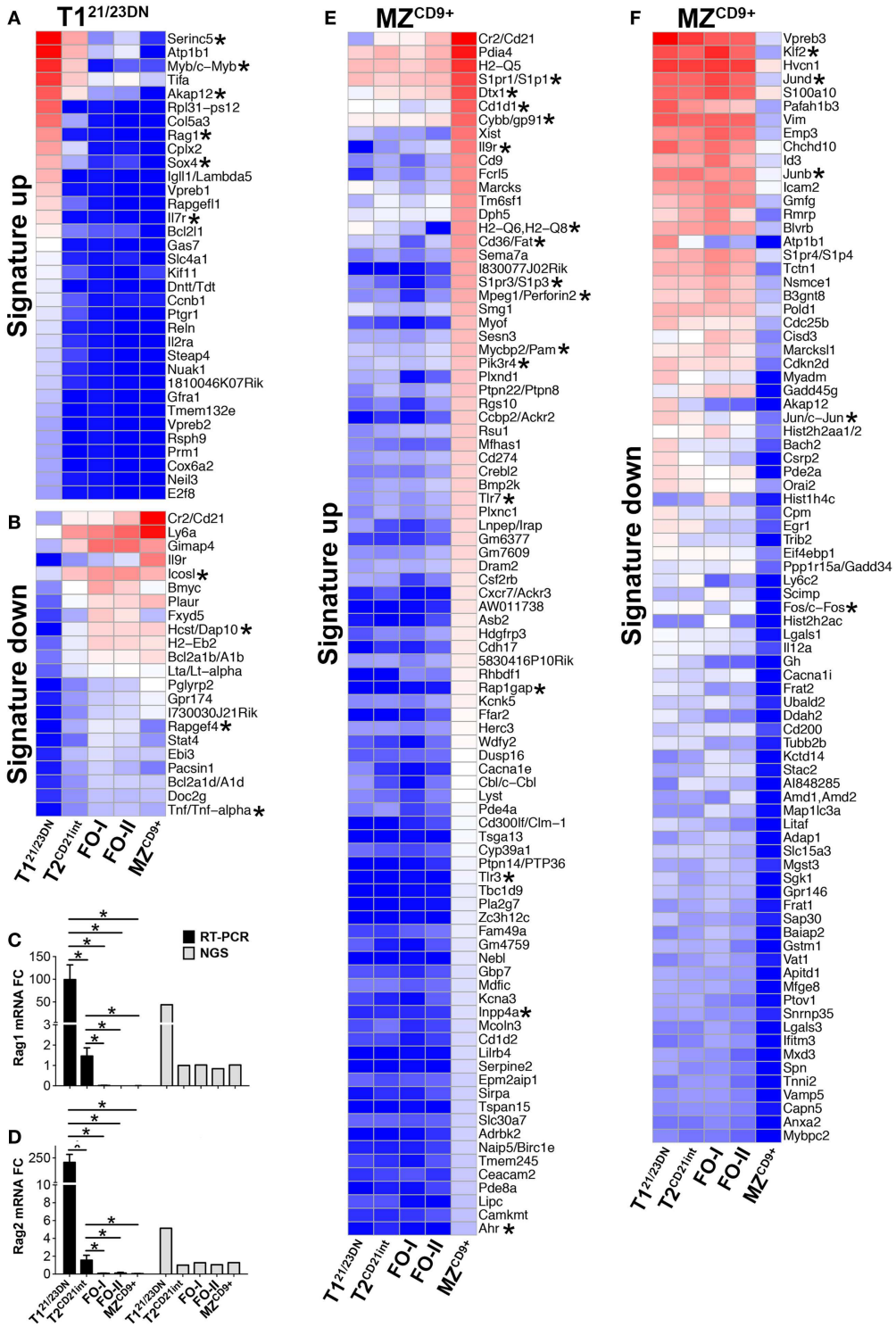

FIGURE 3 | $T1^{21/23DN}$ and MZ^{CD9+} B-cell subsets are highly enriched for signature genes. **(A–D)** RAG1 and RAG2 transcripts are prominent $T1^{21/23DN}$ signature genes. Heatmap displaying *z*-scores of $T1^{21/23DN}$ signature genes (FC > 4) that are uniquely up-regulated **(A)** or down-regulated **(B)**. qRT-PCR analysis validated NGS results showing RAG1 and RAG2 are up-regulated $T1^{21/23DN}$ signature genes. **(C)** RAG1 mRNA expression by qRT-PCR (black bars) and RNA-Seq (gray bars) and **(D)** RAG2 mRNA expression analyzed as in **(C)**. qRT-PCR data represent three independent experiments with FACS-sorted B-cell subsets, where each experiment utilized pooled splenocytes from 2 to 5 mice $*p \leq 0.05$. **(E,F)** MZ^{CD9+} B-cells show highest number of signature genes. Heatmap displaying *z*-scores of MZ^{CD9+} (FC > 4) that are uniquely up-regulated **(E)** or down-regulated **(F)**. Asterisks in heatmaps denote genes discussed in the text. Due to the high number of up-regulated signature genes in MZ^{CD9+} cells, genes below Ahr (57 out of 146) were removed but can be referenced in Table S1 in Supplementary Material.

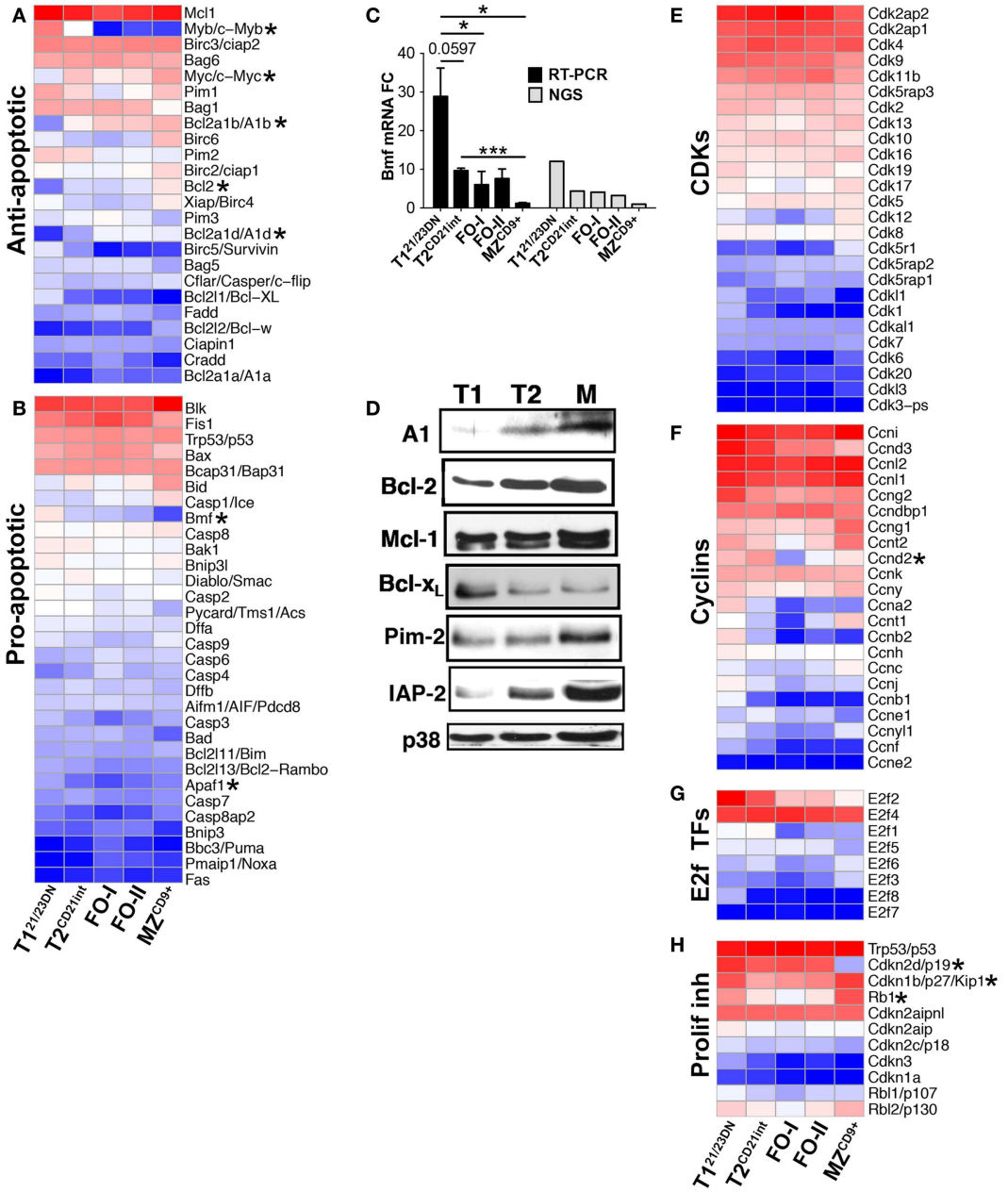

FIGURE 4 | Differentially expressed genes define apoptotic and proliferative potential of transitional and mature B-cell subsets. Heatmap display of NGS data analysis of genes encoding **(A)** anti-apoptotic, **(B)** pro-apoptotic, **(E)** cyclin dependent kinases (CDKs), **(F)** cyclins, **(G)** E2F cell cycle transcription factors, and **(H)** proliferation inhibitors, using *z*-scored NGS gene expression data. Asterisks denote important genes discussed in the text. **(C)** qRT-PCR analysis (black bars) validated NGS results (gray bars) showing upregulation of Bmf in $T1^{21/23DN}$ cells. qRT-PCR data are representative of three independent experiments with FACS-sorted B-cell subsets, where each experiment utilized pooled splenocytes from 2 to 5 mice. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. **(D)** Western blot of anti-apoptotic proteins showing key survival factors such as Bcl-2 increase during differentiation. p38 served as a loading control.

stage-specific genes, we exploited 1581 TRs recently used to define transcriptional architecture of human hemato- and lympho-poiesis (26). A major shift in the expression of TRs accompanied $T1^{21/23DN}$ to $T2^{CD21int}$ differentiation (**Figure 2A**). These included Myb, Sp3, and Sox4 at the $T1^{21/23DN}$ stage whereas Myc, Bmyc, Rarα, Nr4a1/Nur77, and Cebpb were prominent at the $T2^{CD21int}$ stage. These TRs are predicted to regulate key biological processes with Myc alone regulating over 19% of DE genes at the $T2^{CD21int}$ stage (Tables S2 and S3 in Supplementary Material).

We also identified TFs relevant for transitional B-cell maturation, e.g., down-regulation of B-cell differentiation TF Gfi1 coincided with differentiation of transitional into mature B-cells

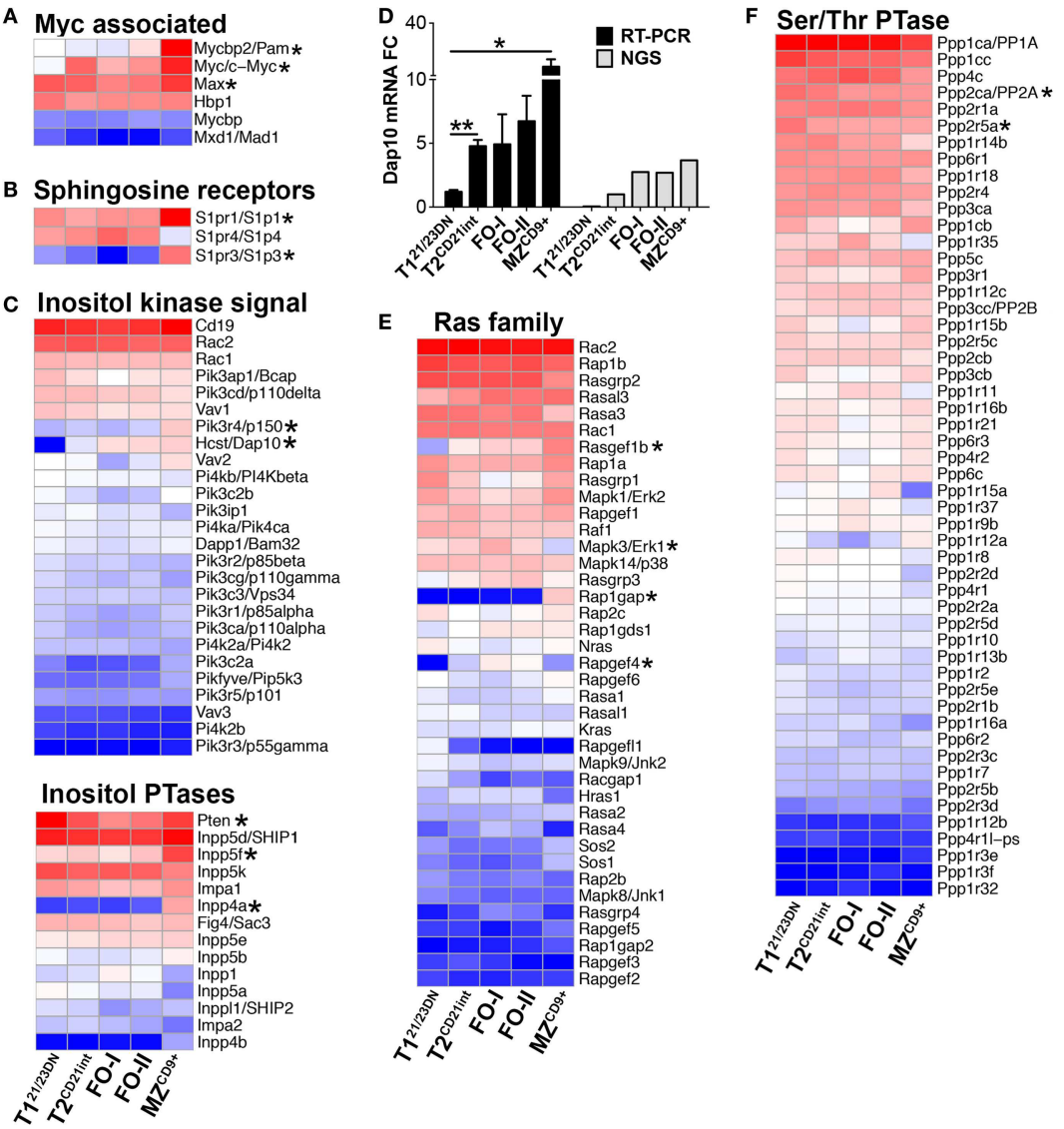

FIGURE 5 | Gene expression profiles predict PI3K/Ras signaling stabilizes Myc during B-cell development. Heatmap display of **(A)** Myc associated genes including Myc binding partners Pam and Max, **(B)** sphingosine receptors possibly mediating Pam/mTOR signaling, **(C)** inositol kinase signaling molecules including Dap10 (bottom) as well as inositol phosphatases (bottom), **(E)** Ras family genes, and **(F)** Serine/threonine phosphatases. **(D)** qRT-PCR analysis (black bars) validated NGS results (gray bars) showing upregulation of Dap10 starting at the $T2^{CD21int}$ stage. qRT-PCR data are representative of three independent experiments with FACS-sorted B-cell subsets, where each experiment utilized pooled splenocytes from 2 to 5 mice. $*P \leq 0.05$; $**P \leq 0.01$.

(**Figure 2D**) (27). Conversely, proliferation-promoting Pou6f1 was enriched over three-fold in mature B-cells (**Figure 2B**) (28). $T2^{CD21int}$ to FO differentiation is regulated mainly by metal-ion binding TFs (Table S3 in Supplementary Material). Further, more TRs were up-regulated in FO-I relative to FO-II consistent with FO-II being less differentiated (**Figure 2G**). GeneGo analysis predicted Tbet, Rar, and Titf/Nkx2-1 TFs were required for differentiation into FO-I whereas Nfatc2 is likely to be essential for FO B-cell function (Table S2 in Supplementary Material). Zfp467 and Stat4 were specifically enriched in FO-I and FO-II (**Figure 2C**) and may therefore selectively regulate FO differentiation and function. Interestingly, Klf2 was predicted to drive FO-II → FO-I terminal differentiation suggesting expression of Klf2 may determine FO-II fate into either FO-I or MZ (Table S3 in Supplementary Material). These data reinforce a T2/FO B-cell differentiation cluster requiring relatively few DE TRs.

$T2^{CD21int}$ to MZ^{CD9+} differentiation required substantial and unique TR rewiring, particularly TFs with zinc-finger and PAS domains (**Figure 2E**; Table S3 in Supplementary Material). Many DE TRs involved in $T1^{21/23DN}$, $T2^{CD21int}$, or FO-II differentiation into MZ^{CD9+} overlapped suggesting commonality in MZ^{CD9+} fate (**Figure 2H**). Signature MZ^{CD9+} TRs included those not

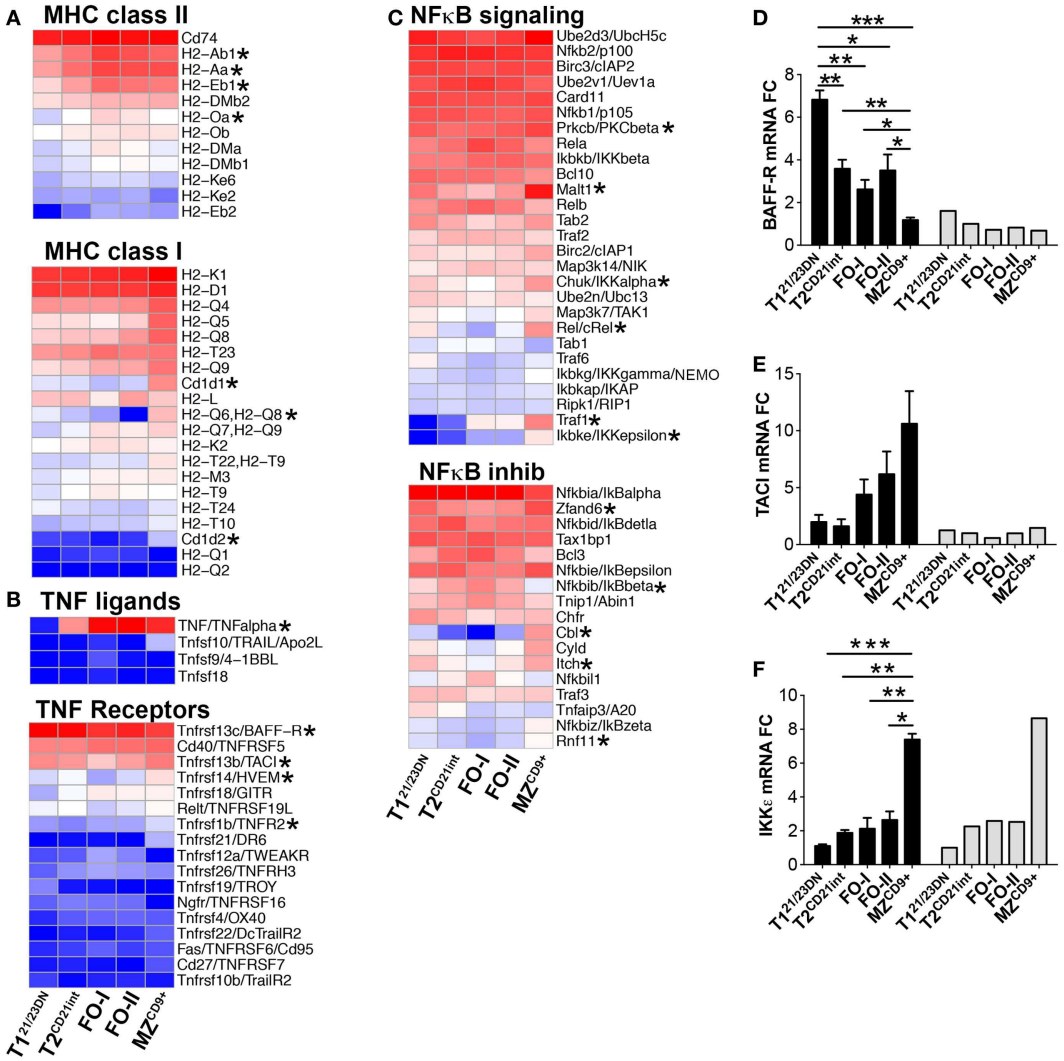

FIGURE 6 | (A,B) Expression of genes associated with immune competence from the $T2^{CD21int}$ stage and **(C–F)** upregulation of genes associated with activated and innate characteristics of MZ^{CD9+} cells. Heatmap display of **(A)** MHC class II (top) and MHC class I (bottom), **(B)** TNF ligands (top) and TNF receptors (bottom), and **(C)** NFκB signaling components (top) and NFκB inhibitors (bottom). qRT-PCR analysis (black bars) validated NGS results (gray bars) of **(D)** BAFF-R, **(E)** TACI, and **(F)** IKKε. qRT-PCR data are representative of three independent experiments with FACS-sorted B-cell subsets, where each experiment utilized pooled splenocytes from 2 to 5 mice. $*P \leq 0.05$; $**P \leq 0.01$.

previously linked to MZ^{CD9+} differentiation and function. One example is Myc binding protein 2 (PAM), which is expressed 4.5-fold higher in MZ^{CD9+} compared to other subsets. We also observed that many TFs [Jund, Junb, Jun, Fos, and Klf2 (29)] were uniquely down-regulated in MZ^{CD9+} (**Figure 2D**). TFs uniquely up-regulated in MZ^{CD9+} cells were predicted to regulate many genes, including those involved in innate immune response (Table S2 in Supplementary Material). Reduced HDAC and distinct HAT expression profile in MZ^{CD9+} cells was consistent with dramatically altered transcriptome (**Figure 2F**). Additionally, we observed Nfat TFs (Nfat5, Nfatc1) as well as Nfat activator Nfam1 were particularly enriched in MZ^{CD9+} cells (Table S1 in Supplementary Material). Thus, we have identified previously known and unknown TRs that contribute to MZ^{CD9+} differentiation and function.

TRANSCRIPTOME ASSOCIATED WITH PERIPHERAL B-CELL TOLERANCE

To systematically define transcriptional programs that regulate alterations in fundamental processes/genes and eventual biological properties of individual B-cell subsets, we identified genes uniquely expressed or repressed termed signature genes (**Figure 3**; Table S1 in Supplementary Material). $T1^{21/23DN}$ were enriched in mitosis factors including cyclins, microtubule motor activity, nucleoside binding, DNA biosynthesis and recombination, and serine/threonine kinases and were deficient in MHCII, B-cell mediated immunity, positive regulation of NFκB, and cytokine and metabolic activity (**Figures 3A,B**; Table S3 in Supplementary Material). Given that DNA recombination is critical for receptor editing, we confirmed that $T1^{21/23DN}$ cells uniquely expressed RAG1 and RAG2 (**Figures 3C,D**). Although previous reports have shown differential RAG expression in splenic transitional B-cells,

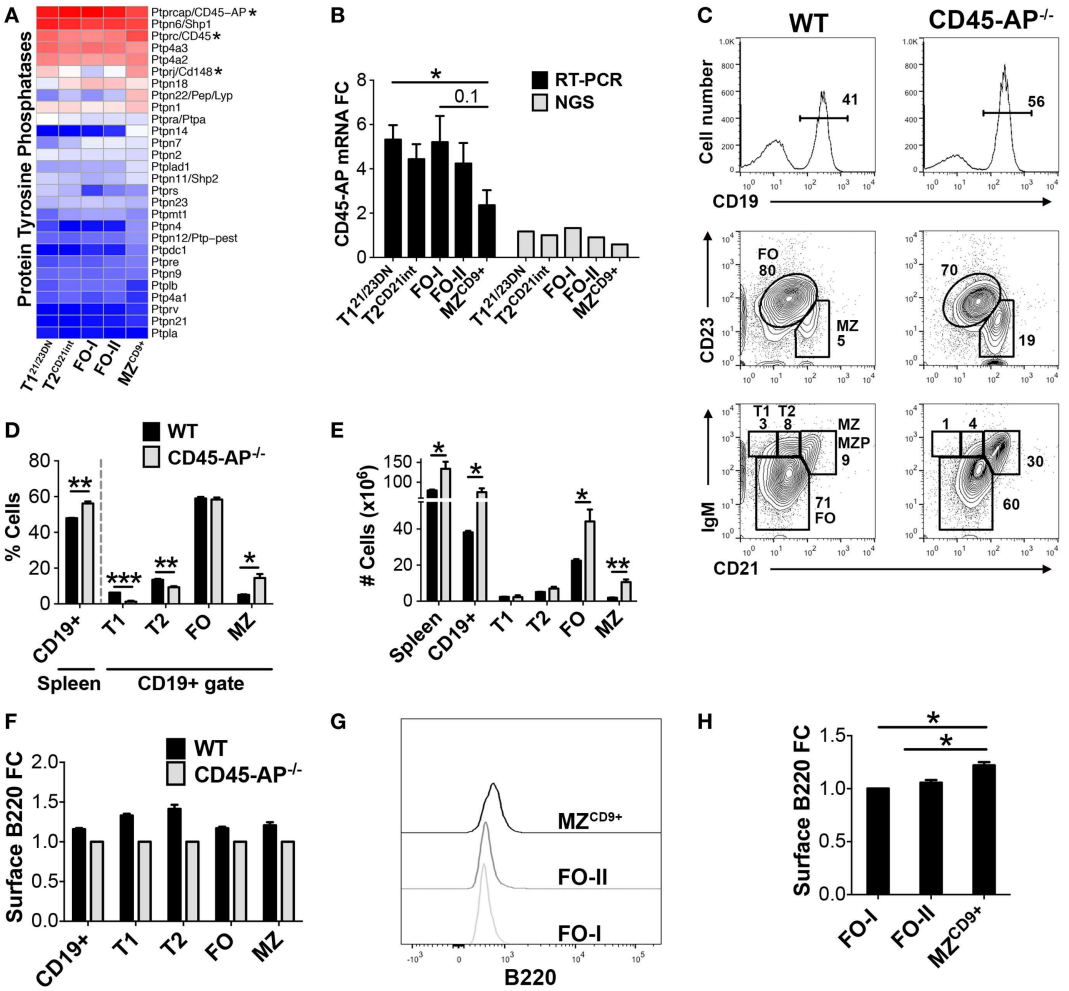

FIGURE 7 | CD45-AP limits premature entry of transitional B-cells into the mature B-cell pool. **(A)** Heatmap display of protein tyrosine phosphatases including tyrosine phosphatase PTPN22, CD45/Ptprc, and CD45 binding partner CD45-AP/Ptprc-ap (astericks). **(B)** qRT-PCR analysis validated NGS results showing CD45-AP is down-regulated in MZ^{CD9+} B-cells. CD45-AP mRNA expression by qRT-PCR (black bars) and RNA-Seq (gray bars). qRT-PCR data are representative of three independent experiments with FACS-sorted B-cell subsets, where each experiment utilized pooled splenocytes from 2 to 5 mice. **(C)** Phenotypic analysis of splenic B-cells isolated from WT and CD45-$AP^{-/-}$ mice. Histogram showing percent of $CD19^+$ B-cells within splenocytes (top panels). $CD19^+$ gated B-cells were further analyzed for transitional (T1 and T2), FO, and MZ B-cells. CD23 versus CD21 staining is used to differentiate MZ ($CD21^{hi}$, $CD23^{lo}$) and FO ($CD21^{lo\text{-}int}$, $CD23^+$). IgM versus CD21 allows for analysis of additional subsets such as T1 (IgM^{hi}, $CD21^-$), T2 (IgM^{hi}, $CD21^{lo\text{-}int}$), and preMZ (MZP). Data are representative of three mice. Bar graph representation of B-cell subset percentages **(D)** and numbers from three mice **(E)**. **(F)** Absence of CD45-AP modestly affects cell-surface display of B220 protein. FCM analysis of WT versus CD45-$AP^{-/-}$ splenic B-cell subsets for the expression of B220. **(G)** MZ^{CD9+} have the highest surface expression of B220. Post-sort surface B220 expression of sorted FO-I, FO-II, and MZ^{CD9+} B-cells (refer to Figure S1 in Supplementary Material) and plotted in **(H)** bar graph. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

we observed much greater FC possibly because our sorting scheme excluded $CD21^+$ and $CD23^+$ cells (30).

Apoptosis and cell cycle control are integral to transitional B-cell tolerance and maturation. Unexpectedly, we did not observe a generalized enrichment of apoptosis and/or cell cycle regulatory genes in any B-cell subset. However, $T1^{21/23DN}$ subset was enriched in select pro-apoptotic genes (Bmf and Apaf1) suggesting their role in negative selection (**Figures 4A–C**). As expected, apoptosis-resistant $T2^{CD21int}$ cells were enriched for anti-apoptotic genes Bcl-2a1b/d and Bcl-2. Bcl-2 and other anti-apoptotic protein levels largely corresponded with the gene expression (**Figure 4D**). $T1^{21/23DN}$ cells also expressed higher cell cycle repressors Rb1 and Cdkn1b whereas $T2^{CD21int}$ cells expressed slightly higher Myc-target gene cyclin Ccnd2, a key regulator of proliferation (31) (**Figures 4E–H**). These findings are consistent with $T1^{21/23DN}$ sensitivity to apoptosis and tolerance checkpoint.

TRANSCRIPTOME ASSOCIATED WITH B-CELL MATURATION AND IMMUNE COMPETENCE

Myc is kept in check in $T1^{21/23DN}$ and up-regulated in $T2^{CD21int}$ and beyond (**Figure 5A**). Due to their significance in stabilizing Myc, we focused on PI3K and Ras signaling (32, 33). We found $T1^{21/23DN}$ cells were significantly deficient for PI3K

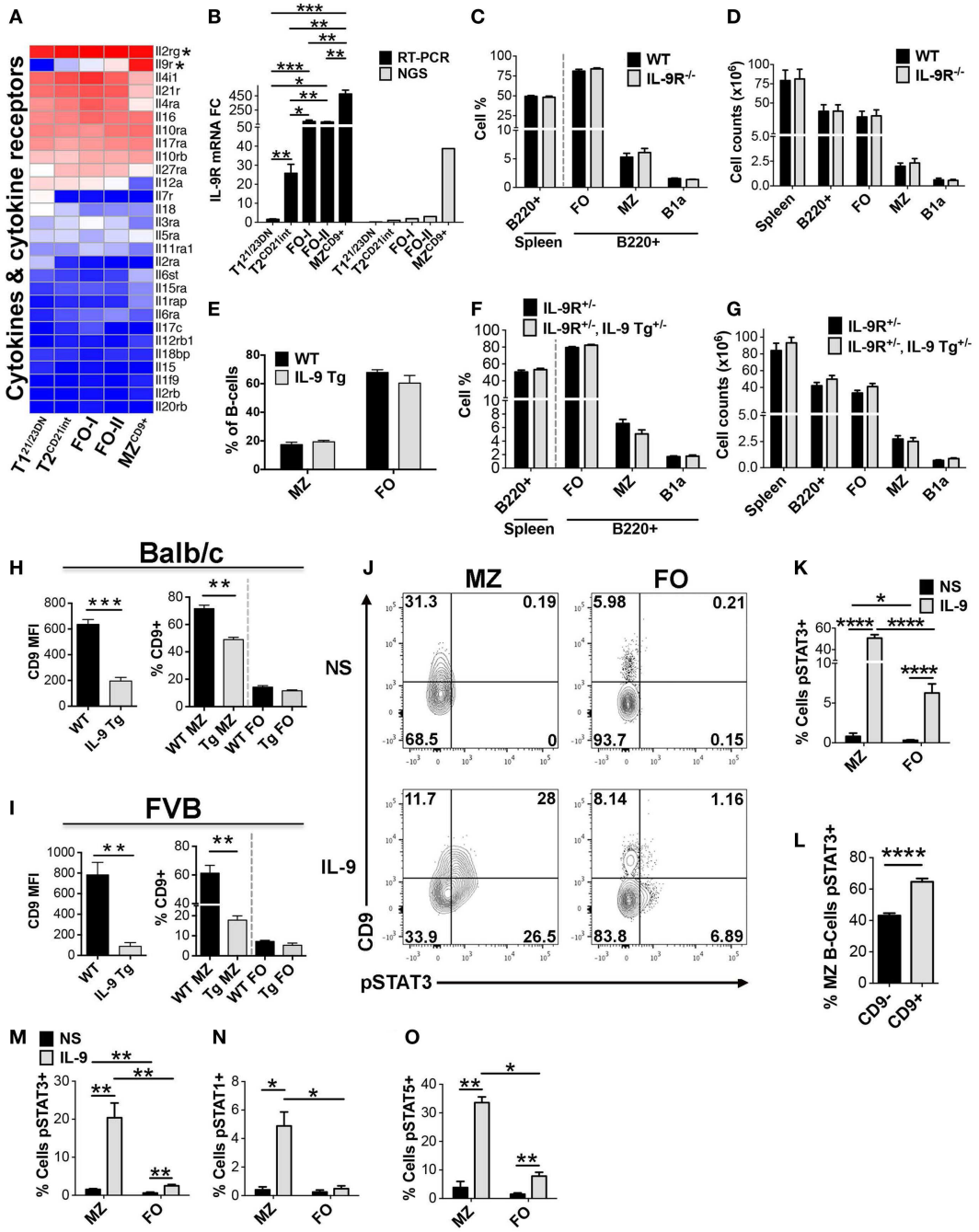

FIGURE 8 | Increased IL-9R expression manifests in enhanced responsiveness of MZ^{CD9+} B-cells to IL-9. Increased IL-9R expression manifests in enhanced responsiveness of MZ^{CD9+} B-cells to IL-9. **(A)** Heatmap display of cytokine and cytokine receptors including IL-9R and its heterodimerization partner IL-2Rg (astericks). **(B)** qRT-PCR analysis validated NGS results showing IL-9R is a MZ^{CD9+} up-regulated signature gene. IL-9R mRNA expression by qRT-PCR (black bars) and RNA-Seq (gray bars). qRT-PCR data are representative of three independent experiments with FACS-sorted B-cell subsets, where each experiment utilized pooled splenocytes from 2 to 5 mice. **(C–D)** IL-9R is dispensable for splenic B-cell development. Bar graph displaying MZ (B220^{+}, CD21^{hi}, CD23^{lo}, IgM^{hi}) and FO (B220^{+}, CD21$^{lo\text{-}int}$, CD23^{hi}, IgM^{+}) B-cell subset percentages **(C)** and numbers **(D)** from Balb/c control versus IL-9R$^{-/-}$ mice. FO represents follicular B-cell populations (FO-I, FO-II, and T2 B-cells). B1a B-cells were gated as (B220^{+}, CD23^{-}, IgM^{hi}, CD5^{+}). Data are representative of six mice. Transgenic over-expression of IL-9 does not affect B-cell development. **(E)** Bar graph displaying MZ (B220^{+}, CD21^{hi}, CD23^{lo}, IgM^{hi}) and FO (B220^{+}, CD21$^{lo\text{-}int}$, CD23^{hi}, IgM^{+}) B-cell percentages from FVB WT control and IL-9 transgenic (Tg5) mice. Data are representative of three mice. **(F,G)** Bar graph displaying MZ (B220^{+}, CD21^{hi}, CD23^{lo}, IgM^{hi}) and FO (B220^{+}, CD21$^{lo\text{-}int}$, CD23^{hi}, IgM^{+}) B-cell subset percentages **(F)** and numbers **(G)** from Balb/c IL-9R$^{+/-}$ control mice and IL-9 transgenic (Tg5) heterozygotes (IL-9R$^{+/-}$, IL-9 Tg$^{+/-}$) mice. Data are representative of six mice. **(H,I)** MZ B-cells display reduced cell-surface CD9 in transgenic mice overexpressing IL-9. Left bar graphs show CD9 mean fluorescence intensity (MFI) of MZ B-cells (B220^{+}, CD21^{hi}, CD23^{lo})

(*Continued*)

FIGURE 8 | Continued
comparing WT versus IL-9 Tg mice. Right bar graphs show the percentage of cells displaying $CD9^+$ positivity comparing MZ versus FO B-cells ($B220^+$, $CD21^{lo-int}$, $CD23^+$). Bar graphs in H and I are representative of three mice. **(J,K)** Short term IL-9 induces STAT3 activation selectively in MZ B-cells. **(J)** FCM analysis of pSTAT3 in FVB splenic MZ ($B220^+$, $CD21^{hi}$, $CD23^{lo}$) versus FO ($B220^+$, $CD21^{lo-int}$, $CD23^+$) B-cells stimulated or non-stimulated (NS) with bacculo-produced IL-9 (3.5 ng/mL) for 15 min at 37°C. FO represents follicular B-cell populations (FO-I, FO-II, and T2 B-cells). Numbers in quadrants represent the percentage of cells. **(K)** Bar graph comparing the percentage of pSTAT3 positive cells in MZ versus FO B-cells. **(L)** Bar graph comparing the percentage of pSTAT3 positive cells in $CD9^+$ versus $CD9^-$ MZ B-cells. Data from **(H–J)** are representative of five mice. **(M–O)** IL-9 induction of pSTAT3 is independent of genetic background. Balb/c wild-type mice were treated as in J and stained for either pSTAT3, pSTAT1, or pSTAT5. pSTAT3 data **(M)** represent three mice whereas pSTAT1 **(N)** and pSTAT5 **(O)** data represent two mice. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$.

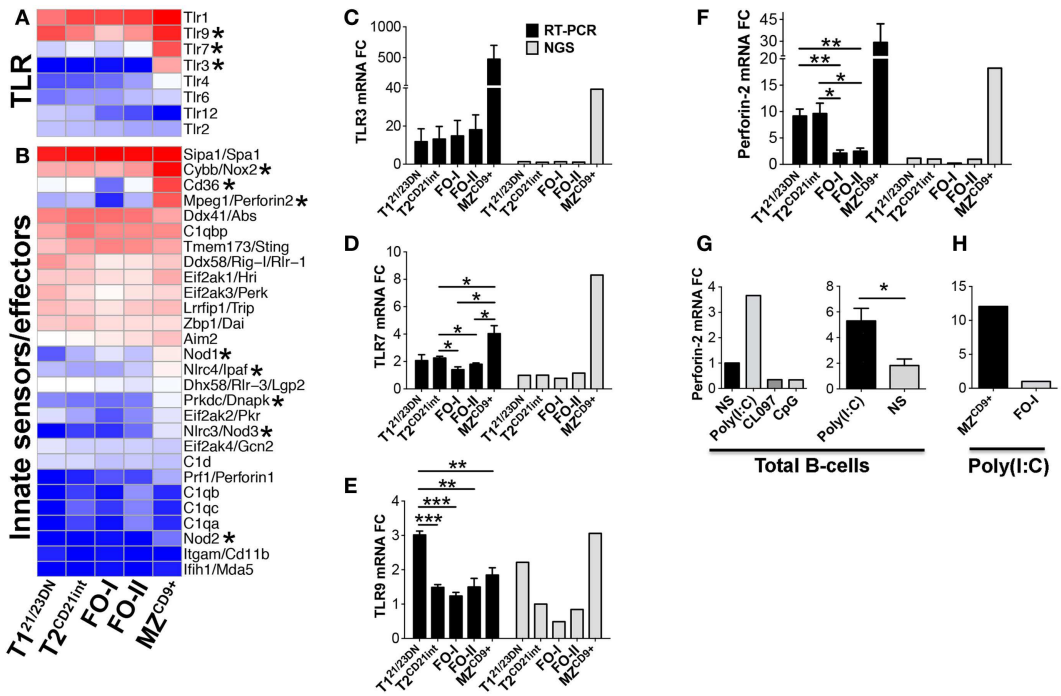

FIGURE 9 | MZ^{CD9+} B-cell subset is poised for innate immune responses. Heatmap display of NGS data showing enrichment of **(A)** TLR and **(D)** innate immune sensors/effector genes. Asterisks denote important genes discussed in the text. qRT-PCR analysis validated NGS results showing MZ^{CD9+} express highest amounts of TLR3, TLR7 and Perforin-2. qRT-PCR (black bars) and RNA-Seq (gray bars) of **(B)** TLR3, **(C)** TLR7, and **(E)** Perforin-2. qRT-PCR data are representative of three independent experiments with FACS-sorted B-cell subsets, where each experiment utilized pooled splenocytes from 2 to 5 mice. **(F)** qRT-PCR of Perforin-2 mRNA fold-change after stimulation with endosomal TLR agonists. B220 positively selected cells (>95% purity) were stimulated 18 h with either TLR3 agonist Poly(I:C), TLR7/8 agonist CL097, or TLR9 agonist CpG at 1 μg/mL and compared to NS. **(G)** qRT-PCR of Perforin-2 mRNA fold-change using B220 positive (>95% purity) or CD43 negative (~90% purity) B-cells stimulated with Poly(I:C) as in **(F)**. Data are representative of four independent experiments. **(H)** qRT-PCR of Perforin-2 mRNA fold-change using FACS derived MZ^{CD9+} or FO-I B-cells treated with Poly(I:C) as in **(F)**. $*P \leq 0.05$; $**P \leq 0.01$.

adaptor protein HCST (DAP10) as well as Ras signaling molecules RasGef1b and RafGef4 (Epac2) (**Figures 5C–E**). Instead, $T1^{21/23DN}$ cells expressed twofold more PI3K antagonist PTEN (34) and highest catalytic and regulatory subunits of Protein phosphatase 2A (PP2A/Ppp2ca, B56alpha/Ppp2r5a) to increase Myc degradation (**Figures 5C,F**) (35). Stunted Myc expression in $T1^{21/23DN}$ cells likely prevents autoreactive B-cell survival and maturation. Differentiation past $T1^{21/23DN}$ stage coincided with a significant upregulation of MHCII genes (H2-Ab1/Aa/Eb1/Oa), a hallmark of immune competence (**Figure 6A**; Table S3 in Supplementary Material). Consistently, $T2^{CD21int}$ were also enriched for Fcer1g, CR2/CD21, complement factors, Icosl and TNFα (**Figure 6B**; Table S3 in Supplementary Material).

In contrast to $T1^{21/23DN}$, Ppp2ca and Ppp2r5a were lowest in MZ^{CD9+}, inversely correlating with the Myc levels (**Figure 5F**). Enhanced Myc along with a distinct transcriptome, including many signature genes, contribute to the activated state of MZ^{CD9+} cells (Table S1 and S3 in Supplementary Material). Consistent with this state, MZ^{CD9+} were enriched for genes that positively regulate NFκB signaling (Rel, Traf1, Ikβkε, Ikkα, Malt1, PKCβ). Additionally, negative NFκB regulator (IκBβ) as well as proliferation inhibitor Cdkn2d were exclusively down-regulated (31, 36) (**Figures 6C,F**). However, to prevent overactivity of Myc, MZ^{CD9+} B-cells also have heightened expression of negative regulators Inpp5f, Inpp4a, PTEN, Rap1gap, down-regulated Mapk3, and increased expression of NFκB negative regulators Zfand6, Itch,

Rnf11, and most notably Cbl (**Figures 5C,E** and **6C**) (31, 37–39). Overall, these data suggest MZ^{CD9+} cells are primed for quick immune responses including TACI-dependent T-independent immune response (**Figure 6E**) (40). Finally, MZ^{CD9+} cells were also enriched for MHCI molecules, especially H2-Q6 suggesting MZ^{CD9+} cells are more suited to present certain types of antigens consistent with their known ability to present glycolipid antigens via CD1d (**Figure 6A**) (13). Interestingly, transcriptome analysis comparing follicular subsets showed FO-I were enriched in histone gene expression while FO-II were enriched for genes involved in lymphocyte and complement activation as well as RNA binding (Figure S1J in Supplementary Material; Table S3 in Supplementary Material). This indicates FO-II share some innate-like qualities with MZ^{CD9+} cells, albeit not as robustly (e.g., CD36 and Perforin-2, **Figure 9B**), and that they are likely capable of context-dependent plasticity (11).

CD45-AP REGULATES MZ B-CELL HOMEOSTASIS

Bioinformatic analysis indicated enhanced regulation of kinase activity in MZ^{CD9+} cells (Table S3 in Supplementary Material). For example, PTPN22 tyrosine phosphatase, a negative regulator of BCR signaling, is expressed highest in MZ^{CD9+} relative to the B-cell subsets analyzed (**Figure 7A**). Of note, polymorphisms in PTPN22 that render B-cells (and T and innate cells) hyper-responsive and are associated with many autoimmune diseases (41, 42). Conversely, CD45 phosphatase is a major positive regulator of BCR-induced kinase activity (15). Kinase activity is critical for BCR signaling strength, which has been proposed to regulate MZ versus FO-cell fate (11). Our NGS and qRT-PCR data revealed that CD45-associated protein (CD45-AP/Ptprc-ap) was 2.5-fold reduced in MZ^{CD9+} relative to FO-I cells (**Figures 7A,B**). CD45-AP is one of the most abundantly expressed genes (FO-I FPKM = 1500). Up to 75% of CD45-AP protein complexes with and potentially positively regulates phosphatase activity of CD45, a key positive regulator of BCR signaling (15). However, the role of CD45-AP in B-cell development and homeostasis remains unknown. Therefore, we analyzed splenic B-cell development in mice deficient for CD45-AP ($CD45\text{-}AP^{-/-}$) (15). FCM analysis of $CD45\text{-}AP^{-/-}$ mice revealed reduced percentage of transitional B-cells (T1 and T2) despite an overall increase in B-cell numbers (**Figures 7C–E**). Although absolute numbers of FO B-cells were increased, their proportion did not. In contrast, both proportions and absolute numbers of MZ B-cells were significantly increased (**Figures 7C–E**). Additionally, absence of CD45-AP slightly reduced cell-surface B220 (CD45) potentially further reducing BCR signaling strength (**Figure 7F**). These results suggest that altered signaling by loss of CD45-AP either facilitates transitional B-cell maturation or survival and/or proliferation of mature B-cell subsets.

SELECTIVE EXPRESSION AND FUNCTION OF IL-9R IN MZ B-CELLS

IL-9R is uniquely enriched in MZ^{CD9+} cells (**Figures 8A,B**), even when compared to other immune cell populations (43). IL-9R interaction with IL-9 regulates inflammation, humoral immunity, and B1 B-cell expansion (17, 44–48). However, IL-9R function in MZ B-cells remains unknown. Therefore, we tested a role for IL-9R in MZ B-cell development and homeostasis using mice with IL-9R gene deletion and transgenic mice over expressing IL-9. Distribution and numbers of splenic B-cell subsets in IL-9R deficient mice were comparable to controls, precluding a role for IL-9R in splenic B-cell development or homeostasis (**Figures 8C,D**). Likewise, IL-9 transgenic (Tg) mice did not show any significant alterations in splenic B-cell populations in two different genetic backgrounds, FVB and Balb/c (**Figures 8E–G**) (17, 48). However, a curious finding was that in IL-9 Tg mice MZ B-cells down-regulated cell-surface expression of CD9 in both genetic backgrounds compared to wild-type control mice (**Figures 8H,I**). These data are consistent with the highest level of IL-9R expression in MZ B-cells, as predicted by our RNA-Seq and RT-PCR data. Taken together, these results demonstrate that IL-9 and IL-9R are dispensable for B-cell development and homeostasis.

Down-regulation of cell-surface CD9 in MZ B-cells by over-expression of IL-9 suggested that IL-9/IL-9R signaling might be consequential in MZ B-cells. Therefore, we sought to experimentally test whether IL-9R in MZ^{CD9+} B-cells transmits intracellular signals. We measured Stat3 phosphorylation by FCM following IL-9 treatment. Compared to non-stimulated cells, low-dose IL-9 induced robust Stat3 phosphorylation primarily in MZ B-cells (**Figures 8J,K**). A much smaller increase was also observed in FO B-cells. Activation of pSTAT3 within MZ B-cells was higher in the $CD9^{+}$ than $CD9^{-}$ fraction (**Figure 8L**). From preferential MZ^{CD9+} B-cell response to IL-9, it seems that this cell population may express highest cell-surface IL-9R protein, consistent with highest IL-9R transcript levels (NGS). Additional experiments showed that in addition to Stat3, IL-9 induced phosphorylation of Stat1 and Stat5 in MZ B-cells. Since, these experiments were carried out with B-cells isolated from Balb/c mice, the results also demonstrate that selective responsiveness of MZ B-cells to IL-9 is independent of the genetic background (**Figures 8M–O**). To our knowledge this is the first report to demonstrate IL-9/IL-9R signaling in MZ B-cells. However, additional experiments are needed to determine the effects of this signaling on MZ B-cell function.

MZ^{CD9+} CELLS SELECTIVELY EXPRESS GENES ASSOCIATED WITH INNATE IMMUNITY

Although MZ B-cells have been proposed to have innate-like properties, a comprehensive transcriptome analysis of these characteristics has not been performed (13, 49). Our analysis of innate immune sensors revealed that MZ^{CD9+} cells are enriched in the RNA sensing molecules Tlr3, Tlr7, and Nlrc3/Nod3, the bacterial peptidoglycan sensors Nod1/2/3 and Nlrc4 and the DNA sensors Tlr9 and Prkdc (**Figures 9A–E**) (50). Innate effector molecules such as respiratory burst oxidase gene Cybb, autophagy associated gene Pik3r4/p150, and the recently identified intracellular bactericidal Perforin-2 are also highly enriched in MZ^{CD9+} cells (**Figure 5C** and **Figures 9B,F**) (51, 52). MZ^{CD9+} cells are also enriched for IKKε, which provides a critical link to innate immune pathways under NFκB control. To our knowledge, this is the first report to suggest a biased use of this innate pathway in MZ^{CD9+} B-cells (**Figures 6C,F**) (53).

We chose TLR3 for functional assessment because it is exclusively expressed in MZ^{CD9+} cells. We show that in total B-cells TLR3 agonist Poly(I:C) was unique among endosomal TLR

agonists by inducing a threefold increase in Perforin-2/MPEG1 transcript (**Figure 9G**). Using sorted cells we showed that MZ^{CD9+}, but not FO-I cells, exclusively induced Perforin-2 (**Figure 9H**). These results show that MZ^{CD9+} cells uniquely and functionally express TLR3, which can endow bactericidal function to MZ^{CD9+} B-cells through Perforin-2 induction.

DISCUSSION

This study is the first to report the mRNA transcriptome obtained by RNA-Seq of mouse splenic B-cell subsets (43, 54–58). Our data support the hypothesis that MZ^{CD9+} subset may arise from multiple precursors. Differentiation from $T1^{21/23DN}$ would theoretically bypass CD23 expression (59). Further support for $T1^{21/23DN}$ to MZ^{CD9+} pathway comes from shared deficiency of N-regions and an increase in TLR9 and Aiolos expression, both of which promote MZ differentiation (60–62). While FO-II could differentiate into MZ, they are most closely related to FO-I. PCA analysis additionally supported FO-II to be an intermediate of FO-I subset, possibly mediated by Klf2.

Enhanced expression of pro-apoptotic gene (Bmf) in $T1^{21/23DN}$ relative to $T2^{CD21int}$ supports the notion that autoreactive B-cell are deleted by mitochondrial pathway of apoptosis at the T1-checkpoint (63). Our novel finding that Serine incorporator (Serinc5) is exclusively expressed in $T1^{21/23DN}$ cells may provide an additional mechanism of cell death by altering membrane composition and BCR signaling (64) (**Figure 3A**). Furthermore, we found expression of genes associated with DNA recombination (RAG1, RAG2, Ligase 4) exclusively in $T1^{21/23DN}$ cells suggesting receptor revision. These data suggest apoptosis and receptor revision contribute to peripheral tolerance at the T1-checkpoint.

We also found that up-regulated $T1^{21/23DN}$ signature gene Akap12 may uphold the $T1^{21/23DN}$ stage by interfering with activation, migration, and proliferation (**Figure 3A**) (65). Consistently, it is dramatically down-regulated in proliferative $T2^{CD21int}$ cells. To our knowledge, this is the first report to identify down-regulation of Akap12 as an indicator of proliferation in splenic B-cells.

Myb to Myc switch from $T1^{21/23DN}$ to $T2^{CD21int}$ was highly consequential as these TFs regulated a substantial proportion of DE genes. We predict Rarα expression contributes to Myb down-regulation (66). Onset of Myc expression in $T2^{CD21int}$ cells profoundly influenced gene expression comprising one-fifth of all DE genes (67) conferring metabolic fitness and likely driving survival, proliferation and differentiation of $T2^{CD21int}$ into mature B-cells (4, 31, 68, 69). Consistently, our data show that MZ^{CD9+} expressed highest levels of Myc as well as Myc binding/regulatory proteins PAM and Max (**Figure 5A**). Consistent with their resting state, FO-I cells express the least Myc of mature subsets, but express highest Bmyc, which we suggest transcriptionally represses Myc (70). Both heightened expression of Bmyc in FO-I and its potential function in B-cells have not been previously reported.

Our data also suggest uncoupling of the BCR from growth, metabolic, and Myc pathways (PI3K and Ras/ERK) contribute to the distinct $T1^{21/23DN}$ cell biology. This may be explained by our novel finding that the PI3K pathway may be may not be fully activated in $T1^{21/23DN}$ cells due to severely reduced levels of the Dap10 adapter for PI3K (71). This genetic data are supported by experimental evidence showing T1 cells only weakly activate the PI3K pathway (via Akt phosphorylation) (3, 5). Additionally, increased expression of negative regulators of BCR signaling PTEN, CD72, and PP2A would further limit the PI3K/Myc pathway in $T1^{21/23DN}$ cells (34, 35, 72). Differentiation into $T2^{CD21int}$ is also accompanied by Bcl-2 transcript and protein. Heightened expression of both Myc and Bcl-2 is a highly potent combination (highest in MZ^{CD9+}) promoting survival during Myc-driven proliferation and frequently occurs in various B-cell lymphomas (73, 74). We suggest that survival at the $T2^{CD21int}$ stage is also supported by TNFα as $T2^{CD21int}$ and mature B-cells express only pro-survival TNFR2 (75). Together, these data point to a previously unknown PI3K- and Myc-driven transcription program, facilitated by TNFα, which distinctly controls metabolic activity in the two transitional B-cell subsets to allow negative selection in $T1^{21/23DN}$ and proliferation and differentiation in $T2^{CD21int}$ B-cells.

With the gain of survival and proliferation potential, $T2^{CD21int}$ cells begin to express MHCII and its transcriptional activator, CIITA as well as the immune modulator Icosl. While the altered gene expression endows $T2^{CD21int}$ cells immune competence, it also prevents inadvertent B-cell mediated T cell activation, which can lead to autoimmune diseases (76). As $T2^{CD21int}$ cells are poised for further differentiation in the splenic follicles, they express LTα and LTβ and facilitate follicular architecture and secondary lymphoid organogenesis (Table S1 in Supplementary Material) (77, 78).

Our analysis of CD45-$AP^{-/-}$ mice revealed an increase in the percentage and numbers of total B-cells due to an increase in mature B-cells, particularly MZ type, similar to $CD45^{-/-}$ mice (79, 80). However, in contrast to CD45, CD45-AP appears to restrict transitional B-cell differentiation, especially to MZ B-cell fate or it is required for maintaining homeostasis within the mature B-cell compartment. Additionally, reduced B220/CD45 surface expression in CD45-$AP^{-/-}$ B-cells suggested that CD45 and CD45-AP are reciprocally dependent for protein stability (81–85). A reduction in CD45 and consequent phosphatase activity would reduce BCR signal strength. Despite conflicting reports concerning CD45-AP's role in receptor signaling (15, 81, 83, 86–89), we propose CD45-AP effects BCR signaling either in transitional cells to prevent their premature entry to the mature B-cell pool or their survival and proliferation. Thus, CD45-AP regulates splenic B-cell homeostasis (79).

Our transcriptome data showing significantly higher IL-9R together with high levels of heterodimer partner IL-2Rγ suggested a potential function for IL-9R in MZ^{CD9+} B-cells. While IL-9R did not play a role in MZ B-cell development, it may play a role in MZ B-cell function as exposure to IL-9 induced phosphorylation of Stat proteins. Activation of Stat proteins, particularly Stat3, is important in host defense (90). Consistently, overexpression of IL-9 has previously been shown to result in increased immunoglobulins before and after immunization (17, 44–46). These findings suggest a potential role for IL-9R-dependent activation of MZ B-cells in immune response (91). Although MZ^{CD9+} B-cells specialize in TI antibody responses, elevated IL-9R suggests that the TD antibody responses may also be differently regulated in this B-cell subset. Consistently, key regulators of antibody response in FO B-cells (IL-4R and IL-21R) are specifically down-regulated in MZ B-cells (Table S1 in Supplementary Material).

A striking finding was that long-term activation of MZ B-cells by IL-9 in transgenic mice dramatically down-regulated CD9 surface levels possibly relating to its function in BCR signaling, migration, adhesion and homing (92–94). However, CD9 deficiency does not alter B-cell development or humoral immunity perhaps due to functional redundancy (95). Taken together, IL-9/IL-9R induction of Stat pathway and down-regulation of CD9 suggests that IL-9R contributes to MZ B-cell function.

Accumulating evidence indicates MZ B-cells function in both innate and adaptive immunity (13) and we identified many DE transcripts with unknown functions in this subset. One such molecule was PAM, which has not been previously linked with MZ B-cell function. This E3 ubiquitin ligase mediates mTOR activation through sphingosine-1-phosphate receptors, of which S1p1 and S1p3 are both highly enriched in MZ^{CD9+} cells (**Figure 5B**) (96, 97). Given that mTOR promotes B-cell activation, maturation, antibody production, and survival, we speculate PAM bridges mTOR activation with sphingosine receptor signaling imparting MZ^{CD9+} with unique functionality (98). We provided evidence that MZ^{CD9+} cells possess unique innate sensing ability through expression of various PAMP receptors such as bactericidal Perforin-2. Further, we showed that TLR3 induction exclusively induced Perforin-2 in MZ^{CD9+} cells (51, 52). Thus, through a unique transcription program, MZ^{CD9+} cells become hardwired to recognize infectious agents and respond quickly to bridge the adaptive immune response.

In summary, our data have identified several genes and gene clusters, which have not previously been linked to specific splenic B-cell subsets. These data revealed potential novel developmental relationships among splenic B-cell populations, and indicated receptor revision in $T1^{21/23DN}$ may contribute to peripheral tolerance. The first major shift in the transcription program accompanied $T1^{21/23DN} \rightarrow T2^{CD21int}$ differentiation, which was dominated by Myc and PI3K/Ras pathways indicating enhanced metabolic activity, survival (via Bcl-2) and proliferation and these alterations were largely shared with mature B-cell subsets. Our analysis also demonstrated that CD45-AP is important for peripheral B-cell homeostasis while IL-9R participates in MZ^{CD9+} cell function. Highly selective expression and function of IL-9R suggests that in MZ^{CD9+} B-cells, TD antibody responses are regulated via mechanisms distinct from FO B-cells. Further, MZ^{CD9+} cells expressed genes that are known to confer innate effector functions as exemplified by expression of PAMPs, TLR3, and Perforin-2. Thus, MZ^{CD9+} B-cells are uniquely suited for TI antibody response, may distinctly regulate TD antibody response and possess broader innate immune capabilities than previously appreciated.

AUTHOR CONTRIBUTIONS

EK, KH, AT, JR, and WK designed research; EK, MH, KH, JL, IC, JW, and DD performed research; EK, DS, ESC, EC, and WK analyzed data; KH edited text; EK and WK wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2015.00030/abstract

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Regulation of germinal center, B-cell memory, and plasma cell formation by histone modifiers

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Understanding the regulation of antibody production and B-cell memory formation and function is core to finding new treatments for B-cell-derived cancers, antibody-mediated autoimmune disorders, and immunodeficiencies. Progression from a small number of antigen-specific B-cells to the production of a large number of antibody-secreting cells is tightly regulated. Although much progress has been made in revealing the transcriptional regulation of B-cell differentiation that occurs during humoral immune responses, there are still many questions that remain unanswered. Recent work on the expression and roles of histone modifiers in lymphocytes has begun to shed light on this additional level of regulation. This review will discuss the recent advancements in understanding how humoral immune responses, in particular germinal centers and memory cells, are modulated by histone modifiers.

Keywords: humoral memory, B-cells, germinal centers, epigenetics, EZH2, MOZ, histone modifiers

INTRODUCTION

Pathogen clearance and formation of immunity requires the activation of B-cells and subsequent differentiation into antibody-secreting cells and memory cells. Humoral memory consists of both memory B-cells and long-lived plasma cells, the latter of which resides mainly in the bone marrow. Together, humoral memory cells are able to clear subsequent infections by the same pathogen more efficiently than responding B-cells during the initial response (1). The mechanisms underlying how memory is formed, and what controls its reactivation, are still unclear. In recent times, transcriptional regulation during B-cell differentiation (2–5) has been the focus of efforts to understand the intrinsic controls that regulate immune cell fates. In contrast, epigenetic regulation during a humoral immune response is relatively unknown. This review will discuss the limited information that is currently known about epigenetic regulators and their importance in the generation and maintenance of immune memory, focusing on the role of histone modifiers within the germinal center (GC).

HUMORAL IMMUNE RESPONSES AND GERMINAL CENTERS

Humoral responses can be broadly categorized into either T-independent or T-dependent responses, with the production of high-affinity antibody and class-switched memory the main outcome of the latter (**Figure 1**). To this end, antigen-activated B-cells that receive T cell help and do not participate in the extrafollicular foci of low-affinity plasmablasts, or become early GC-independent memory (1, 6), can instead form GCs. GCs are divided into a light and dark zone. Within the dark zone, cells undergo multiple rounds of proliferation and adapt their antigen receptor to the immunizing antigen through the process of somatic hypermutation and class-switch recombination (7–11). B-cells then transition to the light zone, in which cells that have a high-affinity antigen receptor will be selected to continue to divide or to differentiate (12–14). In contrast, low-affinity cells and cells that have mutated their receptor to no longer be antigen-specific will die. High-affinity cells that are selected to survive may differentiate into plasma cells or memory cells.

Long-lived plasma cells are generally high-affinity, sessile cells that reside in the bone marrow, relying on extrinsic factors from niche cells for their survival (16). These plasma cells continually secrete high-affinity antibody, resulting in lowering of the amount of an invading pathogen upon re-encounter. Together with memory B-cells, they contribute to maintaining immunity.

REGULATION OF HUMORAL IMMUNE RESPONSES

The activation, proliferation, and differentiation of antigen-activated B-cells during an immune response is orchestrated and regulated at both the cellular and molecular levels. During an immune response, B-cell behavior is regulated by both extrinsic and intrinsic mechanisms. B-cells respond to signals in the microenvironment, including cytokines, cell surface ligand/receptor pairings, and other soluble factors such as chemokines and cell survival molecules (17). For these signals to orchestrate cell behavior in a coordinated manner, cells integrate these signals, resulting in initiation or silencing of genes, which in turn directs cellular behavior.

Transcription factors are molecules that coordinate the expression of a number of genes, thus one transcription factor is often linked to the identity of a cell subset. Different B-cell subsets are associated with particular transcription factors. The transcriptional repressor B cell lymphoma 6 (BCL-6) is expressed in GC B-cells, regulating a program of genes required for GC function and as such is essential for the formation of GCs (18, 19). In contrast, the transcriptional repressor B lymphocyte-induced maturation protein 1 (Blimp-1) is expressed in plasma cells (20).

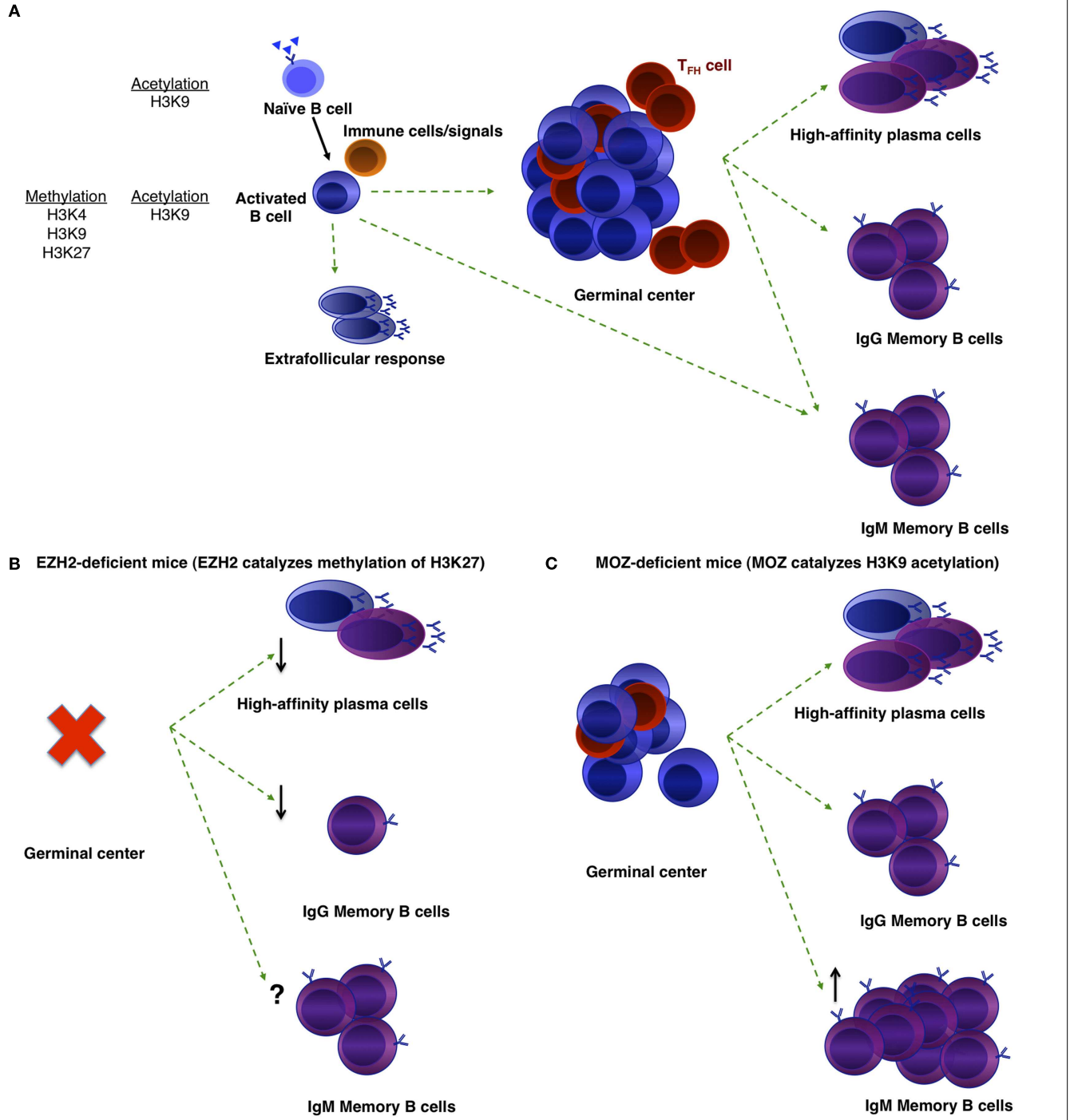

FIGURE 1 | Histone modifications that may regulate B-cell differentiation. **(A)** B-cell differentiation during a T-dependent humoral response. Naïve B-cells that are specific to a foreign pathogen will become activated and receive help from accessory cells. They may become early memory B-cells or extrafollicular plasmablasts, or form a GC. GC B-cells then may either differentiate into long-lived plasma cells or memory B-cells. Within the memory population, there are IgM and IgG memory B-cells. Histone marks in naïve and activated B-cells are noted (15). **(B,C)** Phenotypes of EZH2-deficient **(B)** and MOZ-deficient **(C)** mice after immunization.

BCL-6 and Blimp-1 were previously denoted as "master regulators" of B-cell differentiation, by reciprocally repressing each other (21). However, there are various lines of evidence demonstrating that, similar to the Th1/Th2 paradigm for T cells, the idea of master regulators was a useful concept but too simple to completely explain the genetic programs underlying B-cell differentiation

(22). For example, in the case of memory B-cells, no master regulator transcription factor has been found, and both *Bcl-6* and *Prdm1*, the gene encoding Blimp-1, are downregulated (2). Furthermore, plasma cell differentiation can be induced in the absence of Blimp-1 (23).

Memory B-cells are the centerpiece of the secondary response, in which foreign pathogens are cleared more quickly than a primary response (1). As such, resting memory B-cells have decreased expression of cell cycle inhibitors, correlated with their ability to enter division earlier than naïve B-cells (4). Transcriptionally, naïve and memory B-cells are actually quite similar (3, 4), despite the enhanced survival and proliferative capabilities. Therefore, it is likely there is an additional level of regulation that endows memory B-cells with the ability to respond more efficiently to pathogen infection than naïve B-cells.

EPIGENETIC REGULATION

Genetic regulation also occurs via modification of histones. This is termed epigenetic regulation, i.e., stable inherited modifications of genetic material without altering the DNA sequence. The N-terminal tail of histones can be modified either to promote or inhibit transcription, via creating either an open chromatin structure (euchromatin) or a tightly packed structure (heterochromatin) (24). This is performed by histone modifiers, a group of enzymes such as methyltransferases, acetyltransferases, and histone deacetyltransferases (HDACs). Through these modifiers, histone structure and thus the ability of transcription to proceed is regulated (25, 26). DNA methylation is another form of epigenetic regulation, and recently it was demonstrated that inhibiting DNA methyltransferase 1 (DNMT1) can abrogate GC responses (27) (**Table 1**). However, due to space limitations, DNA methylation will not be discussed further here.

In recent years, epigenetic regulation of B-cell development – especially VDJ recombination (32) – has been revealed. However, much less is known about whether epigenetic modifiers can regulate B-cell differentiation during a humoral response. This mini-review will focus specifically on the current understanding of differential histone modifications during the formation of GC-dependent memory.

HISTONE MODIFICATION PATTERNS IN DIFFERENT B-CELL SUBSETS

Germinal center B-cells and plasma cells have their own unique transcriptional program compared to naïve and memory B-cells (2, 18–20). A large number of gene expression changes occur during differentiation of a naïve B-cell to GC to memory or plasma cell. In addition, an antigen-activated B-cell has the ability to choose any one of those three fates during a response. Therefore, it is likely that regulation of heterochromatin or euchromatin states plays a large role in this adaptability. It could be hypothesized then that the pattern of histone marks is unique to different mature B-cell subsets. Indeed, assessment of H3K4me1, H3K4me3, H3Ac, H3K36me3, H3K27me3, and PolII demonstrated that human naïve and GC B-cells had different patterns of open chromatin (33). Thus, it appears that there is a change in the epigenetic landscape either upon B-cell activation or during the first couple of days during an immune response.

CHANGES TO HISTONE MODIFICATIONS UPON ACTIVATION OF B-CELLS

The immediate epigenetic events that may occur upon activation of an antigen-specific B-cell are unknown. However, preliminary data have shown differences in histone marks between quiescent and activated B-cells (**Figure 1**). Methylation of various histone lysines was found to be decreased in resting cells compared to *in vitro* activated cells (15). For example, H3K4, H3K9, and H3K27 methylation increased after *in vitro* activation, whereas, H3K9 acetylation is present in both quiescent and activated cells (15). In contrast to H3K9 and H3K27 methylation, H3K4 methylation is a permissive mark. Although the authors suggest that histone lysine hypomethylation was a mechanism that endowed B-cells with reprograming potential (15), this has yet to be shown functionally.

Although it is clear that different B-cell subsets have different patterns of histone modifications, there is limited evidence on

Table 1 | Humoral responses in the absence of EZH2, MOZ, p300 (acetyltransferase activity), or DNMT1 [from Ref. (27–31)].

Deletion	Type (target)	GC response	Memory	Plasma cells/Antibody
EZH2	Methyltransferase (H3K27)	– Absent – Reduction in proliferative cells – Higher frequency of cells in G0/G1 – Increase in apoptosis	Decreased IgG1$^+$ memory and affinity	– Decreased IgG1, IgG2b – No change in IgG2a or IgG3 – Decreased plasma cells in vivo – Increased plasmablasts in vitro
MOZ	Acetyltransferase (H3K9)	– Decreased – Dark zone GC B-cells reduced Higher frequency of cells in G0/G1 Decreased BCL6	– Numbers of IgG1$^+$ memory normal but decreased affinity – Increased IgM$^+$ memory	– No change in numbers, but decrease in affinity of plasma cells
p300^{AT}	Acetyltransferase	No change	– Memory response impaired	– No change in IgG1 – IgG2b decreased – SLE-like disease
DNMT1	Methyltransferase (DNA)	– Decreased – Reduction in proliferative cells	Not assessed	Not assessed

the role particular histone modifiers play during the early phase of humoral responses. For example, B-cells from a mouse engineered to have reduced acetyltransferase activity in p300 were still able to respond to T cell-derived stimuli such as anti-CD40, IL-4, and the T-independent stimuli LPS or CpG agonist (28). In contrast, there was a 50% reduction in the ability of these cells to respond to BCR stimulation (28). Because B-cell development is altered in these mice, it is not clear whether this defect is the result of a defect that occurred during B-cell development, as opposed to a direct role upon BcR engagement in the periphery.

An area of great interest currently is whether "bivalency," i.e., the presence of both activating and repressive marks at the same loci, is important for lymphocyte plasticity in identity and function (22). Preliminary studies suggest that bivalency is an important regulator of gene expression during differentiation of naïve to GC B-cells. Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase and a polycomb group member that catalyzes methylation of H3K27 (34). A number of EZH2 target genes in centroblasts that were marked by H3K27me3 were also H3K27me3 marked in naïve B-cells, although likely not by EZH2 as its expression is very low in naïve B-cells (35). A study of bivalent genes in naïve and GC B-cells (with respect to the activating mark H3K4me3 and silencing mark H3K27me3) found that differentiation into GC B-cells resulted in ~1000 new bivalent domains (29). However, the vast majority of these promoters that had dual marks came from the acquisition of H3K27me3 (likely due to upregulation of EZH2) – i.e., already marked H3K4me3 promoters in naïve B-cells (29). As the transcriptional program in GCs is known to involve the large-scale repression of many genes, bivalency may allow GC B-cells to establish the transcriptional program required for the multiple rounds of proliferation and somatic hypermutation that occurs, while retaining the ability to differentiate into centrocytes and eventually plasma cells and memory B-cells. However, the likely complex roles of bivalent domains during B-cell differentiation are yet to be unraveled.

REGULATION OF GCs BY EZH2 AND MOZ

Polycomb group proteins are differentially expressed in the GC in human tonsils. BMI-1 and RING1 downregulation, and ENX and EED upregulation, occur upon differentiation into centroblasts (36). This was then reversed in centrocytes. EZH2 was also found to be upregulated in centroblasts (30, 35, 37). It has also been shown that while *Ezh2* is expressed in plasmablasts, BMI-1 is expressed in plasma cells (38), correlating EZH2 expression with cycling cells in both the GC and in the plasmablast populations. The expression of *Ezh2* is decreased, however, in PC and memory B-cell populations compared to GC B-cells (30).

To investigate the role of epigenetic regulation in B-cell differentiation during humoral responses, a number of groups have conditionally deleted histone modifiers (**Table 1**). Two such enzymes are EZH2 and the histone acetyltransferase monocytic leukemia zinc finger protein (MOZ) (**Figure 1**). EZH2 plays an important role during B-cell development by modulating *Igh* rearrangement (39), and has now been revealed to be essential for GCs (29, 30). The deletion of EZH2, by use of either Cγ1-Cre or CR2-Cre, dramatically reduced GC frequency, with the remaining GC cells $EZH2^{+}$ (29, 30). Both research groups demonstrated the regulation of cell cycle genes by EZH2 (29, 30, 35), although GC B-cells were also found to undergo increased apoptosis in the absence of EZH2 (30).

Although MOZ is a histone acetyltransferase, there were similarities between the phenotypes of MOZ-deficient and EZH2-deficient mice. Deletion of MOZ using *Mb-1*-Cre (in all B-cells) or *Aicda*-cre (specifically in activated B-cells) mice also resulted in a decrease in GC B-cells (31), although not to the extent of EZH2-deficiency (29, 30). This was found to be due to defective proliferation, correlating to a decrease specifically in dark zone B-cells (31). Thus, expression and/or function of EZH2 and MOZ can be localized to the dark zone of the GC. Somatic hypermutation and class-switch recombination is also known to be regulated epigenetically, however, this has been reviewed recently (32) and thus will not be discussed here. Given that a number of other histone modifiers are located either in the dark or light zone (36, 37), future investigations could assess whether these other modifiers regulate particular functions within the light zone.

B cell lymphoma 6 (BCL-6) is absolutely required for GC formation (18, 19). BCL-6 shares some common targets with EZH2 in GC B-cells. EZH2 binds approximately 1800 promoters in GC B-cells (35), and a portion of these were specific to GC B-cells. Within this GC-specific geneset, it appeared that EZH2 targets were involved in cellular proliferation and repression of differentiation (29, 30, 35). Interestingly, EZH2 targets that were not H3K27me3-marked in naïve B-cells were also bound by BCL-6 (35). Approximately half of the genes that were bound by both the polycomb repressor complex 2 and BCL-6 in wild-type GC B-cells were upregulated in EZH2 mutants (30). In contrast, EZH2-deficiency mostly did not affect the expression of BCL-6 targets that lack the H3K27me3 mark (30), and EZH2 does not modulate BCL-6 expression itself (30). In contrast, MOZ-deficient GC B-cells had decreased levels of BCL-6 (31), which may be associated with the gene expression program modulated by MOZ (31).

REGULATION OF PLASMA CELLS BY HISTONE MODIFIERS

Conditional deletion of histone modifiers in B-cells has demonstrated that differentiation of GC cells into plasma cells is epigenetically regulated. In the case of MOZ, deficiency altered the affinity but not numbers of plasma cells, likely due to the reduction of dark zone B-cells (31). Similarly, the GC defect in EZH2-deficient mice resulted in a significant reduction in both numbers and affinity of plasma cells (30). However, when these authors stimulated EZH2-deficient cells *in vitro*, differentiation into plasmablasts was increased in the absence of EZH2. This was correlated to functional repression of the plasma cell genes *Prdm1* and *Irf4* by EZH2 (30), and the reduction of H3K27me3 marking at *Irf4* and *Prdm1* loci upon differentiation (30). In addition to *Irf4* and *Prdm1*, EZH2 appears to regulate the genetic programs associated with differentiation of GC B-cells to plasma cells or memory B-cells (29, 30, 35). Thus, continued EZH2 expression is likely required to maintain the GC phenotype and prevent premature differentiation (35). It is

known that EZH2 mutations are associated with malignant transformations (29, 30, 35, 40), but it is also possible that dysregulation of EZH2 may also play a role in antibody-mediated autoimmune disorders.

Lastly, it is likely that HDACs can also regulate plasma cell differentiation, although previous studies have had contrasting results on whether inhibiting HDACs inhibit or promote differentiation (41, 42). This will be important to determine as HDAC inhibitors are being used to treat lymphocyte malignancies (43–45). Dysregulation of gene expression during B-cell responses can lead to autoimmune diseases, and there is some evidence this could occur as a result of improper histone modifications. Mice lacking acetyltransferase activity in p300 specifically in B-cells develop a systemic lupus erythematosus-like disease (28). Thus, there is future potential to use epigenetic modifiers as treatment for antibody disorders.

REGULATION OF B-CELL MEMORY BY EZH2 AND MOZ

Immune memory is defined as the rapid and robust response that occurs upon secondary infections, clearing invading pathogens more quickly than the primary response. The memory B-cell population is phenotypically and functionally heterogeneous (1, 46, 47). Recently, a number of research groups have postulated that the heterogeneity evident within the memory population allows the pool to undergo specialized functions, i.e., differentiation into plasmablasts whilst being able to self-renew. IgM^+ memory B-cells persist longer than IgG^+ memory B-cells, and are able to initiate a response to secondary infections when IgG^+ memory B-cells are present in low numbers (48, 49). In contrast, switched memory B-cells has been linked to the rapid production of antibody during secondary responses (48, 49). A number of genes expressed in IgM^+ B-cells are silenced when those cells are engineered to signal through the cytoplasmic tail of IgG1 (50, 51). Therefore, regulation of gene transcription programs may be linked with the plasticity of the memory pool, allowing persistence in the presence of rapid activation and differentiation during re-infection.

In the absence of EZH2, GC-derived $IgG1^+$ memory B-cells and antibody produced in a secondary response were significantly reduced (30). It is likely that the reduction in memory formation and function is a result of the absence of functional GCs (29, 30). High affinity $IgG1^+$ memory B-cells were also reduced in the absence of MOZ (31). The latter study also investigated the IgM^+ memory B-cell subset, which has been linked to longevity of the memory population (48, 49). In the absence of MOZ, the make-up of the memory B-cell population was altered such that IgM^+ memory B-cell numbers were increased. It is likely that as a result, secondary GC formation was increased in these mice, whereas, secondary plasmablast formation was decreased (31). Thus, MOZ regulated the composition and functional outcome of the memory compartment. More work is now needed to investigate in detail the role of epigenetic regulation in memory B-cell formation and function.

CONCLUDING REMARKS

Histone modifications are an important component of gene expression regulation. Specifically, in both T and B-cells, during development and during differentiation in the periphery, patterns of histone modifications are unique to different lymphocyte subsets. These modifications likely allow adaptability of cells – i.e., for the ability of an antigen-activated B-cell to undergo differentiation into either a memory B-cell, GC or plasmablast.

The enzymes that catalyze modifications of histones, such as EZH2 and MOZ, have recently been shown to play important roles in formation, maintenance and modulation of B-cell populations. Thus, these new studies demonstrate that programing of B-cell subsets by epigenetic changes influence differentiation decisions during immune responses. However, it is only the beginning for these types of studies. A better understanding of epigenetic regulation of humoral responses will be attained as the targets for each modifier in B-cell subsets, factors involved in facilitating modifications, and interactions between known regulatory complexes are revealed. It will be important to use an integrated approach to identify histone modifications important for B cell generation and function, and the transcriptional networks they regulate. Thus, in addition to ChIP-sequencing and gene-targeted mice, it will be essential to use new methods that can systematically initiate histone marks during B cell responses to unravel the role of particular modifications during memory formation and secondary responses.

Revealing the roles of other histone modifiers has the potential to reveal the molecular mechanisms underlying the production of a memory population that is able to persist in the absence of antigen whilst being poised to respond to subsequent infections. This not only has implications for vaccines and immunodeficiencies that are unable to produce memory cells, it will also result in a wider understanding of how epigenetic regulation controls gene expression during programs of cell differentiation. Understanding these fundamental cellular processes are applicable not only to B-cell and hematopoietic development, but also more generally for developmental processes. It is noteworthy that it is precisely these transcriptional networks that are predictive in disease, particularly autoimmune diseases and cancers.

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HP-1γ controls high-affinity antibody response to T-dependent antigens

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In vitro observations suggest a role for the mouse heterochromatin protein 1γ (HP-1γ) in the immune system. However, it has not been shown if and how HP-1γ contributes to immunity *in vivo*. Here we show that in mice, HP-1γ positively regulates the germinal center reaction and high-affinity antibody response to thymus (T)-dependent antigens by limiting the size of $CD8^+$ regulatory T-cell (T_{reg}) compartment without affecting progenitor B- or T-cell-development. Moreover, HP-1γ does not control cell proliferation or class switch recombination. Haploinsufficiency of *cbx-3* (gene encoding HP-1γ) is sufficient to expand the $CD8^+$ T_{reg} population and impair the immune response in mice despite the presence of wild-type HP-1α and HP-1β. This is the first *in vivo* evidence demonstrating the non-redundant role of HP-1γ in immunity.

Keywords: chromatin remodeling, epigenetics, adaptive immunity, germinal center response, $CD8^+$ regulatory T cells

INTRODUCTION

The adaptive immune system allows jawed vertebrates to distinguish self from non-self, to eliminate infectious agents, and to eradicate tumors. In addition, jawed vertebrates have the unique ability to store long-term immunological memory, thus enabling a rapid and vigorous adaptive immune response against previously encountered microbes. To achieve this outcome, diverse lymphocyte populations and their effector functions must be finely orchestrated and controlled. Dysregulation of any of these processes may result in the development of autoimmune diseases, inability to resolve infections, or failure to control the outgrowth of malignant cells. Therefore, the regulation of the adaptive immune response must occur on many levels, and there still remain novel genes and pathways yet to be uncovered.

The heterochromatin protein 1 (HP-1) family includes members that associate with modified histones, indicating that HP-1 proteins are involved in epigenetic modifications. HP-1 proteins are conserved from the yeast *Schizosaccharomyces pombe* (*S. pombe*) to mammals (1–5). The mammalian HP-1 family consists of three conserved members: HP-1α, HP-1β, and HP-1γ encoded by *cbx-5*, *cbx-1*, and *cbx-3*, respectively (2). Of particular significance to the immune system is the observation that HP-1γ is found associated with the transcription elongation complex containing RNA polymerase II (Pol II) within the coding region of the actively transcribed IL-2 gene in stimulated primary T cells (6). By contrast, during B-cell-development, HP-1γ associates with the silenced κ allele implicating HP-1γ in allelic exclusion (7). In addition, HP-1γ has been found associated with both heterochromatin and euchromatin suggesting that it participates in transcriptional repression and activation, respectively (4, 8, 9). HP-1γ interacts with the methyl groups of H3K9 through the chromodomain (CD) and with the methyl transferase SUV39-H1 and other proteins through the chromoshadow domain (CSD) (2, 3, 10). Despite these crucial *in vitro* observations, it is not understood if and how HP-1γ contributes to the regulation of immunity in mammals *in vivo*. Our interest in HP-1γ stems from efforts to identify novel targets of miR-155 (11). We find that HP-1γ expression is induced in activated mutant B cells suggesting that it might be an miR-155 target.

During a thymus (T)-dependent B-cell response, activated B cells migrate into follicles of secondary lymphoid organs. A fraction of activated B cells mediate a primary antibody (Ab) response through differentiation into plasma cells, others are recruited to the germinal center (GC) reaction (12, 13). In the GC, a specialized structure within the follicle, B cells undergo massive proliferation accompanied by class switch recombination (CSR) and somatic hypermutation (SHM) of rearranged immunoglobulin (Ig) V region genes. SHM leads to the acquisition of mutations that increase Ab affinity to the immunizing antigen (Ag), a process known as affinity maturation (12, 14, 15). The production of high-affinity, isotype-switched Ab is crucial for the clearance of many infectious pathogens and provides the basis for humoral immunity and vaccine efficacy.

Resident GC T follicular helper (T_{FH}) cells make up a specialized subset of effector $CD4^+$ T cells that are pivotal in affinity maturation by selecting activated B cells to enter the GC, regulating GC positive selection, and directing B-cell differentiation to plasma cells and memory B cells (16–18). Within the GC, T_{FH} cells develop in concert with GC B cells (19–24).

Early observations show that a subset of effector $CD8^+$ T cells can suppress T-cell help to B cells (25). Recent studies demonstrate that these $CD8^+$ regulatory T (T_{reg}) cells control GC reaction and high-affinity Ab response to foreign T-dependent Ags as well as self-Ags by limiting the size of the T_{FH} compartment (26, 27). In mice, genetic disruption of the inhibitory interaction between $CD8^+$ T_{reg} cells and their target Qa-1^+ T_{FH} cells results in the development of systemic lupus erythematosus (SLE)-like autoimmune disease and the inability to mount a high-affinity Ab response to T-dependent Ags. These studies reveal the central role that $CD8^+$ T_{reg} cells play in the control of the adaptive immune response. However, mechanisms that regulate the development and/or homeostasis of these cells remain elusive.

In this study, we uncover a novel molecular pathway that regulates the adaptive immune response to T-dependent Ags. We demonstrate that HP-1γ positively controls the GC reaction and high-affinity Ab response. HP-1γ does so by limiting the size of the $CD8^+$ T_{reg} compartment. Haploinsufficiency of *cbx-3* results in the expansion of $CD8^+$ T_{reg} cells and impaired immune response.

RESULTS

B- OR T-CELL-DEVELOPMENT IS NOT AFFECTED BY HP-1γ DEFICIENCY

Although *in vitro* studies suggest a role for HP-1γ in the immune system, it has not been determined if it contributes to immunity *in vivo*. The *cbx-3* (gene encoding HP-1γ) mutant mouse was generated by gene-trapping technology as described previously (10, 28). We found that *cbx-3*$^{-/-}$ mice died perinatally. Because haploinsufficiency of genes involved in epigenetic modifications has been shown to alter cellular functions (29), we asked if haploinsufficiency of *cbx-3* was sufficient to affect the immune system. First we assessed if *cbx-3* deficiency influenced progenitor lymphoid development. A survey of the bone marrow (BM) and thymus showed that progenitor B and T cells developed normally in *cbx-3*$^{+/-}$ mice compared to littermate controls (**Figures 1A,B**). Mature B- and T-cell-development also remained normal in *cbx-3*$^{+/-}$ mice (**Figures 1C,D**). Thus, HP-1γ is not required for progenitor or mature B- and T-cell-development.

HP-1γ DEFICIENCY RESULTS IN IMPAIRED GERMINAL CENTER REACTION

To determine the physiological function of HP-1γ in the adaptive immune response in mice, we immunized littermate control and *cbx-3*$^{+/-}$ mice with the T-dependent Ag 4-hydroxy-3-nitrophenylacetyl hapten conjugated to chicken gamma globulin (NP-CGG) in alum. On day 14 after immunization, the expected frequency of spleen $B220^+CD38^{lo/-}FAS^+$ GC as well as switched $B220^+CD38^{lo/-}IgG_1^+$ B cells was obtained from littermate controls, demonstrating that the GC reaction occurred normally (**Figure 2**). In contrast, the GC response was impaired in *cbx-3*$^{+/-}$ mice. On day 14, the percent of spleen GC B cells in *cbx-3*$^{+/-}$ mice decreased by 2.3-fold compared to control mice ($p = 0.0002$, **Figures 2A,B,F**). Correspondingly, there was a twofold reduction in the percent of switched IgG_1^+ spleen B cells in *cbx-3*$^{+/-}$ mice compared to control mice ($p = 0.0007$, **Figures 2A,C**). Similarly, the frequency of IgG_1^+ GC B cells in *cbx-3*$^{+/-}$ mice was reduced by 2.8-fold compared to wild-type littermate mice (**Figures 2D,E**, $p < 0.0001$). The spleen architecture of wt littermate and mutant mice remained intact; and more peanut agglutinin (PNA) positive GCs were detected in wt littermate mice than in mutant mice on day 14 after immunization with NP (**Figure 2F**). These results demonstrate that HP-1γ positively regulates the GC reaction and production of IgG_1^+ B cells, and haploinsufficiency of *cbx-3* is sufficient to impair these processes. The defect cannot be compensated for by the presence of wild-type HP-1α and HP-1β in *cbx-3*$^{+/-}$ mice suggesting that HP-1γ has a non-redundant function in immunity *in vivo*.

CBX-3$^{+/-}$ MICE FAIL TO MOUNT HIGH-AFFINITY NP ANTIBODY RESPONSE

To determine if the diminished GC reaction in *cbx-3*$^{+/-}$ mice results in defective serum anti-NP Ab response, we measured anti-NP activity in sera obtained from *cbx-3*$^{+/-}$ and wt littermate mice on day 14 after NP immunization. Serum high- and low-affinity responses to NP can be measured by enzyme-linked immunosorbent assay (ELISA) using NP4 and NP25 Ags, respectively. On day 14 after immunization, serum IgG anti-NP25 Ab activity increased in littermate control and mutant mice compared to unimmunized animals, and the level was similar between the two groups. By contrast, the amount of serum anti-NP4 antibodies in littermate control mice was 4.75-fold higher than *cbx-3*$^{+/-}$ mice ($p = 0.006$, **Figure 3A**). Accordingly, the ratio of NP4/NP25 Ab titer was threefold lower in *cbx-3*$^{+/-}$ mice compared to littermate control mice ($p = 0.02$, **Figure 3B**). The low-affinity response was not affected by *cbx-3* haploinsufficiency. Both littermate control and mutant mice produced low amounts of serum IgM Abs against NP, and the majority of IgM antibodies were of low-affinity (**Figures 3C,D**). There was no difference in the production of total pre-immune serum IgG_1 and IgM between wt littermate control and mutant mice (**Figure 3E**). Thus *cbx-3*$^{+/-}$ mice could not mount high-affinity Ab response to NP.

HP-1γ DOES NOT REGULATE B-CELL PROLIFERATION OR CLASS SWITCH RECOMBINATION

To rule out the possibility that reduced GC and Ab responses resulted from defects in proliferation or class switching after Ag encounter, we carried out *in vitro* proliferation/switch assays. Spleen B cells from *cbx-3*$^{+/-}$ mice proliferated and switched as well as littermate control B cells when activated through the B-cell receptor, Toll-like receptor (TLR) 4, or CD40 plus IL-4 (**Figures 4A,B**). Therefore, HP-1γ deficiency results specifically in impaired high-affinity, not total NP Ab response. HP-1γ does not control the IgM response to NP. Because HP-1γ deficiency does not perturb proliferation or switching, the defect in high-affinity Ab response observed in *cbx-3*$^{+/-}$ mice implies that HP-1γ may regulate Ab affinity maturation.

THE T FOLLICULAR HELPER CELL POPULATION IS REDUCED IN *CBX-3*$^{+/-}$ MICE

T follicular helper cells play a crucial role in affinity maturation in part by selecting B cells to enter the GC, regulating GC positive selection, and directing B-cell differentiation to plasma cells and memory B cells. Hence, the high-affinity Ab response defect seen in *cbx-3*$^{+/-}$ mice may arise from inefficient T_{FH} support. Fluorescence-activated cell sorting (FACS) analysis showed that as the immune response proceeded to day 14, the frequency of $TCR\beta^+CD4^+CXCR5^{hi}PD\text{-}1^{hi}$ T_{FH} cells decreased by 1.7-fold in

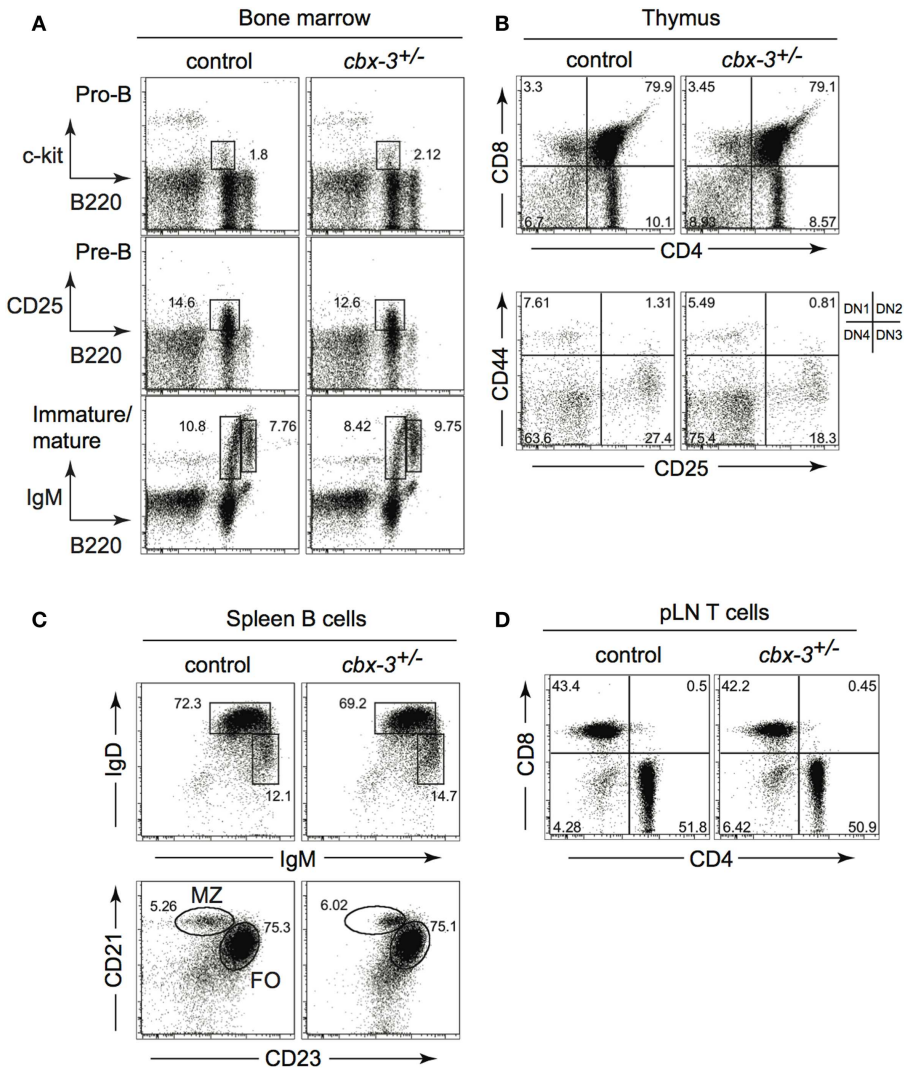

FIGURE 1 | B- or T-cell-development is not affected by HP-1γ deficiency. **(A)** Bone marrow progenitor B-cell development from unimmunized wt littermate and *cbx-3*$^{+/-}$ mice was determined by FACS. Progenitor B cells were gated on the lymphoid population. Pre-B cells were derived from the surface IgM (sIgM) negative lymphoid gate. **(B)** Thymi from unimmunized wt littermate and *cbx-3*$^{+/-}$ mice were analyzed to assess progenitor T-cell-development. Upper plots were derived from the lymphoid gate; lower plots were gated on the CD4$^-$CD8$^-$ population. **(C)** All plots were gated on CD19$^+$ lymphoid population. Frequency of mature (IgD^{hi}IgM^{lo}), marginal zone (CD21^{hi}CD23^{lo}), and follicular (CD21^{lo}CD23^{hi}) spleen B cells was determined by FACS. **(D)** Lymphoid cells from peripheral lymph nodes (pLNs) were gated on the CD3$^+$ population, and analyzed to assess the development of mature CD4$^+$ and CD8$^+$ T cells. Results are representative of three independent experiments with $n = 6$ of each genotype.

cbx-3$^{+/-}$ mice compared to wt littermate control mice ($p < 0.0001$, **Figures 5A,B**). Moreover, we did not detect any differences in *Bcl6*, *Prdm1*, or *Aicda* expression between wt littermate control and mutant mice suggesting that GC and plasma cell differentiation was not affected by HP-1γ deficiency (data not shown). Thus, HP-1γ governs Ab affinity maturation perhaps by controlling the size of the T_{FH}-cell compartment during an immune response to T-dependent Ags.

THE GC REACTION DEFECT IN *CBX-3*$^{+/-}$ MICE IS NOT INTRINSIC TO B OR T_{FH} CELLS

To determine if T_{FH} cells were directly responsible for the GC phenotype observed, we generated *cbx-3*$^{+/+}$/*cbx-3*$^{+/-}$ mixed BM chimeras. Recombinase activating gene 2 and common γ chain double knock out (B6-*Rag2*$^{-/-}$*cγ*$^{-/-}$) mice were reconstituted with a 1:1 mix of either CD45.1 *cbx-3*$^{+/+}$/CD45.2 *cbx-3*$^{+/+}$ or CD45.1 *cbx-3*$^{+/+}$/CD45.2 *cbx-3*$^{+/-}$ BM. Eight weeks after reconstitution chimeric mice were immunized with NP-CGG in alum. On day 14 after immunization, mice were analyzed to determine the frequency of GC B cells, switched IgG_1^+ B cells and T_{FH} cells derived from CD45.2 (control *cbx-3*$^{+/+}$ or *cbx-3*$^{+/-}$) donor BM in each mouse (**Figure 6A**). FACS analysis showed that CD45.2 *cbx-3*$^{+/+}$ and CD45.2 *cbx-3*$^{+/-}$ chimeric mice had similar percentage of GC and IgG_1^+ B cells as well as T_{FH} cells (**Figure 6B**). Therefore, the GC defect observed in *cbx-3*$^{+/-}$ is not intrinsic to B or T_{FH} cells.

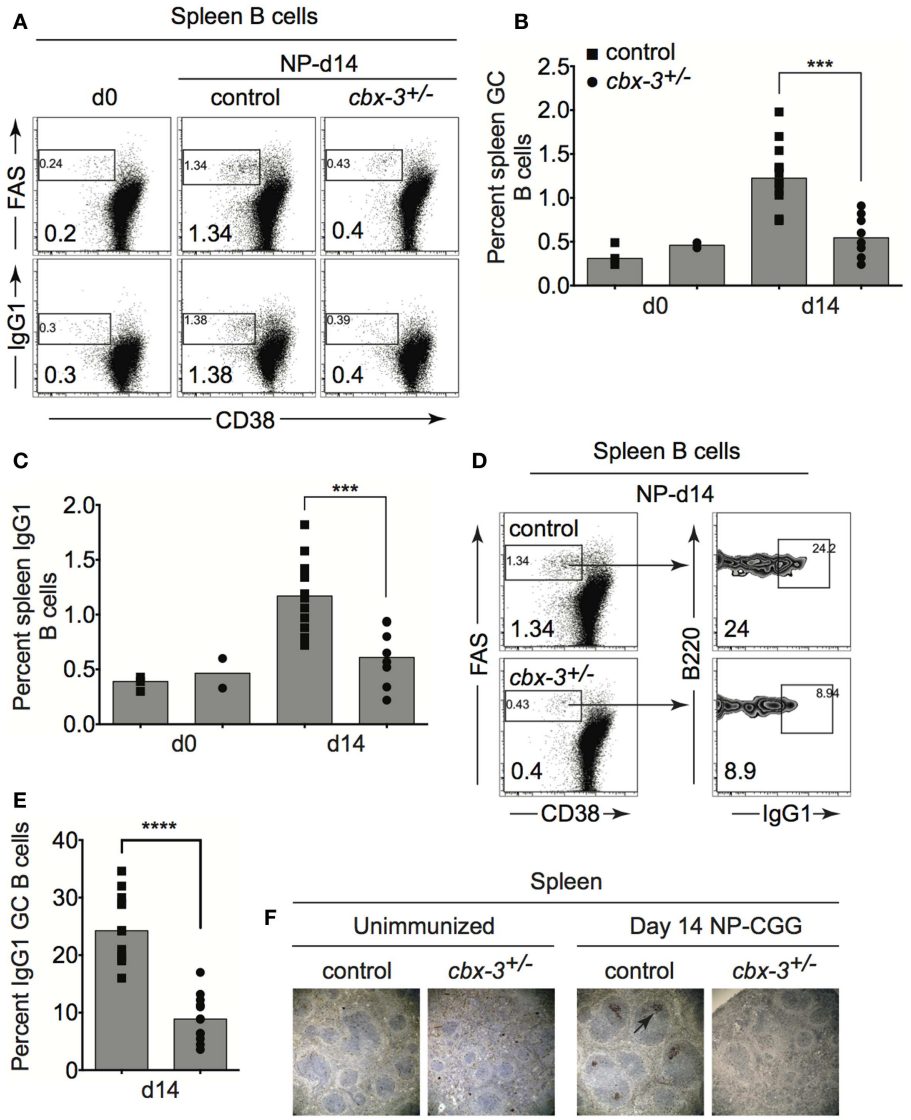

FIGURE 2 | Heterochromatin protein 1γ deficiency results in impaired germinal center reaction. **(A)** Wt littermate and *cbx-3*$^{+/-}$ mice were immunized with NP-CGG in alum. On day 14 after immunization, mice were analyzed by FACS to determine the frequency of spleen CD38$^{lo/-}$FAS^{+} GC and switched CD38$^{lo/-}$IgG$_1^{+}$ B cells from the B220^{+} gate. Numbers in left bottom corners indicate percent cells. **(B)** Plot depicts the compilation of GC B-cell frequency from experiments in **(A)**. Each symbol represents an individual mouse. Bars represent median ***$p = 0.0002$. **(C)** Plot summarizes switched IgG$_1$ B-cell frequency from experiments in **(A)**. Bars represented median ***$p = 0.0007$. Each symbol represents an individual mouse. **(D)** Frequency of IgG$_1^{+}$ GC B cells was determined from **(A)**, gated on B220^{+}CD38$^{lo/-}$FAS^{+} GC B cells. Numbers in left bottom corners indicate percent cells. **(E)** Plot summarizes the percent IgG$_1^{+}$ GC B cells from **(D)**. Bars represent median, ****$p < 0.0001$. Each symbol represents an individual mouse. **(F)** Immunohistochemistry of spleen sections from unimmunized and day 14 NP-immunized mice were stained for PNA (brown) to detect GCs (arrow). Spleens were from mice in **(A)**. Images are shown at 100× magnification. Statistical analysis was performed with GraphPad one-way ANOVA. $N = 8$–12 for each genotype.

CD122^{+}LY49^{+}CD3^{+}CD8^{+} REGULATORY T-CELL COMPARTMENT IS EXPANDED IN *CBX-3*$^{+/-}$ MICE

Recently, Kim and colleagues showed that CD122^{+}Ly49^{+}CD3^{+}CD8^{+} regulatory T (T$_{reg}$) cells served to inhibit the expansion of T$_{FH}$ population during an immune response to foreign Ags as well as to self-Ags (26, 27). Thus it is plausible that reduction in the T$_{FH}$ compartment in *cbx-3*$^{+/-}$ mice may be due to an increase in CD8^{+} T$_{reg}$ cells within the CD8^{+} T-cell compartment. On days 7 and 14 after immunization, compared to wt littermate mice, *cbx-3*$^{+/-}$ mice had 2.3- and 1.8-fold higher frequency of spleen CD8^{+} T$_{reg}$ cells, respectively ($p < 0.0001$, **Figures 7A,B**). Correspondingly, the number of spleen CD8^{+} T$_{reg}$ cells in *cbx-3*$^{+/-}$ mice increased by 1.8- and 1.75-fold on days 7 and 14, respectively ($p < 0.0001$, **Figure 7C**). Next, western blots were carried out to assess the expression status of HP-1γ in mutant CD8^{+} T cells. To our surprise, CD8^{+} and CD4^{+} T cells as well as B

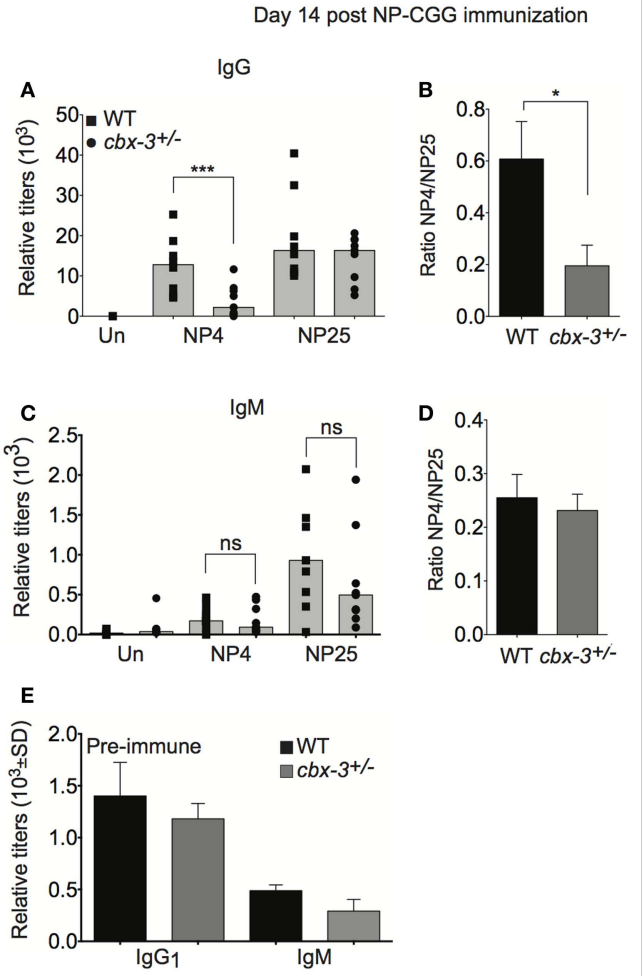

FIGURE 3 | *Cbx-3*$^{+/-}$ mice fail to mount the high-affinity NP antibody response. **(A)** ELISA analysis was carried out to measure high-affinity IgG_1 anti-NP4-BSA and total IgG_1 anti-NP25-BSA antibody activity in sera from mice immunized with NP-CGG in alum. Each symbol represents an individual mouse. Bars represent median. Statistical analysis was performed with GraphPad one-way ANOVA. Day 14, $n = 9$ for each genotype, $^{***}p = 0.006$. **(B)** High-affinity serum IgG_1 anti-NP Ab activity was determined by calculating the ratio of anti-NP4/NP25, $^{*}p = 0.02$. **(C)** Serum IgM anti-NP4 and anti-NP25 antibody activity was analyzed by ELISA to NP-BSA as in **(A)**. **(D)** Plot depicts ratio of IgM anti-NP4 over anti-NP25. **(E)** Total serum IgG_1 and IgM titers of pre-immune sera from **(A)** were measured by ELISA.

cells from *cbx-3*$^{+/-}$ mice expressed much less HP-1γ than control cells despite the presence of one wild-type allele (**Figure 7D**). To ensure that the expansion of CD8$^+$ T_{reg} cells in *cbx-3*$^{+/-}$ mice was intrinsic to the CD8$^+$ T-cell population, mixed BM chimeras were generated as described in **Figure 6A**. Mice were allowed to reconstitute for 8 weeks. On day 14 after immunization, reconstituted mice were analyzed to assess the frequency of T_{reg} cells derived from CD45.2 (control *cbx-3*$^{+/+}$ or *cbx-3*$^{+/-}$) donor BM in each mouse. As shown in **Figure 7E**, CD45.2 *cbx-3*$^{+/-}$ chimeric mice had 4.6-fold more CD8$^+$ T_{reg} cells than CD45.2 *cbx-3*$^{+/+}$ control mice. Thus, the CD8$^+$ T_{reg} population expanded in *cbx-3*$^{+/-}$ mice. These results suggest that HP-1γ limits the size of CD8$^+$ T_{reg} cells during an immune response, and the effects are intrinsic to these cells.

CBX-3$^{+/-}$ CD8$^+$ REGULATORY T CELLS DIRECTLY CONTROL T-DEPENDENT IMMUNE RESPONSE

To investigate if CD8$^+$ T_{reg} cells from *cbx-3*$^{+/-}$ mice directly controlled the Ab response, adoptive transfers into B6-*Rag2*$^{-/-}$ *cγ*$^{-/-}$ recipients were performed. Group 1 (control) recipients received B cells, CD4$^+$ and CD8$^+$ T cells from wt littermate mice; group 2 (experimental) received B cells and CD4$^+$ T cells from wt littermate mice, and CD8$^+$ T cells from *cbx-3*$^{+/-}$ mice; group 3 (control) received B cells and CD8$^+$ T cells from wt littermate mice, and CD4$^+$ T cells from *cbx-3*$^{+/-}$ mice (**Figure 8A**). On day 10 after immunization with NP-CGG in alum, recipients were analyzed to assess the status of the GC response. The percent of GC B cells from group 2 was 2.2-fold lower than groups 1 and 3 ($p = 0.04$ and $p = 0.02$, respectively, **Figure 8B**). Additionally, group 2 recipients had 1.7- and 1.9-fold less T_{FH} cells than groups 1 and 3, respectively (**Figure 8C**). By contrast, the frequency of CD8$^+$ T_{reg} cells from group 2 was three- and fourfold higher than groups 1 and 3, respectively (**Figures 8D,E**). Thus, HP-1γ-deficient CD8$^+$ T cells alone have the propensity to reduce the GC response. The results suggest that HP-1γ positively regulates GC and high-affinity Ab responses to T-dependent Ags by curtailing the ability of CD8$^+$ T cells to inhibit an immune response.

DISCUSSION

The regulation of the adaptive immune response is multilayered, requiring the participation of multiple cells and their proper functions. Here we uncover a novel function for the chromatin-remodeling factor HP-1γ in governing immunity.

Cbx-3 was cloned nearly two decades ago and yet very little is known of its physiological function in the mammalian immune system (30). Our results reveal an essential role for HP-1γ in the control of the adaptive immune response in mice. We demonstrate that HP-1γ has a positive impact on the GC reaction and high-affinity Ab response to T-dependent Ags. Mainly, *cbx-3*$^{+/-}$ mice fail to mount an effective GC reaction and high-affinity IgG_1 Ab response, whereas the low-affinity IgG_1 Ab response remains intact. The GC reaction and high-affinity response defects are accompanied by a reduction in the T_{FH} compartment. The fact that neither low-affinity IgG_1 Ab nor IgM response is affected in *cbx-3*$^{+/-}$ mice indicates that HP-1γ may not be essential for extrafollicular reaction. The presence of wild-type HP-1α and HP-1β proteins cannot override defects in GC reaction and high-affinity Ab response seen in *cbx-3*$^{+/-}$ mice suggests that HP-1γ has a non-redundant regulatory function in immune response to T-dependent Ags. The function of HP-1γ in immune response is not intrinsic to B or T_{FH} cells.

In vitro observations suggest that HP-1γ associates with the silenced κ allele thus may be involved in light chain allelic exclusion during B-cell-development (7). Our results demonstrate that light chain allelic exclusion and B-cell-development in the BM occur normally in *cbx-3*$^{+/-}$ mice. However, our data do not rule out the possibility that other HP-1 proteins, HP-1α and HP-1β, may compensate for HP-1γ deficiency during B-cell-development.

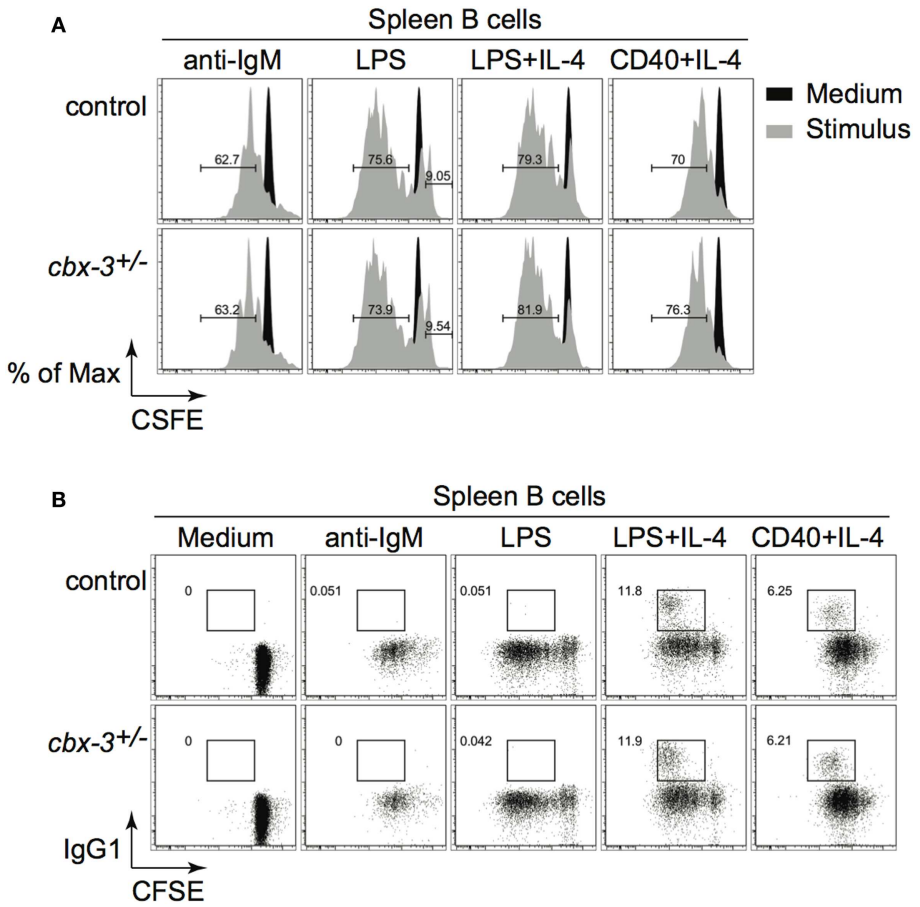

FIGURE 4 | Heterochromatin protein 1γ does not regulate B-cell proliferation or class switch recombination. **(A)** Naïve spleen B cells were labeled with CFSE and stimulated with various stimuli for 3 days. Cell division was determined using the FlowJo Proliferation Platform software. **(B)** IgG_1 switching was determined from the same cultures as in **(A)**. Results are representative of three independent experiments with six mice per genotype. Analysis was performed on cells derived from the live gate.

Recent studies have shown that a subpopulation of effector $CD8^+$ T cells, known as $CD8^+$ T_{reg} cells, control GC reaction and high-affinity Ab response to foreign T-dependent Ags as well as self-Ags by limiting the size of the T_{FH} compartment (27). However, mechanisms that regulate the development or homeostasis of these cells remain elusive. Here, we reveal a novel molecular pathway that controls $CD8^+$ T_{reg} cells in mice after immunization. We show that, through its non-redundant function, HP-1γ limits the size of the $CD8^+$ T_{reg} population thus allowing the immune response to foreign T-dependent Ags to proceed. In mice, HP-1γ deficiency results in the expansion of these cells and reduction of T_{FH} population, which leads to the abrogation of GC reaction and high-affinity Ab response. The level of HP-1γ present in mutant cells is much less than expected despite the presence of one wild-type allele, implying that HP-1γ may also regulate its own expression. HP-1γ deficiency only affects $CD8^+$ T-cell function despite the fact that mutant $CD4^+$ and B cells also express low amounts of HP-1γ, suggesting that in these cells HP-1γ may regulate the expression of genes that are not essential to NP-response.

It remains to be determined how HP-1γ controls the development/homeostasis of $CD8^+$ T_{reg} cells, and if HP-1γ deficiency would alleviate autoimmunity. We speculate that HP-1γ may control the expression and/or function of a transcription factor(s), which governs $CD8^+$ T_{reg} development/homeostasis. HP-1γ does so perhaps by maintaining a chromatin conformation that is unfavorable to the expression and/or function of this putative transcription factor(s). Future genome wide experiments will allow us to map the changing epigenomic landscape in HP-1γ sufficient and deficient $CD8^+$ T cells. These ongoing studies will expand our understanding of mechanisms by which HP-1γ, through its ability to remodel the chromatin, regulates immunity.

In summary, this study shows for the first time that in mice the non-redundant regulatory function of HP-1γ governs GC and high-affinity Ab responses by limiting the pool of $CD8^+$ T_{reg} cells.

MATERIALS AND METHODS

MICE

cbx-3 mutant mice were generated, as described in Ref. (10, 28). Mice were backcrossed to C57BL/6 for 12 generations.

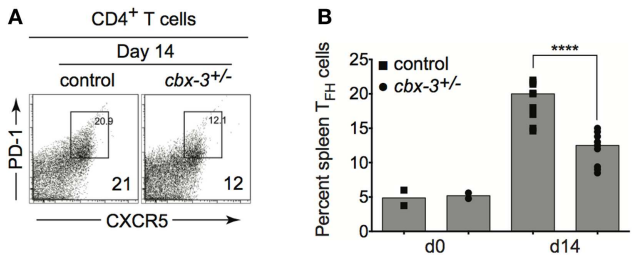

FIGURE 5 | The T follicular helper cell copulation is reduced in *cbx-3*$^{+/-}$ mice. **(A)** The frequency of spleen TCRβ$^+$CD4$^+$CXCR5^{hi}PD-1^{hi} T_{FH} population was determined by FACS analysis. Numbers in right bottom corners indicate the percent cells. CXCR5^{hi}PD-1^{hi} T_{FH} cells were gated on the TCRβ$^+$CD4$^+$ population. **(B)** Plot represents a compilation of the percent of T_{FH} population from **(A)**. Each symbol denotes an individual mouse. Bars represent median. Statistical analysis was performed with GraphPad one-way ANOVA. Day 14, $n = 8$–12 for each genotype, $^{****}p < 0.0001$.

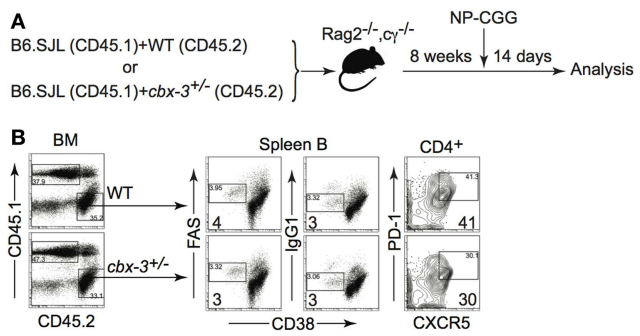

FIGURE 6 | The GC reaction defect in *Cbx-3*$^{+/-}$ mice is not intrinsic to B or T_{FH} cells. **(A)** *cbx-3*$^{+/+}$/*cbx-3*$^{+/-}$ mixed bone marrow chimeras were generated in B6-*Rag2*$^{-/-}$*cγ*$^{-/-}$ recipients at a 1:1 ratio of either CD45.1 *cbx-3*$^{+/+}$/CD45.2 *cbx-3*$^{+/+}$ or CD45.1 *cbx-3*$^{+/+}$/CD45.2 *cbx-3*$^{+/-}$ bone marrow. Eight weeks after reconstitution chimeric mice were immunized with NP-CGG in alum. **(B)** On day 14, spleens were analyzed to determine the frequency of GC B cells, switched IgG_1^+ B cells and T_{FH} cells derived from CD45.2 (control *cbx-3*$^{+/+}$ or *cbx-3*$^{+/-}$) donor bone marrow in each mouse. Numbers in bottom left corners of FACS plots indicate percent GC B cells. Numbers in bottom right corners denote percent T_{FH} cells. CD45.1 and CD45.2 populations were derived from the lymphoid gate. GC and IgG_1^+ B cells were derived from the B220$^+$ gate. CXCR5^{hi}PD-1^{hi} T_{FH} cells were gated on the TCRβ$^+$CD4$^+$ population.

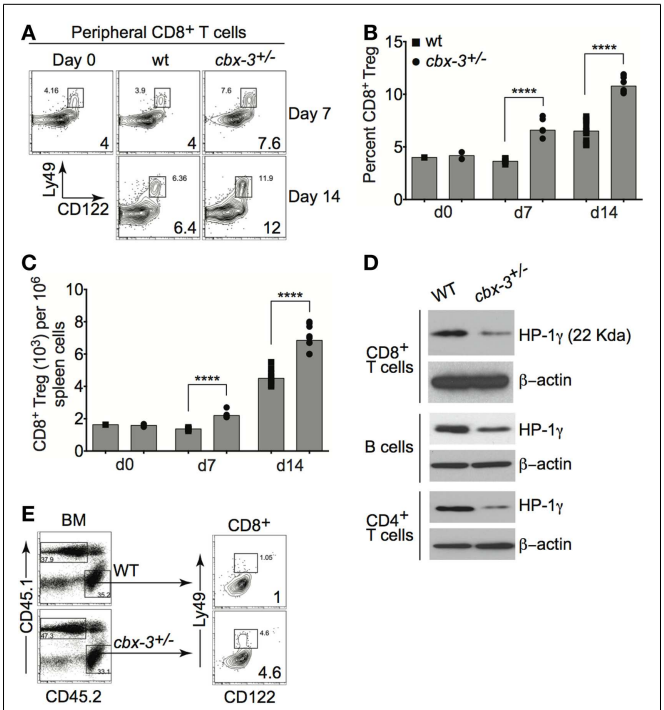

FIGURE 7 | CD122$^+$Ly49$^+$CD3$^+$CD8$^+$ regulatory T-cell compartment is expanded in *cbx-3*$^{+/-}$ mice. **(A)** On days 7 and 14 after immunization, percent of spleen CD122$^+$Ly49$^+$CD3$^+$CD8$^+$ regulatory T-cell (T_{reg}) population from wt littermate and *cbx-3*$^{+/-}$ mice was assessed by FACS. Numbers in lower right corners indicate percent cells. CD122$^+$Ly49$^+$CD8$^+$ T_{reg} cells were gated on the CD3$^+$ population. **(B,C)** Plots represent a compilation of the percent and number of CD8$^+$ T_{reg} population from **(A)**. Each symbol denotes an individual mouse. Bars represent median. Statistical analysis was performed with GraphPad one-way ANOVA. Day 7, $n = 5$ for each genotype; $^{****}p < 0.0001$. Day 14, $n = 8$–12 for each genotype; $^{****}p < 0.0001$. **(D)** Purified CD8$^+$CD44$^-$, CD4$^+$CD25$^-$, and CD43$^-$ B cells were collected from spleen and peripheral lymph nodes. Blots were probed with anti-total HP-1γ (22 kDa) and anti-β-actin (42 kDa). Results are representative of two independent experiments; $n = 4$ mice of each genotype. **(E)** *cbx-3*$^{+/+}$/*cbx-3*$^{+/-}$ mixed bone marrow chimeras were generated as in **Figure 6A**. Percent CD8$^+$ T_{reg} cells were determined by FACS. Numbers in lower right corners indicate percent cells. CD45.1 and CD45.2 populations were derived from the lymphoid gate. CD122$^+$Ly49$^+$CD8$^+$ T_{reg} cells were gated on the CD3$^+$ population.

B6-*Rag2*$^{-/-}$ *Cγ*$^{-/-}$ and B6.SJL mice were purchased from Taconic. All mice were maintained in specific pathogen-free conditions. All mouse protocols were approved by the BIDMC Institutional Animal Care and Use Committee.

FLUORESCENCE-ACTIVATED CELL SORTING

Fluorescence-activated cell sorting was performed on the BD 5-laser LSR II. Analysis was carried with FlowJo software (Tree Star, Inc.). All fluorochrome-conjugated antibodies were purchased from Biolegend or BD Biosciences. The following antibodies were used: ckit-APC (1:200); CD25-PE (1:200); IgM-FITC (1:500); CD8-Pacific blue (1:200); CD8-APC-Cy7 (1:300); CD8-PE-Cy7 (1:200); Ly-49-FITC (1:100); CD44-Pacific blue (1:200); IgD-PE (1:500); CD21-APC (1:200); CD23-PE (1:150); CD19-PE-Cy7 (1:300); B220-Pacific blue (1:300); CD38-APC (1:200); IgG1-FITC (1:50); FAS-PE (1:200); CD4-FITC (1:200); CD4-PE (1:150); TCRβ-Brilliant-Violet 412 (1:200); PD1-PE-Cy7 (1:100); CXCR5-Biotin (1:100); SA-PerCP (1:100); CD45.1-FITC (1:150); CD45.2-PE-Cy7 (1:100); CD45.2-Pacific blue (1:200); CD3-APC (1:200); CD122-Pacific blue (1:200).

T-DEPENDENT IMMUNE RESPONSE

Adult mice (7–8-week-old) were immunized with 50 μg of the T-dependent Ag 4-hydroxy-3-nitrophenylacetyl hapten conjugated to chicken gamma globulin (NP-CGG, BioSearch Technologies) per mouse in alum (Thermo Scientific) (ratio 1:1). Immune sera

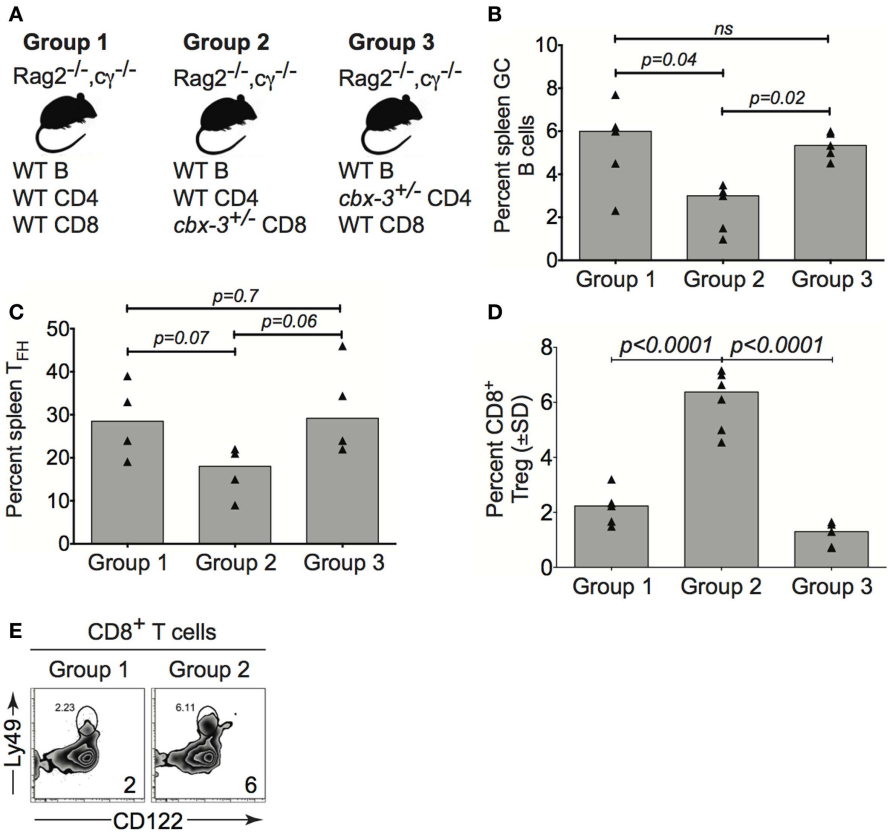

FIGURE 8 | *Cbx-3*$^{+/-}$ CD8$^+$ T cells directly control the T-dependent immune response. **(A)** Purified wt or *cbx-3*$^{+/-}$ spleen and pLN lymphocytes were adoptively transferred into B6-*Rag2*$^{-/-}$ *cγ*$^{-/-}$ recipients: group 1 (control, $n = 5$) received wt B cells, CD4$^+$ and CD8$^+$ T cells; group 2 (experimental, $n = 5$) received B cells and CD4$^+$ T cells from wt littermate mice and CD8$^+$ T cells from *cbx-3*$^{+/-}$ mice; group 3 (control, $n = 5$) received B cells and CD8$^+$ T cells from wt littermate mice and CD4$^+$ T cells from *cbx-3*$^{+/-}$ mice. **(B,C)** On day 10 after NP-CGG in alum immunization, spleens of recipients were analyzed by FACS to determine the frequency of GC (from the B220$^+$gate) and T_{FH} (from the TCRβ$^+$CD4$^+$ gate) populations. **(D,E)** The frequency of CD122$^+$Ly49$^+$CD8$^+$ T_{reg} cells (from the CD3$^+$ gate) was determined by FACS **(E)** and plotted **(D)**. Numbers on bottom right corners indicate percent cells. Statistical analysis was performed with GraphPad one-way ANOVA; ns, not significant.

were obtained at days 7 and 14 after immunization. FACS analysis was performed on the same days.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed using 4 μm thick formalin-fixed, paraffin-embedded tissue sections. Briefly, slides were soaked in xylene, passed through graded alcohols, and put in distilled water. Slides were then pre-treated with 1.0-mM EDTA, pH 8.0, or 1.0 mM citrate (Zymed) in a steam pressure cooker (Decloaking Chamber, BioCare Medical) as per manufacturer's instructions, followed by washing in distilled water. All subsequent steps were performed at room temperature in a hydrated chamber. Slides were pre-treated with Peroxidase Block (DAKO) for 5 min to quench endogenous peroxidase activity, followed by Serum free Protein Block (DAKO) for 20 min. Biotinylated PNA (Vector Laboratories) was applied for 1 h (all diluted in DAKO diluents). Slides were washed in 50 mM Tris–Cl, pH 7.4. Slides were washed again, and detected with anti-streptavidin–HRP Envision + kit (DAKO) as per manufacturer's instructions. After further washing, immunoperoxidase staining was developed using a DAB chromogen (DAKO) and counterstained with hematoxylin. Images were acquired with the Nikon Eclipse E600 and SPOT Insight four camera and software.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Antibody response to NP was determined by ELISA using NP(4)-BSA or NP(25)-BSA (BioSearch Technologies) from days 7 and 14 immune sera. ELISA was performed as described (11).

IN VITRO B-CELL ACTIVATION AND ISOTYPE SWITCH ASSAY

MACS-purified (Miltenyi Biotec) CD43$^-$ or CD19$^+$ B cells were activated *in vitro* at a density of 1–3 × 10^6 cells/ml with 2 μg/ml of anti-CD40 clone HM40-3 (eBiosciences) plus 25 ng/ml of recombinant mouse IL-4 (R&D Systems), 10 μg/ml of goat F(ab′)$_2$ anti-mouse IgM (Jackson Immunoresearch), LPS (20 μg/ml), or LPS + IL-4 (Sigma).

PROLIFERATION ASSAYS AND ANALYSIS

MACS-purified CD43$^-$ B cells labeled with CFSE were activated with indicated stimuli as above for 3 or 4 days. Data were analyzed

using the proliferation platform of the FlowJo software (Tree Star Inc.).

GENERATION OF BONE MARROW CHIMERAS

Bone marrow cells from femurs and tibias of 4-week-old B6.SJL mice (CD45.1) were mixed with either 4-week-old *cbx3*$^{+/-}$ or littermate control mice (CD45.2) at a 1:1 ratio. Mixed BM cells (2×10^6) were injected i.v. into 7-week-old *Rag2*$^{-/-}$*C*γ$^{-/-}$ recipients. Eight weeks after BM reconstitution, recipients were immunized with NP-CGG in alum (ratio 1:1). Analysis was carried out 14 days after immunization.

WESTERN BLOTS

Purified CD8$^+$CD44$^-$ (1×10^6) cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Boston BioProducts) containing protease inhibitor cocktail (Roche) on ice for 30 min. Cells were centrifuged at 14,000 rpm for 15 min at 4°C. Protein concentration was determined by Bio-Rad Protein Assay Kit (Bio-Rad). Ten micrograms of protein extracts were denatured at 95°C for 10 min, separated by SDS-PAGE, and transferred onto PVDF membranes (EMD Millipore). Membranes were probed with antibodies against HP-1γ (Cell Signaling Technology) or β-actin (Sigma Aldrich). Proteins of interest were detected with HRP-conjugated secondary antibodies and visualized with the Pierce ECL Western blotting substrate (Thermo Scientific).

ADOPTIVE TRANSFER

B, CD8$^+$, and CD4$^+$ cells were prepared from spleen and lymph nodes of 7-week-old *cbx3*$^{+/-}$ and wt littermate mice as described (18). 2×10^6 wild-type B cells, 1×10^6 wild-type or mutant CD8$^+$, and 1×10^6 wild-type or mutant CD4$^+$ cells were injected i.v. into 7-week-old *Rag*$^{-/-}$*C*γ$^{-/-}$ recipients. The following day, recipients were immunized with NP-CGG in alum (ratio 1:1). Analysis was carried out 10 days after immunization.

STATISTICAL AND GRAPH ANALYSIS

P values were calculated using one-way ANOVA and graphs were plotted with Prism 6 (GraphPad Software).

AUTHOR CONTRIBUTIONS

Ngoc Ha, Duc-Hung Pham, and Aliakbar Shahsafaei carried out all experiments; Chie Naruse and Masahide Asano generated the *cbx-3* mutant mice and provided advice on their use; To-Ha Thai conceived and directed all research, and along with Ngoc Ha and Duc-Hung Pham prepared the manuscript.

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REVIEW
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Regulation of B cell differentiation by intracellular membrane-associated proteins and microRNAs: role in the antibody response

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B cells are central to adaptive immunity and their functions in antibody responses are exquisitely regulated. As suggested by recent findings, B cell differentiation is mediated by intracellular membrane structures (including endosomes, lysosomes, and autophagosomes) and protein factors specifically associated with these membranes, including Rab7, Atg5, and Atg7. These factors participate in vesicle formation/trafficking, signal transduction and induction of gene expression to promote antigen presentation, class switch DNA recombination (CSR)/somatic hypermutation (SHM), and generation/maintenance of plasma cells and memory B cells. Their expression is induced in B cells activated to differentiate and further fine-tuned by immune-modulating microRNAs, which coordinates CSR/SHM, plasma cell differentiation, and memory B cell differentiation. These short non-coding RNAs would individually target multiple factors associated with the same intracellular membrane compartments and collaboratively target a single factor in addition to regulating AID and Blimp-1. These, together with regulation of microRNA biogenesis and activities by endosomes and autophagosomes, show that intracellular membranes and microRNAs, two broadly relevant cell constituents, play important roles in balancing gene expression to specify B cell differentiation processes for optimal antibody responses.

Keywords: B cell activation and differentiation, plasma cell, memory B cell, intracellular membrane associated proteins, endosome, lysosome, autophagosome, microRNA

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Abbreviations: Ago, argonaute; AID, activation-induced cytidine deaminase; Atg, autophagy-related gene; BCR, B cell receptor; Blimp-1, B lymphocyte-induced maturation protein-1; CSR, class switch DNA recombination; ER, endoplasmic reticulum; GAP, GTPase activating protein; HDI, histone deacetylase inhibitors; Ig, immunoglobulin; KO, knockout; MHC II, major histocompatibility complex II; PI-3K, class III phosphatidylinositol 3-kinase; RA, rheumatoid arthritis; Rab, Ras-related in brain; RISC, RNA-induced silencing complex; SHM, somatic hypermutation; TCR, T cell receptor; TLR, toll-like receptor; VPS, vacuolar protein sorting.

INTRODUCTION

B lymphocytes are critical to immunity by mediating production of neutralization antibodies to infectious pathogens and tumor cells (1). They develop through several highly regulated steps, first as pro-B cells and then pre-B cells in the bone marrow, in which immunoglobulin (Ig) V(D)J DNA recombination occurs, and subsequently as immature B cell stages and then mature B cells in the periphery. V(D)J recombination gives rise to a highly diverse repertoire of the B cell receptor (BCR) for antigens. Unlike immature B cells, which express predominantly IgM-containing BCRs, mature naïve B cells express high levels of IgD on their surface. In secondary lymphoid organs (e.g., spleen and lymph nodes), B cells are organized into the follicular or marginal zone areas (2), in part due to their BCR signaling differences.

Upon antigen encounter, B cells are activated in a T cell-independent or T cell-dependent manner. In T-independent antibody responses and the early T-independent phase of T-dependent antibody responses, B cells differentiate upon dual engagement of BCR (e.g., by repetitive antigenic ligands) and an innate immune receptor, such as a toll-like receptor (TLR) (3). In T-dependent antibody responses, specific B cells are induced through cognate interaction, and engagement of CD40, which is constitutively expressed on B cells, by trimeric CD154 expressed by specific T helper (T_H) cells (4). Activated B cells differentiate in germinal centers, the newly formed specialized microenvironment within secondary lymphoid organs (5). They undergo class switch DNA recombination (CSR) in the Ig heavy chain (IgH) locus to switch their BCR from IgM or IgD to, depending on eliciting stimuli, IgG, IgE, or IgA, which endows an antibody with different biological effector functions without changing antigen specificity (4). B cells also undergo somatic hypermutation (SHM). This inserts point-mutations into Ig V region DNA, thereby providing the substrate for positive selection of antibody mutants with higher affinity to the antigen (6). Finally, B cells differentiate into plasma cells, which secrete large amounts of antibodies, or memory B cells, which can be re-activated for amnestic antibody responses upon second challenge by the same antigen (7–10). Memory B cells and plasma cells are long-lived and play an important role in the protection against re-exposure to microbial and other antigens.

B cell CSR/SHM and differentiation into plasma cells and memory B cells are tightly regulated. Dysregulation of these processes can lead to immune deficiencies, autoimmunity, or B lymphomagenesis. In this review, we will focus on how B cell differentiation is regulated for effective antibody responses by intracellular membranes, particularly the emerging functions of endosomes and autophagosomes. We will also emphasize the induction of proteins associated with these intracellular membranes and the role of these proteins in signaling and induction of gene expression. Furthermore, we will summarize the evidence of the epigenetic modulation of expression of these proteins by microRNAs and the consequence to B cell differentiation – readers are referred to other articles that have extensively reviewed the regulation/dysregulation of immune functions by microRNAs (11–15). Finally, we will discuss the notion that microRNA activities are reciprocally regulated by intracellular membranes, as part of the controlling mechanisms that fine-tune gene expression and buffer molecular aberrancies to ensure the specificity of B cell differentiation and functions.

B CELL REGULATION BY INTRACELLULAR MEMBRANE-ASSOCIATED PROTEINS

Intracellular Membrane Structures and Associated Proteins

Endosomes are internalized lipid vesicles containing extracellular molecules and cell surface components, including fluid, solutes and their carriers, lipids, membrane proteins, and receptor–ligand complexes (16). They regulate various cellular processes by sorting, processing, recycling, storing, and degrading these cargos. Early endosomes, which have tubular and vacuolar domains, are the main sorting station of internalized cargos, most of which are recycled back to the plasma membrane directly or through recycling endosomes (**Figure 1**). Late endosomes form from the vacuolar domains of early endosomes, with many changes in the lipid and protein contents. They mature upon moving from the peripheral cytoplasm to the perinuclear area, where mature late endosomes fuse with each other and eventually fuse with lysosomes or pre-existing hybrid organelle endolysosomes (**Figure 1**). Endosome maturation is regulated by a group of Rab (Ras-related in brain) small GTPases (17). Rab5 is a defining component of early endosomes through various stages, and regulates the conversion of early endosomes to late endosomes (17). The GTP-bound form of Rab5 (Rab5-GTP) recruits Rab7 and is then converted to Rab5-GDP, which dissociates from the membrane (18, 19). Rab7, upon recruitment, can auto-activate to bind GTP and suppress Rab5 binding to GTP (20). Rab7-GTP recruits its own effectors, including RILP, which connects late endosomes to dynein motors for trafficking, and components of the HOPS complex, which promotes late endosome fusion with lysosomes (21, 22). Lysosomes are the terminal degradative compartments. These have a luminal pH of 4.6–5.0 and contain hydrolases that digest the cargo (17). They can also fuse with autophagosomes to form autolysosomes, which carries out degradation of extracellular and intracellular components.

Macroautophagy (referred here as autophagy) is a process that degrades cytoplasmic compartments and organelles and is underpinned by the formation of autophagosomes, which are enclosed double-membrane structures (23). Autophagy was first characterized as a cellular process to counter stress, e.g., by mediating degradation of cytoplasmic organelles/proteins in starved cells for the generation of energy and macromolecules to maintain cell viability (24). Autophagosomes, however, can be induced under physiological stress-free conditions, likely through slightly different pathways, and play important roles in immunity (25, 26). Their induction starts from activation of a protein complex containing ULK1 and the autophagy-related gene (Atg) 13, and formation of a phagophore, a double-membrane structure (**Figure 1**). The nucleation of phagophores requires activation of the class III phosphatidylinositol 3-kinase (PI-3K) complex consisting of the VPS34 (vacuolar protein sorting 34), which is activated by a complex containing Beclin 1, VPS15, and Atg14

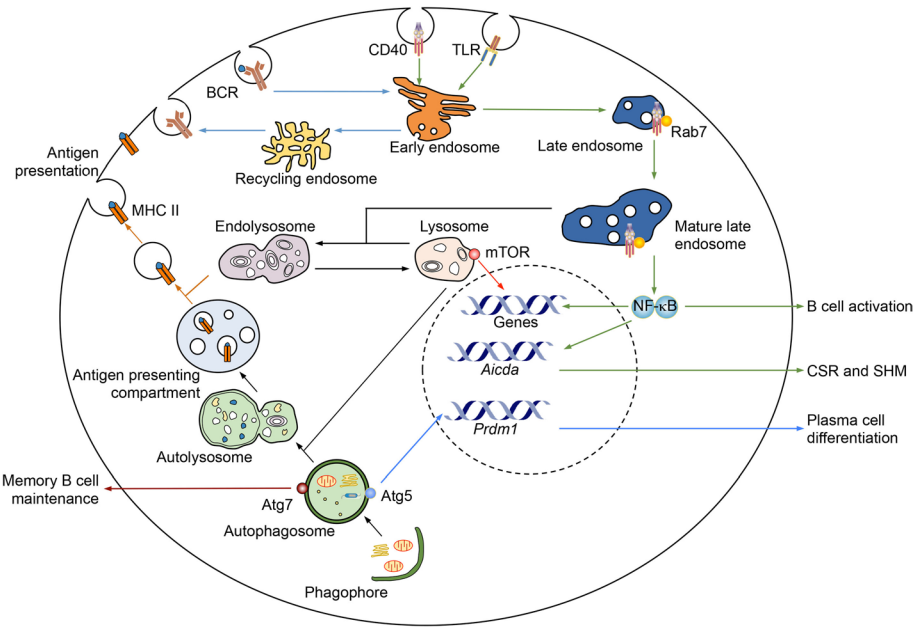


FIGURE 1 | Regulation of B cell differentiation and functions by intracellular membranes and associated proteins. Several B cell processes are regulated by endosomes, lysosomes, and autophagosomes. Upon antigen-triggered internalization, BCR is sorted by early endosomes to either recycle back onto the cell surface or go through antigen processing, which are mediated by endolysosomes, for the MHC II-dependent antigen presentation. Signaling receptors, such as CD40 (as depicted) and TLRs (both surface TLRs and intracellular TLRs), can be internalized and/or sorted by early endosomes to localize to mature late endosomes, as marked by Rab7. A Rab7-dependent process would stabilize the interaction of such receptors and their adaptors (e.g., CD40 and TRAF6), thereby promoting sustained signaling, such as NF-κB activation, for induction of genes important for B cell activation and CSR/SHM, e.g., AID. Lysosomes, as transformed from and maintained by mature late endosomes, can recruit mTOR, which plays an important role in B cell activation. Autophagosomes are important for memory B cell maintenance, i.e., through Atg7, and plasma cell differentiation and survival, e.g., through Atg5. They can also fuse with lysosomes to become autolysosomes, which then transform into a specialized compartment for antigen presentation.

(24). Phosphorylation of ULK1 and Atg13 by the mTOR kinase, which regulates B cell differentiation and CSR (27, 28), inhibits autophagy activation. Inhibition of mTOR, e.g., by rapamycin, promotes autophagosome formation (29). Autophagosomes form after the expansion and final closure of phagophores, and then fuse with either mature endosomes to form amphisomes or lysosomes to form autolysosomes, in which luminal cargos and the inner membrane are degraded (**Figure 1**) – amphisomes can also fuse with lysosomes to form autolysosomes.

Antigen Presentation

In T-dependent antibody responses, B cells uptake antigens through the BCR, process it, and present it through the major histocompatibility complex II (MHCII) to cognate $CD4^+$ T_H cell TCR to prime T_H cells. These, in turn, will prime B cells for full activation, proliferation, and differentiation. It has been long known that antigen processing for MHC II-dependent presentation is mediated by endosomes/lysosomes (30), while MHC I-dependent antigen presentation is mediated by endoplasmic reticulum (ER). Autophagy also plays an important role in MHC II-dependent antigen presentation in B cells and dendritic cells, in which autophagosomes continuously merge with multi-vesicular compartments loaded with MHC II (**Figure 1**). Targeting of the influenza virus matrix protein-1 to autophagosomes by fusion of this protein with LC3, a marker of growing autophagosomes, strongly enhances the presentation of this protein to $CD4^+$ T cells (31). Mice with conditional knockout (KO) of autophagosome-associated protein Atg5 in dendritic cells fail to mount sufficient $CD4^+$ T cell priming after herpes simplex virus infection (32). Conditional KO mice lacking Atg5 in B cells have not been analyzed in this context, but would likely display a similar phenotype.

Protein citrullination is highly relevant to rheumatoid arthritis (RA), as RA patients display high levels of autoantibodies to citrullinated self-proteins (33). Citrullinated peptides, but not unmodified peptides, are presented to T cells by dendritic cells and macrophages through autophagy. In B cells, citrullinated peptides are also presented in a manner dependent on BCR engagement (e.g., triggered by anti-IgM) and autophagosome formation (which can be blocked by 3-methyladenine, an inhibitor of PI-3K and autophagy). Upon brief serum starvation, B lymphoma cells can also present citrullinated peptides through an Atg5- and autophagosome-dependent pathway (34).

B Cell Activation and CSR/SHM

Intracellular membrane-associated proteins regulate activation and differentiation of B cells, in addition to their development and survival – pre-B cell development in the bone marrow and B-1 cell survival in the periphery are defective in mice with B cell conditional KO (*Cd19-cre*) of *Atg5* (35). Upon BCR engagement by antigen, the fast and extensive induction of autophagosomes,

in which the antigen would be rapidly processed, can cause cell death (36). Autophagy-dependent cell death can be overcome by CD40 engagement for full B cell activation (36), suggesting a role of autophagy in controlling self-reactive B cell activation and autoimmunity.

In B cells activated by CD40 engagement or dual TLR/BCR engagement (37), Rab7 is upregulated, suggesting a role of Rab7 and Rab7$^+$ late endosomes in peripheral antigen-dependent B cell differentiation. In conditional KO *Igh*$^{+/C}\gamma^{1\text{-}cre}$*Rab7*$^{fl/fl}$ mice, *Rab7* is ablated only in B cells undergoing *Igh*$^{C}\gamma^{1\text{-}cre}$ Iγ1-Sγ1-Cγ1-*cre* transcription, as induced – like *Igh* germline Iγ1-Sγ1-Cγ1 transcription – by IL-4 in conjunction with CD40 or dual TLR/BCR engagement (38). These mice are normal in B and T cell development, but cannot mount T-independent or T-dependent class-switched IgG1 responses, while maintaining normal IgM levels. *Igh*$^{+/C}\gamma^{1\text{-}cre}$*Rab7*$^{fl/fl}$ B cells are normal in proliferation, survival, and plasma cell differentiation, as well as activation of the p38 kinase and ERK1/2 kinase pathways, but show defective CSR (38). This defect can be rescued by enforced expression of activation-induced cytidine deaminase (AID), which is essential for CSR and SHM. In addition, inhibition of Rab7 activity by a small molecule compound, CID 1067700 (39), reduces CSR and antibody responses in normal B cells/mice as well as autoantibody response and disease symptoms in lupus-prone MRL/*Fas*$^{lpr/lpr}$ mice (40). These findings, together with our demonstration that Rab7 mediates canonical NF-κB activation, as critical to AID induction, outline a novel role of Rab7 in signaling pathways that lead to AID expression and CSR, likely by promoting assembly of signaling complexes along mature endosomes. Such a role of Rab7 is consistent with the proximity of Rab7-containing mature late endosomes to the nucleus (**Figure 1**), as activated NF-κB would have a short path to reach genes, an advantage shared by ER membrane-mediated NF-κB activation, as occurring in BL41 B cells upon CD40 engagement and in Jurkat T cells upon TCR engagement (41). It would also be irrespective of the initial location of engaged immune receptors, e.g., on the plasma membrane (such as CD40, TLR1/2, and TLR4) or in endosomes (TLR7 and TLR9), and their trafficking pattern.

Lysosomes have been recently implicated to regulate signal transduction, serving as the "docking station" for mTOR in various cancer cells (42–44) and likely in B cells. Knock-in mice expressing a hypomorphic mTOR mutation and conditional KO mice with B cells mTOR deficiency are defective in germinal center formation and antibody responses, in concomitant with reduced AID expression and CSR (28). Pharmacological inhibition of mTOR kinase activity results in complicated phenotypes, likely due to non-redundant functions of two mTOR-containing complexes, mTORC1 and mTOC2 (28, 45).

Plasma Cell Differentiation

The differentiation of activated B cells into antibody-secreting plasma cells is associated with changes in gene expression that lead to the loss of the B cell identity and the gain of protein secretion functions. Such changes are mediated by the master transcription factor Blimp-1 (B lymphocyte-induced maturation protein-1), as encoded by *Prdm1* (8). In plasma cells, ER membranes expand and enhance their capacity to fold nascent peptides, a process driven by X-box binding protein-1, to promote antibody secretion (46). Both differentiating plasmablasts (which still proliferate) and terminally differentiated long-lived plasma cells have high autophagic activities. In two independently generated mouse strains with conditional *Atg5* KO in B cells (both through *Cd19-cre*), antibody responses and generation of antigen-specific long-lived plasma cells are defective (47, 48), likely due to impairment in generation of plasma cells (47), plasma cell survival (48), or both – the ER-related secretion function of plasma cells, however, does not seem to be affected (48). In addition, autophagosomes have been suggested to serve as a platform in recruiting ERK and its activation (49), which plays an important role in Blimp-1 induction and plasma cell differentiation (50). Rab7 does not play a major role in the generation of plasma cells, but may help in maintaining plasma cell survival, perhaps also through NF-κB-dependent sustained expression of Blimp-1 (40). Thus, intracellular-associated proteins are important for plasma cell homeostasis and sustainable antibody production.

Memory B Cell Differentiation

As recently shown, memory B cells specifically express high levels of autophagic activities and do so over time, i.e., low/no levels of autophagosome formation (LC3$^+$ puncta) in naïve B cells, germinal center B cells, and newly formed memory B cells, but high levels in memory B cells isolated much later after the primary immunization (51). Atgs, such as *Ulk1*, *Atg14*, *Becn1* (encoding Beclin 1), *Atg5*, *Atg7*, and *LC3*, display similar expression patterns. Furthermore, Atg7 is dispensable for the initial generation of memory B cells, but critical for the long-term survival of memory B cells, likely by preventing apoptosis, as mice with B cell conditional KO of Atg7 (through *Cd19-cre*) can mount normal primary antibody responses, but much reduced secondary antibody responses and SHM upon re-challenging by antigen (51, 52). Finally, memory B cells express high levels of a forkhead family transcription factor FoxO3 and, to a lesser extent, FoxO1, consistent with the important role of these two factors in autophagy gene expression (53). How Atg7 and related intracellular membranes regulate apoptosis and perhaps other metabolic processes for the maintenance of memory B cells remains to be determined. Overall, intracellular membranes play an important role in peripheral antigen-dependent B cell differentiation. And, different membrane structures, likely through their associated proteins, specify different differentiation processes, e.g., Rab7 in CSR/SHM, Atg5 in plasma cell differentiation, and Atg7 in memory B cell differentiation (**Figure 1**).

REGULATION OF B CELL DIFFERENTIATION BY INTRACELLULAR MEMBRANE-ASSOCIATED PROTEINS AND MicroRNAs

MicroRNAs Regulate Peripheral Antigen-Dependent B Cell Differentiation

MicroRNAs are endogenous small non-coding RNAs that regulate adaptive and innate immunity, including B cell differentiation (54, 55). They modulate gene expression through

mainly post-transcriptional mechanisms, such as inhibiting translation of mRNAs and/or promoting their degradation after pairing with the 3′-untranslated region (3′-UTR) of the target mRNA (**Box 1**). A single microRNA can regulate multiple gene targets and, conversely, a single gene can be regulated by multiple microRNAs. Conditional KO of *Dicer*, which is essential for microRNA biogenesis, through *Cd19-cre* leads to impairment in the generation of follicular B cells with concomitant increase in marginal zone B cells (56). This, together with the higher Dicer expression in follicular B cells, supports the notion that selected microRNAs mediate this stage of B cell development. High titers of autoantibodies in female *Dicer* conditional KO mice suggest a role of microRNAs in preventing autoimmune diseases (56).

MicroRNAs regulate the germinal center reaction. As *Aicda* gene expression is restricted to B cells activated by T-independent or T-dependent stimuli, *Aicda-cre* mice have been widely used to generate activated B cell-specific KO of genes. Conditional KO of *Dicer* through *Aicda-cre* dampens germinal center formation, likely through elevated expression of Bim, a pro-apoptotic protein (57). *Dicer* KO B cells are also defective in proliferation and survival, leading to impairment in production of high-affinity class-switched antibodies and generation of plasma cells or memory B cells. The relevant microRNAs missing in these KO B cells would include miR-155, as this microRNA is highly expressed in germinal center B cells and miR-155 KO leads to defective germinal centers (58, 59).

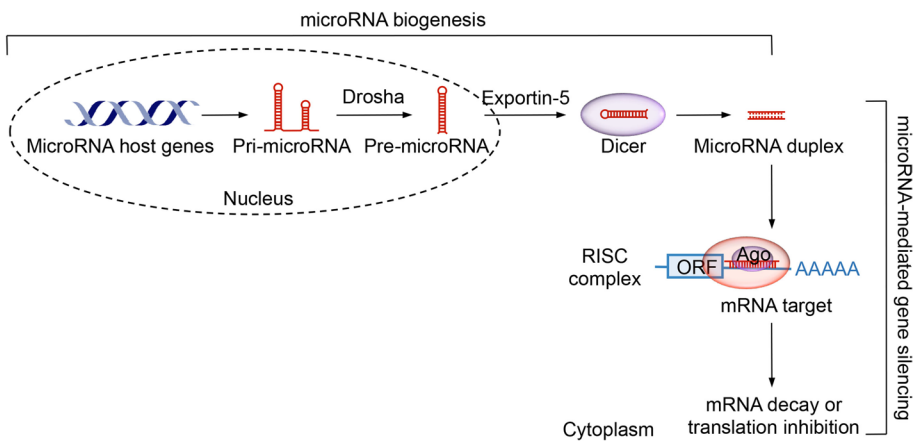
MicroRNAs play vital roles in CSR and SHM. MiR-155 targets the *Aicda* 3′-UTR, as knock-in or transgenic mice with the miR-155-binding site mutated in *Aicda* 3′-UTR show higher level of AID expression with upregulation of CSR and SHM (60). MiR-181b can also target *Aicda* 3′-UTR to regulate AID expression and CSR. Accordingly, miR-181b expression is downregulated in activated B cells, thereby allowing accumulation of AID (61). MiR-210 has been shown to be upregulated in activated B cells and inhibit CSR, likely by downregulating CSR factors – miR-210-deficient mice produce high levels of autoantibodies (62). B cell differentiation into plasma cells is also regulated by microRNAs. Overexpression of miR-125b, which is expressed in centroblasts (activated germinal center B cells that are enlarged and proliferating), represses plasma cell differentiation and Ig secretion by targeting *Prdm1* (63). Finally, histone deacetylase inhibitors (HDI) upregulate miR-155, miR-181b, and miR-361 to silence *AICDA/Aicda*, as well as miR-23b, miR-30a, and miR-125b to silence *PRDM1/Prdm1* in human and mouse B cells (64), but not other CSR/SHM-related genes or microRNAs that are not known to regulate *AICDA/Aicda* or *PRDM1/Prdm1* (65). Importantly, HDI also abolish CSR/SHM and impair the class-switched high-affinity antibody responses in normal mice and pathogenic autoantibody response in lupus-prone mice (64).

MicroRNAs Regulate Intracellular Membrane Functions

MicroRNAs regulate virtually all cellular pathways, including intracellular membrane functions, such as endocytosis (66), lysosome degradation (67), and autophagy (68). They would do so in cancer cells, stem cells, and B cells, which express relevant intracellular membrane structures. For instance, miR-509 can bind to the transcripts encoding Rab5C, one of the three Rab5 isoforms, thereby inhibiting Rab5C expression in pre-B acute lymphoblastic leukemia cells and resulting in a growth defect that can be rescued by Rab5C overexpression (69). Targeting

BOX 1 | MicroRNAs biogenesis and function.

MicroRNAs are a class of endogenous small non-coding RNAs, usually 19-23 nucleotide in length, that regulate gene expression through mainly post-transcriptional mechanisms. In microRNA biogenesis, a microRNA host gene is first transcribed by RNA polymerase II, giving rise to primary microRNA (pri-microRNA) transcripts. These are then processed by a complex composed of Drosha, an RNase III enzyme, and Pasha, a double-stranded RNA binding protein, to precursor microRNAs (pre-microRNAs), a stem-loop structure of 70-100 nucleotides. Pre-microRNAs are transported into the cytoplasm and cleaved by the Dicer RNase III. Resulting microRNA duplexes are incorporated into a RISC complex that also contains Ago. The "lead" functioning strand is guided to match the 3'-UTR of the mRNA target, while the "passenger" strand is degraded. A functional microRNA can pair perfectly or imperfectly with the microRNA recognition sites within mRNA 3'-UTR, leading to transcript degradation and translation inhibition, respectively.



of SUMF1, a cellular sulfatase activator, by miR-95 disrupts sulfatase activities, resulting in an accumulation of sulfated substrates in lysosomes that, in turn, impairs lysosome-mediated cargo degradation – miR-95-mediated lysosome dysfunction also results in defects in autophagy-mediated cargo degradation (67). Knocking down of miR-95 in cells isolated from patients with a severe lysosomal storage disorder called multiple sulfatase deficiency due to hypomorphic *SUMF1* mutations can increase SUMF1 protein levels, suggesting a potential therapeutic intervention for this disease (67). MiR-376b directly targets the 3′UTR of *ATG4C* and *BECN1* mRNA, thereby inhibiting Atg4C- and Beclin 1-dependent autophagy induced by starvation or rapamycin, and an antagomir of miR-376b increases Atg4C- and Beclin 1 expression and autophagy (70). Likewise, miR-20a and miR-106b suppresses ULK1 expression and autophagy, a process that can be de-repressed by nutrient (leucine) deprivation (71). Finally, systems biology approaches have led to the identification of several microRNAs (e.g., miR-130, miR-98, miR-124, miR-204, and miR-142) that can target lysosome-related proteins to modulate the autophagy–lysosomal pathways (72).

MicroRNAs Regulate Intracellular Membrane-Associated Proteins Involved in B Cell Differentiation

The miR-17–92 locus encodes a cluster of microRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a) that play important roles in regulating immune functions (73). These microRNAs are essential for B cell development (74), but dispensable for mature B cell survival (75). Mature B cell-specific miR-17-92 KO mice are defective in producing class-switching IgG2c antibodies (75). Lymphocyte-specific overexpression of miR-17-92 increases proliferation and survival of B cells and germinal center B cell differentiation, eventually leading to lymphomagenesis (76). Among microRNAs in the miR-17-92 cluster, miR-17 targets transcripts encoding TBC1D2, which acts as a GTPase activating protein (GAP) of Rab7 to downregulates Rab7 activity (66) – TBC1D2 is also an effector of Rab7-GTP and would participate in the negative feedback-loop that controls Rab7 activities. MiR-17-dependent upregulation of Rab7 activities may explain, at least in part, the role of miR-17-92 in class-switched antibody responses. MiR-17 also targets Atg7 (77), suggesting a role of miR-17-92 in memory B cell differentiation, in addition to its inhibition of plasma cell homing to the bone marrow (75).

As we have recently reported (64), both miR-23b and miR-30a are upregulated by HDI in B cells to target the *Prdm1* 3′-UTR, thereby playing a role in mediating HDI repression of B cell differentiation into plasma cells (**Figure 2**). These microRNAs would also modulate plasma cell differentiation and/or functions by downregulating autophagic proteins, such as Atg5, Atg12, Beclin 1, and others factors (78–80) (**Table 1**). Another member of the miR-30 family, miR-30c, is predicted to target both Rab7 and AID, consistent with the notion that one microRNA can target multiple factors in the same pathway to maximize its influence on the outcome of that pathway (**Figure 2**, a). Other examples supporting this notion include miR-302b, which can target IRAK4 and likely Rab7, both of which are important for TLR-induced NF-κB activation (38, 81). In addition, miR-155 and miR-181b downregulate CSR by targeting AID. They may also inhibit plasma cell differentiation by virtue of their ability to target the 3′-UTR of transcripts encoding autophagic proteins, such as Atg5 and possibly Rheb and Rictor. MiR-93 targets the *AICDA* 3′-UTR and would regulate AID expression in activated B cells, as it does in the MCF7 breast cancer cell line (82), which, like several cancer cell lines, expresses elevated levels of AID, a potent DNA mutator and tumorigenesis factor (83). MiR-93 would also downregulate plasma cell differentiation by inhibiting autophagy and likely do so by targeting Atg16L1 (82), SQSTM1 (84), and possibly a battery of other autophagic proteins, such as ULK1, ATG14 and RB1CC1 (**Table 1**). Finally, consistent with the notion that a single factor can be regulated by multiple microRNAs, Rab7 would be regulated by both miR-30c

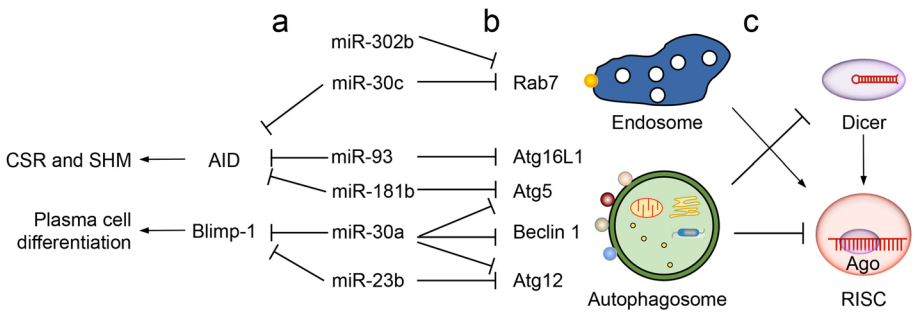


FIGURE 2 | Cross-regulation of intracellular membrane-associated proteins and microRNAs in B cell differentiation. (a) Regulation of multiple intracellular membrane-associated proteins as well as AID (critical for CSR/SHM) and Blimp-1 (driving plasma cell differentiation) by a single microRNA. For example, miR-30a regulates Atg5, Beclin 1 and Atg12 in autophagy and Blimp-1, miR-30c regulates Rab7 (in the endosome pathway) and AID, as well, miR-93 and miR-181b regulate AID as well as Atg16L1 and Atg5, respectively. (b) Regulation of one intracellular membrane-associated protein by several microRNAs. For example, Rab7 is regulated by both miR-30c and miR-302b, and Atg12 is regulated by both miR-30a and miR-23b. (c) Regulation of microRNA activities by endosomes and autophagosomes. Endosomes provide a structure to support the assembly of RISC in microRNA-mediated gene silencing. Autophagosomes promote degradation of Dicer and Ago, thereby downregulating microRNA biogenesis and functions, respectively.

TABLE 1 | MicroRNAs target intracellular membrane-associated proteins and factors critical for B cell differentiation and functions.

MicroRNAs	Intracellular membrane-associated protein target	Specific factor target	Relevant B cell function	Reference
miR-17-92	TBC1D2, Atg7	Bim, Pten	B cell development, Germinal center reaction	(66, 73, 76, 84)
miR-23b	Atg12, Atg2B[a], RAB11FIP2[a]	Blimp-1	Plasma cell differentiation	(64, 85)
miR-30[b]	Rab7[a]	AID[a]	CSR	(64, 79, 80)
	Atg5, Beclin 1, Atg12[a]	Blimp-1	Plasma cell differentiation	
miR-155	Rheb, Rictor	AID SHIP-1	CSR, SHM, Germinal center reaction	(60, 86, 87)
miR-181b	Atg5	AID	CSR	(61, 88)
miR-93	Atg16L1, SQSTM1, Atg14[a], RB1CC1[a], ULK1[a]	AID	CSR	(82, 84, 89)
miR-302b	Rab7[a], RABGAP1[a], Rab9A[a]	IRAK4	NF-κB activation	(81)
miR-9	Atg14[a], ULK2[a]	NF-κB	CSR	(90)
miR-10a	RB1CC1	BCL6	Germinal center reaction	(91, 92)
miR-146a	SQSTM1[a]	TRAF6	NF-κB activation	(93)

[a]*High confident targets predicted by miRDB (www.miRDB.org) (94).*
[b]*Rab7 and AID are predicted to be targeted by miR-30c, with others being targets of miR-30a.*

and miR-302b (**Figure 2**, b), suggesting an (indirect) regulation of NF-κB induction and AID activation by these microRNAs.

As suggested by a recent study (95), intracellular membrane proteins would evade microRNA regulation by using mRNA transcripts with short 3′-UTRs for their production. Proteins can be encoded by mRNAs with alternative 3′-UTRs, the longer ones of which function as scaffold to interact with the RNA-binding proteins HuR and SET and facilitate the plasma membrane localization of membrane-associated proteins (95, 96) – HuR plays an important role in germinal center formation, CSR, and antibody responses, possibly though facilitating protein localization to the plasma membrane (97). When encoded by transcripts with shorter 3′-UTRs, which contain much fewer microRNA targeting sites and are less susceptible to microRNA-mediated silencing, these membrane-associated proteins would display a different subcellular localization, shifting from the plasma membrane to intracellular membrane structures.

Overall, the regulation of B cell differentiation and intracellular membrane-associated proteins by microRNAs would be deeply intertwined to achieve highly fine-tuned antibody responses.

CONCLUSION AND PERSPECTIVES

Intracellular membrane structures regulate, in addition to cellular homeostasis and basic metabolic processes, B cell differentiation in antibody responses and likely other cell type-specific functions. Different intracellular membranes are responsible primarily for distinct B cell differentiation processes, such as Rab7$^+$ mature late endosomes in AID induction and CSR/SHM, Atg5$^+$ autophagosomes in plasma cell differentiation and survival, and Atg7$^+$ autophagosomes for memory B cell maintenance. Nevertheless, these lipid-containing micro-domains would also cross-talk, e.g., through their partially shared biogenesis pathways, thereby regulating multiple processes. Indeed, Rab7 would regulate plasma cell survival, in addition to its major role in CSR/SHM, and the collaboration of different membranes in regulating B cell differentiation is suggested by the role of ER in endosome biogenesis (98).

Specific functions of selected intracellular membrane-associated proteins would reflect their induction in differentiating B cells, as epitomized by the upregulation of Rab7 in activated B cells and elevated levels of Atg7 as memory B cells survive over the time. This notion emphasizes the importance of the profiling of expression of intracellular membrane-associated proteins and biogenesis of intracellular membrane structures in B cells toward our full understanding of B cell differentiation fate decision. As such, a comprehensive analysis of the role of intracellular membranes in signal transduction, which is ultimately responsible for gene regulation, is intriguing, as it would not only support the emerging paradigm that membrane organization specifies the signal output, but also reveal feedback-loop regulation mechanisms that contribute to the tight regulation of B cell differentiation.

As suggested by the critical dependence of endosomes on Rab5 expression levels (99), intracellular membranes are sensitive for their maintenance to the depletion of relevant factors, making microRNA, powerful regulators of intracellular membrane functions and, therefore, B cell differentiation. Interestingly, intracellular membranes would regulate B cell differentiation in part by modulating microRNA activities, as suggested by an important role of endosomes in promoting formation of an RNA-induced silencing complex (RISC) and downregulation of Dicer and Ago by autophagy (**Figure 2**, c). Ago2, a component of RISC, localizes to mature endosomes and RISC-bound mRNAs accumulate in GW bodies, which are discrete cytoplasmic foci associated with mature endosomes (100). Blocking endosomes maturation (by deletion of ESCRT complexes) results in loss of GW bodies and impairment in microRNA-mediated gene silencing, while accumulation of mature endosomes (through depletion of a tethering factor HPS4) enhances microRNA-mediated silencing (101, 102), all pointing to a positive role of endosomes in microRNA functions. By contrast, the autophagic pathway degrades Dicer and Ago that are not bound with any pre-microRNA and microRNA, respectively, in a manner dependent on the autophagy receptor NDP2, which interacts with LC3 (103). Dicer accumulates in cells deficient of the critical autophagy components Atg5, Atg6, or Atg7 (104), suggesting that B cell-specific conditional KO of *Atg5* and *Atg7* may display elevated levels of microRNAs that would, in turn, downregulate factors important for plasma cell differentiation (e.g., Blimp-1) and memory B cell maintenance.

Collectively, intracellular membrane structures and microRNAs would regulate important B cell processes. The reciprocal regulation of intracellular membranes and microRNAs would fine-tune at any time the gene expression necessary to achieve

the specificity of B cell differentiation processes. Intracellular membranes can quickly regenerate upon replenishment of important relevant factors that were previously downregulated by microRNAs. This buffers the effect of a broad spectrum of aberrant molecular events, which frequently occur in highly proliferating B cells, and would play an important role in preventing disastrous overexpression of proto-oncogenes (such as Myc and BCL6) and the DNA mutator AID and, therefore, B cell lymphomagenesis (105–107).

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