PTEN-Regulated AID Transcription in Germinal Center B Cells Is Essential for the Class-Switch Recombination and IgG Antibody Responses

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PTEN-regulated AID transcription in germinal center B cells is essential for the class-switch recombination and IgG antibody responses. The germinal center (GC) is the region where antigen-activated B cells undergo proliferation and differentiation responses to differentiate into either plasma cells or memory B cells. Somatic hypermutation (SHM) and class-switch recombination (CSR) occur during the proliferation of GC B cells (GCBs). Activation-induced cytidine deaminase (AID) is responsible for both CSR and SHM in GCBs. Here, we show that ablation of PTEN through the Cγ1-Cre mediated recombination significantly influences the CSR and SHM responses. The GCs fail to produce the IgG1 B cells, the high affinity antibodies and nearly lost the dark zone (DZ) in Ptenfl/fl Cγ1Cre/+ mice after immunization, suggesting the impaired GC structure. Further mechanistic investigations show that LPS- and interleukin-4 stimulation induced the transcription of Cγ1 in IgM-BCR expressing B cells, which efficiently disrupts PTEN transcription, results in the hyperphosphorylated AKT and FoxO1 and in turn the suppression of AID transcription. Additionally, the reduced transcription of PTEN and AID is also validated by investigating the IgM-BCR expressing GCBs from Ptenfl/fl Cγ1Cre/+ mice upon immunization. In conclusion, PTEN regulated AID transcription in GCBs is essential for the CSR and IgG antibody responses.

Keywords: PTEN, germinal center, IgG1⁺ B cells, class-switch recombination, somatic hypermutation

INTRODUCTION

The germinal center (GC) is the region where antigen-activated B cells undergo proliferation and differentiation responses to differentiate into either plasma cells or memory B cells. Somatic hypermutation (SHM) and class-switch recombination (CSR) occur during the proliferation of GC B cells (GCBs) (1–3). SHM and CSR account for the generation of high affinity and class-switched B cells in humoral immunity (4). There are eight sets of CH exons at the Igh locus in mice, which are constituted as 5′-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3′. During the CSR, the assembled V(D)J exons from Cμ encoded IgM-expressing B cells is juxtaposed next to one of the sets of the downstream Cγ exons, converting IgM-expressing B cells to different IgH sub-clades (e.g., IgG3, IgG1, and IgG2b), which are, respectively, encoded by different Cγ genes (e.g., Cγ3, Cγ1, and Cγ2b) (5). Activation-induced cytidine deaminase (AID), as the B cell-specific factor, is required for the CSR (5). During GC responses, AID produces C:G to U:G and even C:G to A:T mismatches (7), which then triggers the mismatch and base-excision repairs. Furthermore, the generation of DNA double-strand breaks (DSBs) at switch regions between Sμ and a downstream S region leads to a rearranged Cγ locus and the deletion of the intervening sequence (8, 9). The repair of the AID induced DSBs
via nonhomologous end-joining (NHEJ) eventually completes the CSR by rejoining the two broken S regions (10, 11).

Previous studies suggested that the phosphatidylinositol-3-kinase (PI3K) and AKT signaling can both regulate the Ig gene rearrangement during B cell development and the CSR during GC responses (12–18). Phosphatase and tension homolog (PTEN) is known to negatively regulate PI3K-mediated growth, survival, proliferation and cellular metabolism of B cells (16, 17, 19–22). Thus PTEN deficiency alters B1 marginal zone B (MZB) and follicular B (FOB) cell subsets in Ptenfl/fl CD19-Cre mice (16, 17). Further study revealed that imbalanced PTEN and PI3K signaling impaired the pHc recombination in pro-B cells in Ptenfl/fl mb1-Cre mice (12). Recently, emerging efforts have been placed to investigate the molecular mechanism of PTEN- and PI3K-tuned AKT signaling in regulating the strength of GC responses (14, 15, 23). B cell specific deficiency of PTEN in Ptenfl/fl mb1-Cre mice leads to the severe defects of B cell development at the bone marrow stage due to failed VDJ recombination (12). The loss of the mature naïve B cell population in Ptenfl/fl mb1-Cre mice prevented the assessment of the function of PTEN in GCB-mediated CSR and antibody responses. As a solution, PTEN was recently knocked out in mature B cells in Ptenfl/fl hCD20TamCre mice, which demonstrated the importance of PTEN in regulating GC responses (23). Although mature B cell specific deficiency of PTEN in Ptenfl/fl hCD20TamCre mice excluded the B developmental defects as in the case of Ptenfl/fl mb1-Cre mice, the usage of Ptenfl/fl hCD20TamCre mice cannot explicitly separates the function of PTEN in mature B cell activation and proliferation upon antigen stimulation versus that in GC responses since GCBs were differentiated from activated mature naïve B cells after antigen stimulation. Here, to precisely assess the GC responses since GCBs were differentiated from activated mature naïve B cells after antigen stimulation versus that in mature B cells in Ptenfl/fl mb1-Cre mice, which demonstrated the importance of PTEN in regulating GC responses (23). Although mature B cell specific deficiency of PTEN in Ptenfl/fl hCD20TamCre mice excluded the B developmental defects as in the case of Ptenfl/fl mb1-Cre mice, the usage of Ptenfl/fl hCD20TamCre mice cannot explicitly separates the function of PTEN in mature B cell activation and proliferation upon antigen stimulation versus that in GC responses since GCBs were differentiated from activated mature naïve B cells after antigen stimulation. Here, to precisely assess the function of PTEN in GCB-mediated humoral responses in vivo, we used a mouse model with a PTEN deletion only in specific subsets of GCBs. Our results reveal that PTEN regulated AID transcription in GCBs is essential for the CSR and IgG antibody responses.

MATERIALS AND METHODS

Mice, Cell Culture
C57BL/6J (B6) background Ptenfl/fl mice (a kind gift from Dr. Wei Guo, Tsinghua University) were mated to Cy1-Cre transgenic mice (a kind gift from Dr. Tomohiro Kurosaki, Osaka University and Dr. Klaus Rajewsky, Max Delbrück Center) in which expression of Cre is controlled by the endogenous promoter of the B cell-specific gene Cy1. Offspring carrying Cy1-Cre and two copies of the floxed Pten allele or Cy1-Cre plus two copies of the WT Pten allele were used in the analyses as homozygous mutant (Ptenfl/fl Cy1Cre) or WT (Ptenfl/+ Cy1Cre) mice, respectively. All mice were maintained under specific pathogen-free conditions and used in accordance of governmental and institutional guidelines for animal welfare. Primary B cells were negatively isolated from the spleen of Ptenfl/+ Cy1Cre or Ptenfl/fl Cy1Cre mice as previously reported (24). Single cell suspensions were cultured in RPMI-1640 medium supplemented with 10% FBS, 50 μM β-mercaptoethanol (Sigma-Aldrich), penicillin/streptomycin antibiotics (Invitrogen) and Non-Essential Amino Acids (Invitrogen). B cells were stimulated for 4 days using 10 μg/mL LPS (Sigma) alone or LPS plus 50 ng/mL interleukin-4 (IL-4) (R&D) or 1 μg/mL anti-CD40 (eBioscience) alone or anti-CD40 plus 50 ng/mL IL-4 (R&D) in order to drive primary B cells class-switch in vitro.

Immunization, ELISA Assay, and Immunohistochemistry
For mice GC Flow cytometry analysis, 6-week-old Ptenfl/+ Cy1Cre and Ptenfl/fl Cy1Cre mice were injected intraperitoneally with 1 × 10⁸ sheep red blood cells (SRBCs, Bioren, China) or emulsified BSA in Alum adjuvant then analysis at day 7 after the immunization. Qβ virus-like particles (VLPs) were expressed in E. coli strain JM109 with exogenous expression plasmid pQ10 and then purified. The CpG contained VLPs were obtained by packaging VLPs with CpG ODN G10 in vitro as described (25). 6-week-old Ptenfl/+ Cy1Cre and Ptenfl/fl Cy1Cre mice were injected intraperitoneally with 10 μg VLP in 400 μL PBS for the immunization. Mice were analyzed at day 7 or day 14 after immunization. For NP-antigen specific T-cell-dependent immunization, 6-week-old Ptenfl/+ Cy1Cre and Ptenfl/fl Cy1Cre mice were injected on footpad with 10 μg NP33-KLH in 20 μL PBS and boost at day 35. Mice were bled at the indicated days after immunization.

To detect VLP or NP-specific IgM, IgG, IgG2a, IgG2c, and IgG3 in immunized mice (6 Ptenfl/+ Cy1Cre and 6 Ptenfl/fl Cy1Cre), 2 μg/mL VLP, 5 μg/mL NP33-KLH or 5 μg/mL NP30-BSA were coated on maxisorb plates (Nunc) and incubated overnight at 4°C. All these plates were blocked with 0.3% gelatin in PBS buffer (2 h at 37°C), followed by addition of pre-diluted mice serum into each well and incubated at 37°C for 1 h. 1:10,000 diluted HRP conjugated goat anti-mouse IgM, IgG, IgG2a, IgG2b, IgG2c, and IgG3 were used to detect VLP or NP-specific antibodies also the innate immune antibodies. Then followed the protocol as published before for the final readout results (26).

For immunohistochemistry 6-week-old Ptenfl/+ Cy1Cre and Ptenfl/fl Cy1Cre mice were injected intraperitoneally with 1 × 10⁸ SRBC in PBS. Spleen sections were frozen from day 7 after immunization in OCT compound (Sakura Finetek). After that spleen sections were stained with Alexa Fluor 488 anti-mouse/human CD45R/B220 (#103225, BioLegend), PE anti-mouse FAS (#152608, BioLegend), and APC anti-mouse CD3ε (#100312, BioLegend) by following the protocol described by Sandrine Sander (14).

Western Blotting
Primary splenic B cells or cultured cells were lysed by 2× Lysis buffer. Proteins were extracted by 10% Bis-Tris PAGE (Life technologies) with the cocktail of proteinase inhibitors (Life technologies) in it, transferred to polyvinylidene fluoride (PVDF). The electroblotted membranes were blocked by TBST containing 5% non-fat milk (BD) and were probed with anti-PTEN, anti-β-actin, anti-p-AKT 473, or anti-p-FoxO1/3a primary antibodies overnight at 4°C then immunoblotted with HRP-conjugated secondary antibodies (Dako). PTEN (138G6) Rabbit antibody (#9559), Phospho-AKT (Ser473) rabbit antibody (#4060), phospho-FoxO1 (Thr24)/FoxO3a (Thr32) rabbit antibody (#9464), and β-actin (13E5) rabbit antibody (#4970) were purchased from...
Cell Signaling Technology. AID rabbit antibody was a kind gift from Dr. Feilong Meng (Shanghai Institute of Biochemistry and Cell Biology, China).

**RT-PCR**

Pure Pten
t
Cy1Cre+/ mice and Pten
Cy1Cre+/ mice splenic B cells were stimulated for 4 days with 10 µg/ml LPS and 50 ng/mL IL-4. Pre-incubated IgM-BCR expressing B cells were sorted by using FACSAria III Cell Sorter (BD). SRBC immunized Pten
t
Cy1Cre+/ and Pten
Cy1Cre+/ mice IgM-BCR expressing GC Bs were first enriched by CL-7 marker (GL-7 monoclonal antibody, Biotin, eBioscience; streptavidin microbeads, Miltenyi Biotec) by using the LS columns (Miltenyi Biotec). On the basis of enrich, cells were sorted by using FACSAria III Cell Sorter (BD) after labeling with IgM-BCR expressing GC Bs dyes (anti CD19-APC, anti-FAS-PE, Alexa Fluor 488 goat Fab anti mouse IgM µ chain and ef450-streptavidin for GL-7).

Total RNA was extracted using TRIzol (GIBCO) according to the manufacturer’s instructions and was subjected to the reverse transcription to make cDNA. For PCR of post-switch Cγ1, transcript was amplified using the following primers pair: (Cγ1, 429 bp): 5’-GGG CCT TCC AGA TTG TTG AG-3’ (Cγ1 forward), 5’-GGA TCC AGA GTT CCA GTT CAC T-3’ (Cγ1 reverse). For amplification of the PTEN (387 bp) transcript, the primer pair of 5’-TTG AAG ACC ATA ACC CAC CAC AG-3’ (PTEN forward) and 5’-GGC AGA CCA CAA ACT GAG GAT TG-3’ (PTEN reverse) was used. Forward primer for AID (349 bp): 5’-GGG GTG AGT CCA ACC GCT GT-3’. Reverse primer for AID: 5’-GGC TGA GTG TGG TCC ATA TCA G-3’. Forward primer for control GAPDH (566 bp): 5’-TGT GTC CGT CGT GGT TCC AGT TG-3’. Reverse primer for GAPDH: 5’-TTG CTG TTG AAG ACC TCA CAG GAG-3’. PCR conditions were 94°C for 3 min, 94°C for 1 min, 60°C for 45 s, 72°C for 45 s, 30 cycles (from step 2 to step 4), and 72°C for 10 min for final extension.

**Flow Cytometry**

Cells were preincubated with FcBlock (anti-CD16/32, eBioscience) to minimize nonspecific staining. Cells were then stained with cocktails of various mAbs (anti-CD19, -B220, -IgG1, -IgM, -IgD, -CD43, -CD93, -CD21, -CD23, -CD5, -FAS, -CL-7, -CD3ε, -CXCR4, or anti-CD86) conjugated with different fluoroscein proteins. All antibodies were purchased from BD, eBioscience or BioLegend. Labeled cells were detected by 5 lasers Fortessa (BD), all data were analyzed with Flowjo software. Cell sorting was performed by using FACSAria III Cell Sorter (BD), following the protocols provided by BD.

**Microscopy Instruments**

Confocal images were captured using Olympus FV-1000 microscope equipped with 10 × objective lens and a 405, a 473, a 549, and a 635 nm laser. The acquisition was controlled by FV10-ASW4.0 software.

**Image Process and Statistical Analyses**

Images were analyzed by Image Pro Plus (Media Cybernetics) software following our previous protocols (27). Statistical tests were performed with Prism 5.0 (GraphPad). Two-tailed t tests were used to compare end-point means of different groups. Statistical significant results ($p$) are indicated as: *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

**RESULTS**

**Normal Development and Homeostasis of B Cells in Ptenfl/fl Cγ1Cre+/ Mice**

To detect the PTEN function in GC, we generated the Pten
Cy1Cre+/ mice by breeding Pten
Cy1Cre+/ mice to C1-Cre mice. In Pten
Cy1Cre+/ mice, PTEN would only be knocked out in B cells with Cy1 transcription, which are ideally the IgG1-BCR expressing B cells (Figure 2A). We avoided to breed Pten
Cy1Cre+/ mice with AicdaCre+/ mice since the Pten
AicdaCre+/ mice have been reported to develop severe submucosal hair loss, skin thickening and the manifestation of squamous papillomas (28).

We first examined the development and homeostasis of B cells in Pten
Cy1Cre+/ mice and confirmed that PTEN deletion in IgG1-BCR expressing B cells did not affect B cell development in the bone marrow and peripheral lymphoid organs (Figure 1B). Further flow cytometry analysis of the splenic IgM and IgG1-BCR expressing B cells showed comparable amounts of surface IgM or IgG1 BCRs in Pten
Cy1Cre+/ versus Pten
t
Cy1Cre+/ control mice (Figure 1C).

**Impaired Antibody Responses in Ptenfl/fl Cγ1Cre+/ Mice**

Pten
Cy1Cre+/ mice showed B cell normal development that allowed us to examine the humoral responses upon the immunization with either the T cell-dependent (TD) antigen NP33-KLH or the Qβ VLPS as reported (25). Age and gender matched Pten
Cy1Cre+/ and Pten
Cy1Cre+/ mice were undergone footpad injection with 10 µg NP33-KLH in 20 µL PBS and boost at day 35 for the TD antigen immunization (Figure 2A). For Qβ virus immunization, 6-week-old Pten
Cy1Cre+/ and Pten
Cy1Cre+/ mice were immunized intraperitoneally with 10 µg VLP in 400 µL PBS (Figure 2B). ELISA analyses showed that the IgM antibody responses upon the induction by both NP-KLH and VLP were significantly higher in the Pten
Cy1Cre+/ mice compared to the control Pten
t
Cy1Cre+/ mice (Figures 2A, B). However, the production of not only IgG1 but also IgG2b and IgG3 were significantly blunted in the Pten
Cy1Cre+/ mice (Figures 2A, B). We hypothesized that the decreased IgG antibody responses may be due to a damaged CSR reaction within the GC of Pten
Cy1Cre+/ mice.

**Damaged CSR in Ptenfl/fl Cγ1Cre+/ Mice**

To test whether or not PTEN deletion in IgG1+ B cells will damage the CSR within the GC reaction, GCs were induced by the immunization of SRBC in both Pten
Cy1Cre+/ and Pten
t
Cy1Cre+/ control mice. Flow cytometry analyses of the splenic B cells at day 7 after SRBC injection unexpectedly demonstrated an increased but not decreased levels of GCBS in Pten
Cy1Cre+/ mice than the control Pten
t
Cy1Cre+/ mice even though the size of the spleen of both types of mice was comparable (Figures 3A, B). Remarkably, further analyses showed that the IgM-BCR expressing GCBS were obviously increased while the class-switched IgG1-BCR...
expressing GCBs were almost lost in SRBC immunized \(Ptene^{fl/}\) \(Cy1^{Cre+}\) mice GC (Figures 3C, D). Similar results were also obtained in the immunized \(Ptene^{fl/}\) \(Cy1^{Cre+}\) and \(Ptene^{+/}\) \(Cy1^{Cre+}\) mice by the utilization of alum adjuvant-precipitate BSA (Figures S1A, B in Supplementary Material). These results suggested that PTEN deletion in IgG1+ B cells significantly damaged the CSR within GCs.

To further verify the above observation of the impaired CSR within the GC of \(Ptene^{fl/}\) \(Cy1^{Cre+}\) mice, we purified the splenic B cells and performed an in vitro CSR assay following the published protocols (13, 16). Clearly, B cells from \(Ptene^{fl/}\) \(Cy1^{Cre+}\) mice failed to undergo CSR to form IgG1-BCR expressing B cells in the presence of lipopolysaccharide (LPS) alone or LPS plus IL-4 after 4 days of stimulation, respectively (Figures S2A, B in Supplementary Material). Similar results were acquired in the presence of other CSR-driven reagents of anti-CD40 antibodies or anti-CD40 plus IL-4 (Figures S2A, B in Supplementary Material).

**Abnormal GC Structure and SHM in \(Ptene^{fl/fl}\) \(Cy1^{Cre+}\) Mice**

It is known that GC contains the light zone (LZ) and the dark zone (DZ). GCBs undergo consecutive and cyclic phases of proliferation and SHM in the DZ, followed by the migration to the LZ, where they capture and internalize antigen for the acquisition of survival signals from follicular helper T cells (3, 29, 30). We thus quantified the formation of LZ and DZ within the GC and found that DZ and LZ compartmentalization was severely disturbed in the \(Ptene^{fl/}\) \(Cy1^{Cre+}\) mice upon the immunization by both SRBC and VLP (Figures 4A-C). Moreover, we observed that the number of...
DZ B cells in Ptenfl/fl Cγ1Cre/+ mice GCs was dramatically decreased, consistent with a recent study showing that upon FoxO1 ablation or induction of PI3K activity, GCs lost their DZ, owing at least partly to downregulation of the chemokine receptor CXCR4 (14, 15). Since an essential step in the selection of high affinity GCBs is the recruitment of LZ GCBs into the GC DZ (31, 32), it is not a surprise that the SHM in GCBs was significantly damaged in Ptenfl/fl Cγ1Cre/+ mice upon the immunization by TD antigen NP33-KLH (Figure 4D). Lastly, it should be noted that even though the GC DZ formation was impaired in the Ptenfl/fl Cγ1Cre/+ mice, the size of the GCs was normal in the spleen section as detected by immunofluorescence and the number of GCBs per spleen section was even higher in these PTEN KO mice (Figures 5A,B), which are consistent with the results that Ptenfl/fl Cγ1Cre/+ mice exhibited an increased levels of GCBs than the control Pten+/+ Cγ1Cre/+ mice upon immunization (Figure 3A; Figure S1B in Supplementary Material).

**Repression of AID Induction in GCBs from Ptenfl/fl Cγ1Cre/+ Mice**

Our observations suggested that Cγ1 transcription-mediated PTEN KO impairs antibody responses, CSR activity, as well as the DZ and LZ compartmentalization and SHM within the GC. We next investigated the molecular mechanism accounting for...
Fig. U3 | Damaged class-switch recombination in Ptenfl/fl Cγ1Cre/+ mice. (A) The statistical comparison of the percentage of germinal center B cells (GCBs) from splenocytes at day 7 after sheep red blood cell (SRBC) immunization (left). Each symbol represents an individual animal (n = 8 for each group). The data represent the mean ± SD from at least three independent experiments. Two-tailed t-tests were performed for statistical comparisons. Flow cytometry analysis the increased GCBs number in the Ptenfl/fl Cγ1Cre/+ mice spleen (right). Values indicated the percentage of cells in the gated population. (B) Macroscopic appearance of spleen from day 7 after SRBC immunization of Pten+/+ Cγ1Cre/+ and Ptenfl/fl Cγ1Cre/+ mice. Data were given from one representative of at least two independent experiments. (C) Representative flow cytometry analysis of IgM, IgG1-BCR expressing GCBs from Pten+/+ Cγ1Cre/+ and Ptenfl/fl Cγ1Cre/+ mice at day 7 after SRBC immunization. Data were given from one representative of at least two independent experiments (n = 4 for each group). The cells were pregated in GCBs. (D) The statistical quantification of the flow cytometry data of IgM (left) and IgG1 (right) as shown in (C). Each symbol represents an individual animal (four mice for each group). The data represent the mean ± SD. Two-tailed t-tests were performed for statistical comparisons.
Abnormal germinal center (GC) structure and somatic hypermutation (SHM) in Ptenfl/fl Cγ1Cre/+ mice. (A) Representative flow cytometry analysis of GC light zone (LZ) and dark zone (DZ) splenocytes from Pten+/+ Cγ1Cre/+ and Ptenfl/fl Cγ1Cre/+ mice at day 7 after SRBC immunization. Data were given from one representative of at least two independent experiments (n = 4 for each group). The cells were pregated in GCBs. (B) The statistical quantification of the flow cytometry data of LZ (left) and DZ (right) splenocytes as shown in (A). Each symbol represents an individual animal (four mice for each group). The data represent the mean ± SD. Two-tailed t-tests were performed for statistical comparisons. (C) Representative flow cytometry analysis of GC LZ and DZ from Pten+/+ Cγ1Cre/+ and Ptenfl/fl Cγ1Cre/+ mice at day 14 after VLP injection (left). Statistical comparison for LZ (middle) and DZ (right) cells percentage of total GCBs was also shown. The data represent the mean ± SD of six mice per group in three independent experiments. Two-tailed t-tests were performed for statistical comparisons. (D) The affinity maturation of the NP-specific IgM, IgG1, IgG2b, and IgG3 antibodies in Pten+/+ Cγ1Cre/+ versus Ptenfl/fl Cγ1Cre/+ mice as determined by the ratio NP8/NP30. The data represent the median ± interquartile range from three independent experiments with six mice per group at the indicated time point and were analyzed with Kruskal–Wallis test. *p < 0.05; **p < 0.01.

the lack of CSR and SHM in the B cells from Ptenfl/fl Cγ1Cre/+ mice. It is well known that AID, which is specifically induced in the GCBs, is a crucial enzyme responsible for both CSR and SHM (6). Thus, we hypothesized that the PTEN expression level in these Ptenfl/fl Cγ1Cre/+ mice shall be influenced before the CSR reaction to drive the switch of IgM-BCR to IgG-BCR expressing B cells, which might subsequently impair the function of AID. To test this hypothesis, splenic B cells from Ptenfl/fl Cγ1Cre/+ and Pten+/+ Cγ1Cre/+ mice were stimulated with LPS and IL-4 to induce CSR in vitro. To specifically examine the B cells without effective CSR, we sorted the IgM-BCR expressing B cells from the LPS- and IL-4 stimulated splenic B cells (Figure 6A). WB of these stimulated IgM-BCR expressing B cells detected the reduced PTEN and AID protein expression in the cells derived from Ptenfl/fl Cγ1Cre/+ mice compared to the Pten+/+ Cγ1Cre/+ control mice (Figure 6B). Meanwhile, these IgM-BCR expressing B cells
also showed hyper-phosphorylated AKT and FoxO1 (Figure 6B). These results suggested that the AID transcription was affected by the hyper-phosphorylation of AKT and FoxO1 since AKT are known to inhibit the expression and function of AID (16, 23). Indeed, RT-PCR assay demonstrated that the level of PTEN and AID mRNA was markedly reduced in the stimulated IgM-BCR expressing B cells from Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice compared to those B cells from the Pten<sup>−/−</sup> Cy<sup>1Cre</sup> control mice (Figure 6C, top and Figure 6D). The transcription of Cy1 was also detected in both the murine control and KO IgM-BCR expressing B cells, which readily explained the Cy1-mediated PTEN deletion in the IgM-BCR expressing B cells in Pten<sup>−/−</sup> Cy<sup>1Cre</sup> mice (Figure 6C, top and Figure 6D). We further validated these conclusions by utilizing purified GC B cells in vivo from SRBC immunized Pten<sup>−/−</sup> Cy<sup>1Cre</sup> and Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice. We sorted the IgM-BCR expressing GC B cells from Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice also detected the significantly reduced transcription of PTEN and markedly reduced AID (Figure 6C, bottom and Figure 6D). The transcription of Cy1 was also detected in IgM-BCR expressing GC B cells from both Pten<sup>−/−</sup> Cy<sup>1Cre</sup> and Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice (Figure 6C, bottom and Figure 6D), which readily explained the Cy1-mediated PTEN deletion in the IgM-BCR expressing GC B cells. All these results demonstrated that the PTEN expression level in Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice was significantly impaired in GC B cells. Thus, PTEN regulated AID transcription through PI3K-AKT signaling pathway in GC B cells controls the CSR, IgG antibody response, and SHM.

**DISCUSSION**

We investigate the function of PTEN in regulating the strength of GC responses by employing a mouse model with the ablation of PTEN through Cy1-Cre mediated recombination. Upon immunization, we found significantly higher IgM antibody responses and drastically lower IgG1 antibody responses in the Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice compared to the control Pten<sup>−/−</sup> Cy<sup>1Cre</sup> mice. Mechanistically, we found that the ablation of PTEN leads to the abnormal GC responses as demonstrated by: (i) severely disturbed compartmentalization of DZ and LZ; (ii) significantly decreased amount of IgG1-BCR expressing B cells; and (iii) the SHM in Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice than the control Pten<sup>−/−</sup> Cy<sup>1Cre</sup> mice. Moreover, an in vitro CSR assay for the purified splenic B cells from Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice also indicates the blunted CSR under a variety of differentiation-promoting conditions. Interestingly, the results of the impaired GC function in this report are different from the PTEN deletion in Pten<sup>fl/fl</sup> CD19<sup>−/−</sup>, Pten<sup>fl/fl</sup> mb1<sup>−/−</sup> or Pten<sup>fl/fl</sup> hCD20tamCre mice in the published studies (13, 16, 23), the deletion of Pten<sup>fl/fl</sup> loci in Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice shall only be effective upon the transcription of Cy1-cre gene, which shall be ideally only available in the IgG1-BCR expressing B cells. Thus, theoretically, only the IgG1 antibody responses shall be affected in Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice upon immunization. However, an unexpected observation in this report is that the production of not only IgG1 but also IgG2b and IgG3 were significantly blunted in the Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice. All these intriguing results were explained by the further mechanistic investigations show that LPS- and IL-4 stimulation robustly induced the transcription of Cy1 in IgM-BCR expressing B cells, which efficiently disrupt the transcription of PTEN and AID in the stimulated splenic IgM-BCR expressing B cells from Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice. There are eight sets of C<sub>δ</sub> exons organized as 5′-VDJ-Cμ-Cδ-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3′ at the IGH locus in mice. Upon the CSR, the assembled V(D)J exons from Cμ encoded IgM-expressing naïve B cells is juxtaposed next to one of the sets of downstream C<sub>δ</sub> exons, allowing the production of different IgH classes (e.g., IgG3, IgG1, and IgG2b) (5). Thus, the transcription of Cy1 in IgM-BCR expressing B cells and the subsequent disruption of PTEN and AID expression explained the poor production of not only IgG1 but also IgG2b and IgG3 in the Pten<sup>fl/fl</sup> Cy<sup>1Cre</sup> mice.
The observation of the pre-transcription of \(\gamma_1\) in IgM-BCR expressing B cells is consistent with the examination of the \(\gamma_1\) reporter mice, which reported the \(\gamma_1\) reporter gene expression in 85–95% of the GCB fraction 10–14 days after immunization with SRBC (33). Not a surprise, hyper-phosphorylated AKT and FoxO1 are observed as a result of the drastically reduced...
expression of PTEN. The reduced transcription of PTEN and AID are also confirmed by investigating the IgM-BCR expressing GCB cells in vivo from SRBC immunized Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ mice. The hyper-phosphorylated AKT and FoxO1 in turn influence the regulation of cell growth, survival, proliferation, cell cycle and cellular metabolism were also reported (16, 19–22, 34, 35). Thus, it is also of interest to investigate how these events can influence the formation of GC structures.

In conclusion, our research provides an alternative mechanistic explanation for the significantly impaired CSR in PTEN deficient GCBs in addition to the recent published studies showing that constitutive PI3K activation or ablation of FOXO1 impairs AID targeting to particular switch regions, which leads to the partly lost CSR (14, 15). Our results demonstrate that PTEN regulated AID transcription in GCBs is essential for the CSR and IgG antibody response, and SHM.

ETHICS STATEMENT

All animal protocols used in this study are approved by the IACUC (Institutional Animal Care and Use Committee) of Tsinghua University and performed in accordance with guidelines of the IACUC. The laboratory animal facility has been accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International). The assurance identification number is 15-LWL2 and was issued by Dr. Zai Chang, the vice chair of IACUC of Tsinghua University, Beijing, China.

AUTHOR CONTRIBUTIONS

WL and JW conceived, designed, and drafted the article; JW and SL performed experiments and laboratory analysis; BH and MY supported the materials; JW and WL wrote the manuscript; WL, HQ, ZD, BH, MY, and JW reviewed and approved the manuscript final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00371/full#supplementary-material.

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**FIGURE 6 |** Repression of activation-induced cytidine deaminase (AID) induction in germinal center B cells (GCBs) from Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ mice. (A) Flow cytometry sorting of in vitro induced IgM-BCR expressing splenic B cells from Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ and Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ mice. Pure splenic B cells from control and KO mice (three mice for each group) were stimulated with LPS plus interleukin-4 (IL-4) for 4 days before sorting. (B) The expression levels of PTEN, p-AKT, p-FoxO1/3a, and AID were determined in Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ and Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ IgM-BCR expressing B cells (left). Reduced PTEN, AID expression and enhanced p-AKT and p-FoxO1/3a expression were observed in Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ IgM-BCR expressing B cells. The in vitro induced IgM-BCR expressing B cells were isolated by the FACSriaIII Cell Sorter as described in (A). Statistical comparison of protein expression level was also shown in right. Ratio values of different proteins from Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ IgM-BCR expressing B cells were normalized to that of the Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ IgM-BCR expressing B cells. Data were given from one representative of at least three independent experiments. *p < 0.05; **p < 0.01. (C) The mRNA levels of AID, C<sup>γ<sub>1</sub></sup>Cre/+ IgM-BCR expressing splenic B cells that was induced by LPS and IL-4 in vitro and the sheep red blood cell immunized Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ IgM-BCR expressing GCBs. The PTEN mRNA level was reduced and the AID mRNA expression level was nearly undetectable in Pten<sup>fl/fl</sup> C<sup>γ<sub>1</sub></sup>Cre/+ mice. The in vitro induced IgM-BCR expressing B cells were isolated by the FACSriaIII Cell Sorter as described in (Figure S3 in Supplementary Material). Data were given from one representative of at least three independent experiments. *p < 0.01; ***p < 0.001.
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