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Histone Arginine Methylation by PRMT7 Controls Germinal Center Formation via Regulating Bcl6 Transcription

Zhengzhou Ying,†‡§ Mei Mei,*‡§ Peizhun Zhang,*∥ Chunyi Liu,*† Huacheng He,*†‡ Fei Gao,‡ and Shilai Bao*

B cells are the center of humoral immunity and produce Abs to protect against foreign Ags. B cell defects lead to diseases such as leukemia and lymphomas. Histone arginine methylation is important for regulating gene activation and silencing in cells. Although the process commonly exists in mammalian cells, its roles in B cells are unknown. To explore the effects of aberrant histone arginine methylation on B cells, we generated mice with a B cell–specific knockout of PRMT7, a member of the methyltransferases that mediate arginine methylation of histones. In this article, we showed that the loss of PRMT7 led to decreased mature marginal zone B cells and increased follicular B cells and promoted germinal center formation after immunization. Furthermore, mice lacking PRMT7 expression in B cells secreted low levels of IgG1 and IgA. Abnormal expression of germinal center genes (i.e., Bcl6, Prdm1, and Irf4) was detected in conditional knockout mice. By overexpressing PRMT7 in the Raji and A20 cell lines derived from B cell lymphomas, we validated the fact that PRMT7 negatively regulated Bcl6 expression. Using chromatin immunoprecipitation–PCR, we found that PRMT7 could recruit H4R3me1 and symmetric H4R3me2 to the Bcl6 promoter. These results provide evidence for the important roles played by PRMT7 in germinal center formation. The Journal of Immunology, 2015, 195: 1538–1547.

The immune system plays essential roles in protecting mammals against invading pathogens. Both the mouse and the human immune systems are broadly categorized into innate and acquired immunity, and the latter is further divided into humoral and cellular components. B cells produce Abs to protect against pathogens and constitute the critical components of humoral immunity. Defects in B cell development can lead to deficient Ab production, malignancy, and allergy. In all adult mammals, B cells are derived from hematopoietic progenitor cells that reside in the bone marrow. Early B cell developmental stages take place in the bone marrow concomitant with the functional rearrangement of the Ig gene segments. When the surface IgM (sIgM) molecule is expressed, B cells leave the bone marrow and migrate to the spleen, where they pass through transitional type I and II B cell stages to differentiate into naive follicular B (FO-B) and marginal zone B (MZ-B) cells (1).

Upon encountering Ags, naive FO-B cells are activated and proliferate to form a histological area termed the germinal center (GC). The GCs, which constitute a crucial portion of the humoral immune response to T cell–dependent Ag stimulation, are the main sites in which class-switch recombination (CSR), somatic hypermutation (SHM), and Ag selection occur. Clonal expansion of the cells in GCs exhibits an extremely high rate of proliferation and apoptosis that is followed by terminal differentiation into plasma cells that produce high-affinity Abs (2). However, the combination of a high proliferative rate, clonal expansion of B cells, and hypermutational machinery that requires the attenuation of the DNA damage-sensing and replication checkpoints comes with a risk (3, 4). The majority of B cell lymphomas, which include ~95% of newly diagnosed human lymphomas, arise from GC-B cells (5). These statistics clearly indicate the requirement for the accurate regulation of GC-B cell development. Upon activation, the accurate expression of transcriptional regulators for GC-B cells guarantees the balance of proliferation, apoptosis, and terminal differentiation; B cell lymphoma 6 (BCL6) functions as a master regulator of these processes (6, 7).

Although emerging evidence has demonstrated that epigenetic events contribute to B cell development (8, 9), the roles of epigenetic regulators in GC formation is not understood. Recently, two epigenetic regulators (EZH2 and MOZ) involved in the histone modification were shown to play roles in the epigenetic changes involved in the formation of GCs (10–13). Therefore, additional histone codes encoded by various epigenetic factors involved in the process are worth defining. Histone arginine methylation by protein arginine methyltransferases (PRMTs) commonly exists in mammals and has been studied for decades. However, compared with histone lysine methylation, the influence of histone arginine methylation on gene regulation has remained largely unexplored. The physiological functions of histone arginine methylation play
roles in numerous processes, such as cell differentiation, embryonic development, and carcinogenesis (14, 15). In mammals, nine members of the PRMT family catalyze mono-, asymmetric di-, and symmetric dimethylated arginine residues (16). Unlike the other members, PRMT7 is unique owing to its controversial catalyzed arginine residues. Initially, Miranda et al. (17) identified PRMT7 as a member of the PRMT family that catalyzed the formation of ε-N3-monomethyl-arginine residues on synthetic peptides in vitro, whereas later in the year another team (18) reported that PRMT7 could methylate both peptides and proteins to form symmetric dimethylated arginine residues. In vivo, PRMT7 has been found to interact with the mammalian testis-specific CCTC-binding factor-like protein to control genetic imprinting (19). In addition, PRMT7 methylated the Sm proteins that constitute the spliceosome and seemed to mediate RNA splicing (20). It was also suggested that PRMT7 was associated with sensitivity to a number of DNA damage agents (21–23). Knockdown of PRMT7 in cell lines demonstrated that PRMT7 played roles in the DNA damage response by upregulating the genes involved in DNA repair (24). Furthermore, it has also been revealed that PRMT7 antagonized MLL4-mediated differentiation by methylating the histones located on MLL4 target genes (25). These results suggest that PRMT7 may play an important role in mammalian development. However, the function of PRMT7 in the DNA damage response and its potential impact on the formation and maintenance of GCs have yet to be investigated.

To investigate whether PRMT7 was required for B cell development, we generated mice with a conditional deletion of PRMT7 in B cells. In this study, we found that PRMT7 deficiency in B cells impaired late B cell differentiation in the spleen and promoted GC hyperplasia. We also addressed the regulation of Bcl6 by PRMT7 in vivo and validated this finding in GC-B cell–derived lymphoma cell lines. These results provide new evidence for epigenetic regulators of GC formation and Ab production, and demonstrate the roles of histone arginine methylation mediated by PRMT7 in the occurrence of B cell lymphoma.

Materials and Methods

Mouse and immunization

All animal experiments were approved by the Animal Care and Use Committee of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Mice containing a floxed Prmt7 allele were generated by gene targeting; then, the mice were crossed with Zp3-Cre mice, and the positive female offspring (Zp3-Cre/Prmt7fl/fl) were mated to male wild-type (WT) to generate Prmt7fl/fl mice. Cd19-Cre/Prmt7flo/flo mice were obtained by crossing floxed Prmt7 mice with Cd19-Cre mice (26). The corresponding homozygotes were sequentially obtained and genotyped by PCR analysis. The primers are listed in Supplemental Table I. All mice were produced in a mixed C57BL/129sv background and maintained in a specific pathogen-free facility in accordance with Chinese Academy of Sciences guidelines. Unless specified, Cd19-Cre/Prmt7flo/flo (conditional knockout of PRMT7 in B cells [PRMT7-CKO]) mice and their litterate counterparts were used at 8–10 wk of age for the experiments.

For GC induction, 8- to 10-wk-old mice were injected i.p. with 5 × 10^6 SRBCs (Ratite Biotec) in PBS. GCs and PCs were analyzed on day 10 after immunization. Serum samples were collected from these mice prior to sacrifice to measure Ab concentrations.

To assess Ag-specific T cell–dependent responses, 8- to 10-wk-old mice were immunized i.p. with 100 μg trinitrophenyl-conjugated keyhole hemagglutinin protein (TNP-KLH) (Biosearch Technologies) in PBS supplemented with 50% (vol/vol) CFA (Sigma-Aldrich). Blood samples were collected from the mice before immunization (day 0) and at days 7 and 14. Then, a booster immunization was performed with 100 μg TNP-KLH in 100 μl PBS supplemented with 50% (vol/vol) IFA (Sigma-Aldrich). Blood samples were collected on days 21 and 28. The titers of TNP-specific serum Ab isotypes were determined by ELISA, as described below.

Flow cytometry

The following Abs/reagents were used for staining: B220–PerCP-cy5.5, Gr1–APC, CD3–PE, slgM–APC, CD43–PE, Gr1–APC, CD3–PE, CD23–FITC, CD21/35–PE, CD19–PE-Cy7, CD138–FITC, and CD95–PE were purchased from eBioscience. Ki67 (Abcam), DAPI (Beyotime), 7-amino-acetoxyfluorocyanin D (BD Bioscience), and peanut lectin (PNA)–FITC (Sigma–Aldrich) were also purchased. Single-cell bone marrow or spleen cell suspensions were prepared and stained (27) for analysis and sorting using a FACS aria II flow cytometer (BD Biosciences). Further analysis was conducted with FlowJo software (TreeStar) for gating or Modifit software (Verity) for cell cycle analysis. For intracellular staining, B cells were prestained with membrane markers, fixed, and permeabilized with Cytofix/Cytoperm solution (BD Bioscience), and stained with intracellular markers.

Immunohistochemistry and immunofluorescence

Briefly, 8-μm paraffin sections were prepared, de-waxed, and rehydrated. Ag retrieval by microwave treatment was also performed. For immunohistochemistry (IHC), after the endogenous peroxidase was blocked using 3% hydrogen peroxide in deionized water and permeabilized with 0.5% Triton-X100, the sections were stained with Abs against CD95 (eBioscience) and Ki67 (Abcam). For immunofluorescence, the sections were prestained with Sadan black B to avoid tissue autofluorescence, and then stained with an Ab against PCNA (Sigma–Aldrich).

Images were acquired on a Nikon 90i microscope. Fluorescence emission was detected as red, green, and blue signal channels. The software programs Photoshop CS4 and Illustrator CS4 (Adobe Systems) were used for image analysis.

ELISA assay

Serum concentrations of total IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA were assessed using the Clonotyping System–HRP (SouthernBiotech) with the Mouse Ig Isotype Panel (SouthernBiotech) as a positive reference standard according to the manufacturer’s instructions.

For the determination of TNP-specific Abs, 96-well ELISA plates (Thermo Fisher Scientific) were coated with 10 μg/ml TNP–BSA (Biosearch Technologies) in coating buffer (Na2CO3–NaHCO3, pH 8.0) at 4 °C overnight, washed, and blocked with 2% normal goat serum in PBS. Different dilutions of serum were added to the plates and incubated for 2 h at room temperature. After washing, isotype-specific HRP–conjugated goat anti-mouse Ig Abs (Southern Biotech) were added for 1 h at room temperature. Following incubation with TMB solution (Thermo Fisher Scientific), HRP activity was measured at OD 450 nm.

RNA sequencing

Total RNA of sorted splenic B cells was isolated with TRIzol (Invitrogen). After passing quality control, the RNA was treated and sequenced by the Novogene Bioinformation Institute as previously described (28). The RNA-sequencing (RNA-Seq) data have been submitted to the National Center for Biotechnology Information Sequence Read archive (accession number: SRP052846, http://www.ncbi.nlm.nih.gov/sra).

Real-time PCR

Total RNA was isolated with TRIzol (Invitrogen) and treated with RNase-free DNase I (Promega). Reverse transcription was performed according to the manufacturer’s instructions (Promega). The cDNA was amplified using SYBR Green PCR Master Mix (QIAGEN) and primers for qRT-PCR. The primers were designed using Primer Premier 5 software or retrieved from the PrimerBank database (http://pga.mgh.harvard.edu/primerbank/); all primer sequences are listed in Supplemental Table I.

Western blotting

Tissues and cell lysates were prepared in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2 mM EDTA, and 0.1% Nonidet P-40) supplemented with proteinase inhibitor mixture (Roche). A total of 50–100 μg proteins were loaded onto an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The samples were blotted with the following Abs: anti-PRMT7 and anti-GAPDH (created by the Animal Center of the Institute of Genetics and Developmental Biology), anti-tubulin (Sigma–Aldrich), anti-H4R3me1 (Novus), anti-H4R3me2 (Active Motif), anti-H3R2me2s (Upstate Biotechnology), and anti-H3 (Upstate Biotechnology). The Abs were detected with an HRP–conjugated secondary Ab (Pierce) and developed with an ECL signal detection kit (GE Healthcare) according to the manufacturer’s instructions.
Plasmid construction, cell culture, and lentiviral infection

To construct pCDH-GFP or pCDH-GFP-PRMT7, first the plasmids encoding PRMT7 were constructed using pEGFP-C1 (Clontech). Then, the GFP or GFP-PRMT7 fragments from the corresponding pEGFP-C1 and pEGFP-C1-PRMT7 plasmids were inserted into the pCDH-CMV-MCS-EF1-Puro vector (System Biosciences). The human B cell leukemia- and lymphoma-derived Nalm6, U266, Z138, Raji, and Oci-ly10 and mouse lymphoma-derived A20 cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS, U266 was kindly provided by Prof. Guangbiao Zhou (Institute of Zoology, Chinese Academy of Sciences). The human embryonic kidney 293 cell line and its derivative 293T cell line were maintained in DMEM supplemented with 10% FBS. Lentiviral supernatants were produced by cotransfection of the packaging vectors pMDL and pAX2 and the target vectors pCDH-GFP-PRMT7 or pCDH-GFP into 293T cells as described. For infection, Raji cells or A20 cells (5 x 10^5 cells per milliliter) were cultured in 8 ml medium and 2 ml viral supernatants supplemented with polybrene (0.5 μg/ml) for 8 h. At 2 d later, the medium was replaced with new medium containing puromycin (2 μg/ml). After 1 wk of selection, the cells were harvested and used for RNA isolation or the chromatin immunoprecipitation (ChIP) assay.

ChIP assay

The ChIP assay was performed as described in the Millipore ChIP protocol. Briefly, A20/GFP and A20/PRMT7ov cells were fixed with 1% formaldehyde and lysed with lysis buffer (50 mM Tris at pH 8.0, 10 mM EDTA, 1% SDS, 0.2 mM PMSF, and protease inhibitor mixture). The cell lysates were sonicated with an ultrasonic machine (Cole-Parmer, 4710 series) for 15–20 min with cycles of 15-s pulses and 1-min pauses to shear the DNA. Chromatin was analyzed by agarose gel electrophoresis. The DNA fragment size did not exceed 500 bp. After preclearing with protein G beads (Millipore) for 2 h at 4˚C, the supernatant was incubated overnight at 4˚C with Abs against PRMT7 (created by the Animal Center of the Institute of Genetics and Developmental Biology), H4R3me2s (Active Motif), and GFP (created by the Animal Center of the Institute of Genetics and Developmental Biology). The blocked protein G beads were added into the mixture and incubated for 2 h at 4˚C. The complex was washed twice consecutively with low-salt buffer, high-salt buffer, LiCl buffer, and TE buffer. After eluting with elution buffer, the cross-links were reversed and purified. The DNA sequences of the target genes were analyzed by qRT-PCR using the primers listed in Supplemental Table 1.

Statistical analysis

Results were expressed as the means ±SD. The statistical significance between the two groups was analyzed by Student t test. The p value < 0.05 indicates statistically significant differences.

Results

PRMT7 was preferentially expressed in immune tissues

Previously, we reported that arginine methylation was associated with B cell leukemia; another two reports also provided data on roles during B cell development and lymphoma genesis (29–31). Given that histone arginine methylation was a common post-translation modification and that arginine methylation deficiency by PRMTs knockout in mice led to abnormal development (14), we generated PRMT7-knockout mice by gene targeting (Supplemental Fig. 1A). The Prmt7^−/− mice were born in mendelian numbers, and no obvious defects were observed during fetal development. However, we found that most Prmt7^−/− mice died within 5–10 d after birth (data not shown). This finding further implicated the essential roles of PRMT7 for normal development. To evaluate a possible relationship between PRMT7 expression and normal B cell differentiation, we determined that PRMT7 was broadly expressed in tissues in normal adult mice and highly expressed in lymphoid tissues, including bone marrow, spleen, and GALT (Fig. 1A). Quantitative real-time PCR (qRT-PCR) measurement of Prmt7 mRNA levels among isolated B cell subtypes from the bone marrow and spleens of adult mice exhibited similar expression levels throughout the process of B cell differentiation, and slightly higher expression in FO-B and GC-B cells (Fig. 1B).

FIGURE 1. Expression pattern of PRMT7 in organs and lymphoid cells. (A) Expression pattern of PRMT7 in adult mouse tissues was identified. Three separate adult mice were used for this experiment. (B) The Prmt7 mRNA level was determined by qRT-PCR using RNA from various B cell subtypes sorted by FACS from adult mice. The qRT-PCR was repeated three times in triplicate. Gapdh was used as an internal control. BM, bone marrow.

Thus, the above results indicated that the arginine methylation mediated by PRMT7 might be required during the B cell lineage differentiation, committed differentiation, and terminal differentiation stages.

B cell differentiation in the bone marrow of PRMT7-CKO mice appeared normal

To identify the roles of PRMT7 in normal B cell differentiation, we generated B cell–specific PRMT7 conditional deletion mice by crossing Prmt7^floxt/lox mice with Cd19-Cre mice (PRMT7-CKO). The genomic arrangement of exon 5 was specifically detected in lymphoid tissues (i.e., bone marrow, spleen, and GALT) in the PRMT7-CKO mice by PCR (Fig. 2A). Moreover, PRMT7 protein expression was dramatically decreased in sorted B220^+ cells from the bone marrow of WT and PRMT7-CKO mice (Fig. 2B).

Next, we investigated whether PRMT7 was required for B cell committed differentiation in the bone marrow, using PRMT7-CKO mice generated as described above. The obtained PRMT7-CKO mice were fertile, exhibited mendelian numbers, and showed a similar overall phenotype versus that of their littermate controls. Unexpectedly, the frequency and number of B cell subpopulations (including pro-B [B220^+slgM^−CD43^+], pre-B [B220^+slgM^−CD43^+], immature B [B220^+slgM^−], and mature B [B220^+slgM^+]) cells in the bone marrow of PRMT7-CKO mice were comparable to what was observed in the WT mice (Fig. 2C–E). Furthermore, the similar frequency of mature B (B220^+slgM^+) cells detected in the peripheral blood of control and PRMT7-CKO mice revealed that the transition of B cells from the bone marrow was not affected by the loss of PRMT7 (Fig. 2F, 2G). This result may be due to the incomplete deletion of Cd19-Cre in the early lymphoid progenitors of mice, which was found in transgenic models involving deletion of floxed alleles with Cd19-Cre (26). In fact, we also detected that the efficiency of Cre-mediated deletion of PRMT7 in pro-B and pre-B cells from the bone marrow of PRMT7-CKO mice was only ~50% by qRT-PCR, whereas no Prmt7 mRNA was observed in immature B and mature B cells (data not shown). The roles of PRMT7 in early lymphoid progenitors and B cell progenitors require further study. However,
these results indicate that changes in PRMT7 expression did not affect immature B and mature B cell differentiation.

**Mature B cells were impaired in the spleens of PRMT7-CKO mice**

Although no obvious defects were noted in early B cell subpopulations of PRMT7-CKO mice, the impact on late B cell differentiation in the spleen might be aberrant owing to diverse roles and more efficient deletion of PRMT7 with 

**Cd19**-Cre. First, we detected the protein expression of PRMT7 in splenic B cells (CD19\(^+\)) and non-B cells (CD19\(^-\)) to validate the deletion of PRMT7. The result showed that indistinguishable PRMT7 expression was detected in splenic B cells in PRMT7-CKO mice, whereas equal expression was found in splenic non-B cells between the WT and PRMT7-CKO mice (Fig. 3A). Subsequently, we observed mild splenomegaly in 8-wk-old PRMT7-CKO mice (Fig. 3B). Surprisingly, only a slight increase in total splenic B cell numbers was found in the PRMT7-CKO mice despite the fact that the total splenic cell numbers were increased when the enlarged spleens were observed (Fig. 3C). This result suggests that other cell types in the spleens also expanded. In this study, we found that myeloid cell and T cell numbers were increased in the PRMT7-CKO mice (Supplemental Fig. 2A, 2B). Moreover, additional significant splenomegaly was observed in PRMT7-CKO mice during the 5th and 10th months (Supplemental Fig. 2C–E). These results indicated that loss of PRMT7 impaired splenic B cell development and function.

To define exactly the impaired B cell subpopulation, we performed flow cytometric analysis on PRMT7-CKO mouse splenocytes. The detailed analyses of FO-B cells (CD19\(^+\)sIgM\(^+\)CD21/35\(^\text{med}\)) and MZ-B cells (CD19\(^+\)sIgM\(^+\)CD21/35\(^\text{hi}\)) showed that the frequency of the FO-B cells was almost normal despite the lack of

**FIGURE 2.** B cell differentiation in bone marrow was normal in PRMT7-CKO mice. (A) Genomic DNA extracted from various tissues of the PRMT7-CKO mice was analyzed by PCR. cre and loxp indicated the transgenic fragments, whereas Prmt7\(^\text{D}\) indicated the fragment after genomic arrangement of Prmt7. (B) Decrease in PRMT7 expression in the B220\(^+\) cells sorted from the bone marrow of WT and PRMT7-CKO mice was validated by Western blotting. (C) Single bone marrow cells were isolated for representative flow cytometric analysis of pre-B cells (top panel, gated as sIgM\(^-\), B220\(^+\)CD43\(^-\)), pre-B cells (bottom panel, gated as CD43\(^-\), B220\(^-\)sIgM\(^+\)), immature B cells (bottom panel, gated as CD43\(^-\), B220\(^+\)sIgM\(^-\)) and mature B cells (bottom panel, gated as CD43\(^-\), B220\(^+\)sIgM\(^+\)) from the bone marrow of WT and PRMT7-CKO mice. (D and E) Frequency (D) and number (E) of B cell subpopulations were analyzed in the bone marrow of WT (n = 4) and PRMT7-CKO mice (n = 4). Bars show the mean (±SD) values. (F) Flow cytometric analysis of mature B cells (gated as CD43\(^-\), B220\(^+\)sIgM\(^+\)) from the peripheral blood of WT and PRMT7-CKO mice. (G) B cell frequencies in the PBLs of control (n = 4) and PRMT7-CKO mice (n = 4) as shown in (F). Bars represent the mean (±SD) values. BM, bone marrow; cKO, PRMT7-CKO mice.
PRMT7, whereas the frequency of the MZ-B cells was diminished by \(~2\)-fold (Fig. 3D top, 3E); this result suggested that PRMT7 was required for MZ-B cell development. The reduction in the MZ-B cell frequency could be due to a decrease in the MZ-B cell precursor population or the inability of MZ-B cells to situate and remain in the marginal zone niches for maturation (32). For this reason, we assessed MZ-B precursor cells (CD19+sIgM+CD21/35hiCD23hi) and mature MZ-B cells (CD19+sIgM+CD21/35hiCD23hi). A strikingly decreased frequency in MZ-B cells was observed in PRMT7-CKO mice, whereas no apparent difference was observed in the numbers of MZ-B precursor cells (Fig. 3D bottom, 3E). Cell counts demonstrated that the number of FO-B cells was slightly increased compared with the WT mice, whereas the number of mature MZ-B cells was significantly decreased in the PRMT7-CKO mice (Fig. 3F). These findings established that although the B cells deficient for PRMT7 could translocate to the spleen and differentiate into the FO-B cell and MZ-B precursor cell stages, there was a differentiation bias between the two groups. In contrast to FO-B cells, which require T cell help to promote effective primary immune responses and Ab class switching, MZ-B cells are T cell independent and critical for the human and mouse responses to blood-borne microorganisms. Thus, the defects in MZ-B cells indicated that the mutants were more susceptible to pathogen infection. Therefore, we assessed whether the function of FO-B cells in PRMT7-CKO mice was affected owing to their roles in high-affinity Ab production and the high incidence of lymphoma.

**Loss of PRMT7 promoted GC formation after SRBC immunization**

FO-B cells are activated and rapidly proliferate to form GCs upon encountering a foreign Ag. To detect the possible effects of PRMT7 on GC formation, we immunized PRMT-CKO mice and their counterparts with SRBCs. We isolated spleens for flow cytometric analysis or section preparation on the 10th day after immunization, when the number of GC-B cells nearly reached its peak. The flow cytometry results revealed significantly increased proportions and numbers of GC-B cells (CD19+PNA+CD95+) in the PRMT7-CKO mice (Fig. 4A–C). Next, we demonstrated that more B cells underwent proliferation via flow cytometric analysis using an Ab against the proliferation marker Ki67 (Fig. 4D–F). To visualize the cells in situ, we stained spleen sections via IHC with Abs against Ki67 and CD95. Consistent with results from the flow cytometric analysis, a marked increase in Ki67- and CD95-positive cells was observed in the white pulps in spleens from PRMT7-CKO mice (Fig. 4G). Furthermore, immunofluorescence staining for another cell proliferation marker (PCNA) also showed a significant accumulation of PCNA+ cells in the spleens from PRMT7-CKO mice (data not shown). These results demonstrated that the loss of PRMT7 in...
splenic B cells promoted GC formation after immunization with SRBCs. After selection by follicular helper T cells in the presence of follicular dendritic cells, GC-B cells undergo Ig class switching and differentiate into B plasma cells or memory B cells. The plasma cells secrete various high-affinity Igs, such as IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3. To visualize the major Ig-positive cells in PRMT7-CKO mice, we stained the spleen sections with IgG1, IgA, and IgM. Of interest, we found that IgG1- and IgA-secreting cells were significantly decreased, whereas the numbers of IgM-secreting cells in PRMT7-CKO mice were similar to those in their counterparts (Fig. 4H), thereby demonstrating the important roles of PRMT7 in GC-B cell differentiation into Ab-secreting cells.

Lack of PRMT7 decreased IgG1 and IgA production

Because GC-B cells might differentiate into plasma cells, and then migrate to bone marrow, we investigated the number of plasma cells (B220+/CD19+) in the bone marrow after immunization with SRBCs by flow cytometric analysis. Of note, although GC formation was promoted in PRMT7-CKO mice, flow cytometric analysis revealed that the plasma cells were diminished (Fig. 5A, 5B); this result was indicative of impairment of plasma cell differentiation. To measure the capability of plasma cells differentiated from PRMT7-deficient B cells to produce Abs, we collected sera from PRMT7-CKO mice and tested the samples by ELISA. Consistent with the IHC results shown in Fig. 4H, the concentrations of IgG1 and IgA were significantly decreased in sera from PRMT7-CKO mice (Fig. 5C), which indicated that the GC-B cells with impaired functions were largely expanded. The results confirmed that the differentiation of cells secreting the IgG1 or IgA subtypes was inhibited in the PRMT7-CKO mice.

To assess the Ag-specific T cell–dependent response, we immunized PRMT7-CKO mice or WT mice with TNP-KLH and

FIGURE 4. GC formation was enhanced in PRMT7-CKO mice after immunization with SRBCs. (A) Briefly, WT and PRMT7-CKO mice were immunized by i.p. injection with SRBCs. At 10 d later, single-cell splenocytes were isolated and stained for flow cytometric analysis of GC-B cells (gated as CD19+ PNA+Fas+). (B and C) Frequency (B) and total number (C) of GC-B cells from the spleens of WT (n = 8) and PRMT7-CKO mice (n = 8) obtained from the analysis of results shown in (A). *p < 0.05 by Student t test. Bars represent the mean (±SD) values. (D) Proliferating B cells (gated as CD19+, Ki67+) in the spleens from WT and PRMT7-CKO mice were also examined by flow cytometric analysis. (E and F) Frequency (E) and number (F) of proliferating B cells from the WT (n = 4) and PRMT7-CKO mice (n = 4). *p < 0.05 by Student t test. Bars represent the mean (±SD) values. (G) IHC staining of spleen sections with Ki67 and Fas was used to visualize the proliferating cells and GC-B cells. (H) Ab-secreting cells in the spleen were also stained with IgG1, IgA, and IgM.
measured the concentration of anti-TNP–specific IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA Abs at various time points post primary and secondary immunization. Similar to the results obtained with basal Ig levels, anti-TNP–specific IgG1 and IgA Abs were obviously decreased in the PRMT7-CKO mice (Fig. 5D). Strikingly, PRMT7-CKO mice showed impaired production of IgM Abs (Fig. 5D). These data demonstrated that PRMT7 was required for the generation of Ag-specific T cell–dependent humoral responses.

PRMT7 directly binds to the promoter of Bcl6 to inhibit its expression

To characterize the pathway in which PRMT7 took part, we isolated splenic B cells from PRMT7-CKO and WT mice and performed an RNA-Seq experiment. A total of 123 differentially expressed genes, which included 115 upregulated genes and 8 downregulated genes, were detected (Supplemental Fig. 3A). Reduced expression of PRMT7 has been reported to contribute to resistance to DNA-damaging agents (21, 22, 24, 33). Gene Ontology analysis of the differentially expressed genes indicated that PRMT7 was involved in the regulation of chromatin organization, the response to DNA damage stimulus, and DNA repair (Supplemental Fig. 3B). This finding indicated that the defects in resting PRMT7-CKO B cells were caused by differences in gene expression in the absence of PRMT7.

Pathways involved in chromatin remodeling and the DNA damage response are vital for the differentiation and transformation of activated GC-B cells. Moreover, BCL6, a master regulator of GC maintenance, has been reported to be able to repress DNA damage–induced apoptosis and tolerate physiological DNA breaks (34). Thus, we investigated whether Bcl6 expression was affected by the loss of PRMT7. For this experiment, we sorted the GC-B cells and examined the Bcl6 mRNA levels, using qRT-PCR. The results showed that Bcl6 expression was significantly elevated, whereas the expression of Prmt7 was largely diminished in GC-B cells from PRMT7-CKO mice (Fig. 6A). Next, we examined BCL6 target genes that mediated plasma cell differentiation (i.e., Irf4 and

FIGURE 5. Humoral responses were impaired in PRMT7-CKO mice. (A and B) At 10 d after immunization with SRBCs, single-cell bone marrow specimens were isolated and stained for flow cytometric analysis of plasma cells (B220med/CD138+). Frequency (A) and total number (B) of plasma cells in WT (n = 5) and PRMT7-CKO mice (n = 5) obtained from the analysis results shown in (A). *p < 0.05 by Student t test. Bars represent the mean (±SD) values. (C) Sera were collected from WT (n = 8) and PRMT7-CKO mice (n = 8). The concentrations of IgG1, IgA, IgG2a, IgG2b, IgG3, and IgM were determined by ELISA. *p < 0.05 by Student t test. Bars represent the mean values. (D) WT (n = 5) and PRMT7-CKO (n = 5) mice were immunized with TNP-KLH. Anti-TNP–specific Ab titers on different days were determined by ELISA. *p < 0.05 by Student t test. Bars represent the mean (±SD) values.
and found that they were consistently downregulated in PRMT7-deficient GC-B cells (Fig. 6A). However, IRF4 has also been reported to be a critical factor in GC formation based on its deletion in mature B cells (38, 39). For this reason, we analyzed the expression of Irf4 in PRMT7-deficient resting mature B cells and found that it was not altered (Supplemental Fig. 3C). Thus, the loss of PRMT7 could induce Bcl6 upregulation and subsequently inhibit plasma cell differentiation via repressing Irf4 and Prdm1 in GC-B cells.

As the master gene maintaining the GC phenotype, Bcl6 is mainly expressed in GC-B cells and is highly expressed in human GC-B cell lines of lymphoma origin (6). Remarkably, a low level of Prmt7 expression was detected in GC-B cell lines of lymphoma origin (Raji) compared with the other human B cell lymphoma origin cell lines (Fig. 6B). To investigate whether the expression of Bcl6 was associated with PRMT7 in Bcl6-positive cell lines, we overexpressed PRMT7 via lentivirus transfection in human Raji cells and mouse A20 cells (Supplemental Fig. 3D). In contrast to the knockout of Prmt7, the overexpression of Prmt7 in cells led to a notable decrease in Bcl6 expression (Fig. 6C, 6D). Furthermore, cell cycle analysis showed that more PRMT7-overexpressing cells were arrested at the G1 stage compared with the control cells (Fig. 6E, 6F).

Although neither the overexpression nor knockout of Prmt7 had a significant effect on global H4R3me levels (Supplemental Fig. 3E, 3F), PRMT7 can specifically regulate H4R3me levels at some genes (24, 25). To determine whether PRMT7 binds to the Bcl6 gene, we performed a ChIP experiment using a spleen chromatin. Notably, PRMT7 was enriched at the Bcl6 gene (Fig. 6G), indicating that PRMT7 might regulate the transcription of the Bcl6 gene by altering its chromatin H4R3me levels. In this regard, we overexpressed PRMT7 in A20 cells and detected H4R3me2s levels at the Bcl6 promoter. ChIP experiments showed that H4R3me2s levels in PRMT7-overexpressing cells were markedly decreased compared with the control cells (Fig. 6H, 6I).

**FIGURE 6.** PRMT7 regulated Bcl6 expression via altering methylated H4R3 levels on its promoter. (A) GC-B cells after immunization with SRBCs were sorted by FACS, and mRNA levels of Prmt7, Bcl6, and their downstream genes were analyzed by qRT-PCR. Each qRT-PCR experiment was repeated in triplicate in three WT and PRMT7-CKO mice. *p < 0.05 by Student t test. Bars represent the mean (±SD) values. (B) Expression pattern of PRMT7 in various B cell lymphoma–derived cell lines. (C and D) Raji cells and A20 cells were infected with lentiviruses containing pCDH-GFP and pCDH-GFP-PRMT7, respectively. Bcl6 mRNA levels in control and PRMT7-overexpressing Raji cells (E) and A20 cells (F) were measured by qRT-PCR. (E and F) Control and PRMT7-overexpressing A20 cells were collected. DAPI staining for DNA content and flow cytometry were used to determine the percentage of cells in the G1, S, and G2 phases. *p < 0.05 by Student t test. Bars represent the mean (±SD) values. (G) For ChIP analysis, chromatin isolated from spleen cells was subjected to immunoprecipitation (IP) using Abs specific for PRMT7. Each ChIP experiment was repeated twice in triplicate. *p < 0.05 by Student t test. Bars represent the mean (±SD) values. (H) ChIP assay was conducted using chromatin from A20/GFP and A20/PRMT7-ov using either IgG or anti-H4R3me2s. Each ChIP experiment was repeated twice in triplicate. *p < 0.05 by Student t test. Bars represent the mean (±SD) values. (I) Relative fold enrichment of Bcl6 using chromatin from A20/PRMT7-ov cells over their counterparts from A20/GFP cells using anti-H4R3me1, anti-H4R3me2s, or anti-GFP. Each ChIP experiment was repeated twice in triplicate. *p < 0.05 by Student t test. Bars represent the mean (±SD) values. A20, mouse B cell lymphoma cell line; 293T, human embryonic kidney cell line; Z138, human mantle lymphoma cell line; Nalm6, human pre-B leukemic cell line; Oci-ly7, human non-Hodgkin lymphoma cell line; PRMT7ov, PRMT7-overexpressed groups; Raji, human Burkitt lymphoma cell line; U266, human myeloma cell line.
increased at the Bcl6 gene, compared with control cells, whereas the H4R3me2s levels at the Actb gene remained steady (Fig. 6H, Supplemental Fig. 3G). We also measured whether H4R3me1 and PRMT7 directly bound to the Bcl6 promoter. In accordance with the H4R3me2s results, H4R3me1 and PRMT7 were enriched at Bcl6 in PRMT7-overpressing cells (Fig. 6I). We examined the H4R3me levels at different regions of the Bcl6 gene and found that there were preferable regions for their enrichment (Supplemental Fig. 3H–K). Thus, we concluded that PRMT7 could mediate H4R3me levels at the Bcl6 gene and negatively regulate Bcl6 expression, thereby further modulating the downstream target genes to control GC formation and plasma cell differentiation.

Discussion
Derived from hematopoietic progenitor cells, B cells go through serial stages prior to maturation in the bone marrow and are activated in secondary lymphoid organs such as the spleen and lymph node. However, the role of arginine methylation, a common posttranslational modification that results in the methylation of ~2% of the arginine residues in mammalian cell nuclei (40), has rarely been studied in B cell development (30, 41, 42). Prmt7, which encodes an arginine methyltransferase, has been associated with DNA repair and cell differentiation. Nevertheless, the roles that PRMT7-mediated arginine methylation plays in mammalian development are unknown. The expression patterns of PRMT7 in mouse tissues (as well as the data from the Immunological Genome Project database) showed the preferential expression of PRMT7 in hematopoietic progenitor cells and B cells. We also discovered an abnormal frequency and number of hematopoietic progenitor cells and B cells in PRMT7-deficient mice (data not shown). However, we observed that PRMT7 was not required for immature and mature B cell differentiation in mice with the B cell–specific PRMT7 deletion. A similar case was reported in mice deficient for the lysine methyltransferase G9a, which showed delayed hematopoietic progenitor cell lineage commitment without apparent defects in early lymphocyte development (43, 44).

Although PRMT7 was not required for B cell committed differentiation in bone marrow, mild splenomegaly was observed in the PRMT7-CKO mice; this result suggested that PRMT7 played an important role during B cell development in the spleen. Splenomegaly is commonly associated with infection, inflammation, and hematological disorders. In this study, we found that there was only a minor increase in total B cell numbers, whereas myeloid cells and T cells increased significantly in the enlarged spleens in 8-wk-old mice. The similar phenomenon, which is that the expanded myeloid cells and T cells are induced in reaction to autoimmunity and inflammation, was found in mice lacking genes involved in NF-κB activation (45–47). Previously, NF-κB was also reported to be regulated by other PRMTs (48–50), suggesting that arginine methylation plays unique roles in NF-κB activation. In addition, it has been reported that histone arginine deimination, which antagonizes arginine methylation, is a response to a wide range of inflammatory stimuli (51, 52). Thus, we speculate that PRMT7-mediated arginine methylation may be able to regulate genes involved in mammalian immune and inflammatory responses, and the deletion of PRMT7 in B cells might impair B cells and induce an immune deregulation reminiscent of sterile inflammation. However, a further relation between PRMT7 and inflammation is an intriguing question to be answered in future studies.

Then, we analyzed the frequency and number of MZ-B cells and FO-B cells in the spleen and found that PRMT7-CKO mice had decreased MZ-B cell and slightly increased FO-B cell numbers in comparison with WT mice. MZ-B cells are the determinant of host defense against blood-borne pathogens; mice with decreased MZ-B cells will be susceptible to infection by these pathogens. In the mouse, MZ-B cells arise from transitional B cells via the MZ-B cell precursor. Our results suggest that signals contributing to the homing of the normal precursor of MZ-B cells may be impaired by PRMT7 deletion. MZ-B cells stall in the MZ, whereas FO-B cells continue to home to follicles. The retention of MZ-B cells requires integrin-mediated adhesion and signaling, and both poor and excessive densities of integrins and their ligands have been reported to retard cell motility (53). In fact, our RNA-Seq data showed that integrin α4 and the dedicator of cytokinesis, which were involved in the integrin pathways, were elevated in splenic B cells from PRMT7-CKO mice.

One major molecular function of PRMT7 uncovered to date is the response to DNA damage, and reduced expression of PRMT7 has been reported to contribute to resistance to DNA-damaging agents (23, 24, 33). During B cell development, events such as V(D)J recombination, CSR, and SHM lead to chromatin remodeling and DNA damage that make the B cells susceptible to transformation. Defects in the response to DNA damage and genomic instability have been shown to result in immunodeficiency disorders (54). Thus, misregulation of the genes involved in the DNA damage response may be an important reason why resting B cells are impaired in the absence of PRMT7. Moreover, activated GC-B cells, which are the central cells that occur in the CSR and SHM and differentiate into plasma cells with the help of T cells and DC cells, express BCL6 to repress DNA damage–induced apoptosis and tolerate physiological DNA breaks (34). Treatment of Burkitt’s lymphoma cell lines (representing transformed GC B cells) with topoisomerase II inhibitors that sensitized tumor cells through the formation of double strand breaks induced Bcl6 downregulation (55). Bcl6 is the master gene involved in the control of GC formation and terminal differentiation; indeed, GCs do not form in the absence of Bcl6, whereas constitutive expression of BCL6 leads to increased GC formation. Coincidently, cells with decreased PRMT7 expression conferred a resistant phenotype to various topoisomerase II inhibitors (21). On the basis of our data, PRMT7 deficiency could evidently promote GC formation and repress plasma cell differentiation after immunization with SRBCs. In summary, these results imply that PRMT7 might be associated with the regulation of Bcl6. We observed that Bcl6 mRNA levels were increased in PRMT7-deficient GC B cells, whereas Irf4 and Prdm1, which are required for plasma cell differentiation, were repressed in the PRMT7-deficient GC-B cells. In addition, we measured the concentration of Igs in serum and found that the production of IgG1 and IgA seemed to be reduced. Using ChIP-PCR, we also found that PRMT7 was enriched in the promoter region of Bcl6. In contrast, its expression was repressed in PRMT7-overexpressing A20 cells, and PRMT7-mediated H4R3 methylation levels were also apparently increased in the region. Taken together, these results suggest that PRMT7 controls GC formation by regulating the expression of Bcl6 through the methylation of histone H4R3 sites on its promoter.

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Disclosures
The authors have no financial conflicts of interest.
References


Figure S1 Schematic representation of the *Prmt7* alleles

**Figure S1 Schematic representation of the *Prmt7* alleles**

Schematic diagram for exon-intron structure of the *Prmt7* allele (*Prmt7*) and targeting strategy to generate genetic ablation of *Prmt7* gene. The *Prmt7* allele contains eighteen exons (black boxes), and exon 5 is targeted. The PRMT7 targeting allele (*Prmt7*') contains the Pgk-Neo cassette (grey box), a pair of Loxp sequences (arrowheads) and Frt sequences (grey oval). The Pgk-Neo cassette is removed by mating with CAG-Flp mice to generate the *Prmt7* allele. The *Prmt7*/+ mice are mated with Zp3-Cre mice to obtain the *Prmt7* allele.
Figure S2 Splenomegaly in PRMT7-CKO mice

(A - B) Leukocytes in the spleen were analyzed by FACS. Frequency (A) and total number (B) of leukocyte subsets from spleens of 8-week-old WT (n = 4) and PRMT7-CKO mice (n = 4) obtained from the analysis results shown in (A). *P < 0.05 by Student's t-test. Bars represent the mean (±SD) values.

(C) Photographs of spleens from 5- and 10-month old WT and PRMT7-CKO mice, respectively.
(D) Average spleen weight of 5-month-old WT (n = 3) and PRMT7-CKO mice (n = 3) and
10-month-old WT (n = 3) and PRMT7-CKO mice (n = 4). *P < 0.05 by Student's t-test. Bars
represent the mean (±SD) values.

(E) H&E staining of the spleens from WT and PRMT7-CKO mice.
Figure S3 Enrichment of methylated H4R3 in Bcl6 regions

A

B

GO Analysis
- cell proliferation
- DNA repair
- response to DNA damage stimulus
- chromosome organization
- DNA metabolic process
- cell cycle
- regulation of RNA metabolic process
- regulation of transcription

C

WT
PRMT7-CKO

Relative mRNA level in resting B cell

0.0
0.5
1.0
1.5

Irf4
Prmt7

D

GFP PRMT7ov GFP PRMT7ov

Raji

A20

GAPDH

PRMT7

E

GFP PRMT7ov

H4R3me1

H4R3me2s

H4

H

CD19+ cells
CD19- cells

F

WT cKO WT cKO

PRMT7 H4R3me1 H4R3me2s H3R2me1 H3R2me2s H3

G

Input IgG anti-H4R3me2s Actb

Input IgG anti-H4R3me2s

Bcl6

H3

I

GFP PRMT7ov

Fold enrichment (H4R3me1 over IgG)

0 5 10 15 20

1 2 3 4 5

Bcl6 regions

J

Fold enrichment (H4R3me2s over IgG)

0 5 10 15 20

1 2 3 4 5

Bcl6 regions

K

Fold enrichment (PRMT7 over IgG)

0 0.5 1.0 1.5 2.0 2.5 3.0

1 2 3 4 5

Bcl6 regions
Figure S3 Enrichment of methylated H4R3 in Bcl6 regions

(A) Total mRNA was isolated from sorted splenic B cells from one WT and two PRMT7-CKO mice. Then, RNA-seq experiments were performed and comparative expression heat maps drawn based on all significant differentially expressed genes. RPKM, reads per kilobase of exon model per million mapped reads.

(B) Gene ontology analysis of the differentially expressed genes.

(C) Relative mRNA expression of Irf4 in resting mature B cells sorted from the WT and PRMT7-CKO mice. *P < 0.05 by Student's t-test. Bars represent the mean (±SD) values.

(D) Validation of the overexpression of PRMT7 in the Raji and A20 cell lines. ★ indicates endogenous PRMT7, and ☆ indicates exogenous GFP-PRMT7.

(E - F) Identification of global arginine methylation levels of various histone sites in PRMT7ov cells (E) and PRMT7-CKO splenic cells (F).

(G) Validation of the ChIP-PCR assay shown in Figure 6H using anti-H4R3me2s by PCR.

(H) Diagram showing the validated regions of Bcl6 by ChIP-PCR: 1, -1542~1399 bp; 2, -453~362 bp; 3, -59~52 bp; 4, 1538~1652 bp; and 5, 7690~7795 bp.

(I – K) Fold enrichment of H4R3me1 (I), H4R3me2s (J), and PRMT7 (K) over IgG in the Bcl6 regions shown in Figure S6H. Each ChIP experiment was repeated twice in triplicate.

*P < 0.05 by Student's t-test. Bars represent the mean (±SD) values.
### Table S1 Primers for qRT-PCR and ChIP-PCR

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a: primers used for checking Prmt7a allele; b: primers used for Prmt7b allele; c: primers used for Prmt7c allele.