AID assists DNMT1 to attenuate BCL6 expression through DNA methylation in diffuse large B-cell lymphoma cell lines

Abstract

The BCL6 proto-oncogene encodes a transcriptional repressor, which is required for germinal centers (GCs) formation and lymphomagenesis. Previous studies have been reported that the constitutive expression of BCL6 leads to diffuse large B cell lymphoma (DLBCL) through activation-induced cytidine deaminase (AID) mediated chromosomal translocations and mutations. However, other DLBCLs (45%) without structural variants were characterized by abnormally high level of BCL6 expression through an unknown mechanism. Herein, we report that deficiency in AID or methyltransferase 1 (DNMT1) triggers high level of BCL6 expression. AID-DNMT1 complex binds to −0.4 kb −0 kb region of BCL6 promoter and contributes to generate BCL6 methylation which results in inhibition of BCL6 expression. The proteasome pathway inhibitor MG132 induces accumulation of AID and DNMT1, causes decreased BCL6 expression, and leads to cell apoptosis and tumor growth inhibition in DLBCL cell xenograft mice. These findings propose mechanistic insight into an alternative cofactor role of AID in assisting DNMT1 to maintain BCL6 methylation, thus suppress BCL6 transcription in DLBCL. This novel mechanism will provide a new drug selection in the therapeutic approach to DLBCL in the future.

Keywords: Activation-induced cytidine deaminase, DNA methyltransferase 1, BCL6 repression, DNA methylation, Diffuse large B-cell lymphoma

Introduction

The affinity maturation in humoral response is critical for effective host defense against microbial infections and tumors. The process depends on two B lymphocyte differentiation mechanisms: Ig somatic hypermutation (SHM) and class switch DNA recombination (CSR) [1–4]. Antigens stimulate naïve B cells to develop to be germinal center B cells (GCBS), which leads to upregulation of activation-induced cytidine deaminase (AID) expression [5,6]. The functions of AID could be summarized as following. Firstly, the deamination of AID converts cytosine in WGCW and WRC (W = A/T, R = A/G) motifs to uracil and produces U:G mismatch, then the error-prone repair cascade replaces the uracil in U:G mismatch to cause point mutations and double strand breaks (DSBs) for SHM and CSR, respectively [7–9]. Secondly, the deaminated 5mC by AID is recognized as a T and the generated T:G mismatch is finally corrected by C:G in error-prone repair pathway, which would implicate that AID involved DNA demethylation beyond DNA editing [10–14]. Third, AID prone to interact with some gene transcription regulatory factors (such as RAN PolII, Spr5), suggesting a alternative cofactor role of AID in regulating gene expression [15–17]. Thus, studies on AID are of considerable interest not only because of its central role in the generation of effective humoral immunity, but also because of its function in DNA methylation diversity or as a cofactor in GCBS, which potentiates AID mediated gene expression by epigenetic modifications or alternative cofactor role in B cell lymphoma.

AID’s function is not restricted to Ig loci, about 25% of highly expressed non-Ig genes in GCBS are mutated by AID because AID targeted hotspot motif lacks strict specificity [18,19]. Among these non-Ig genes, the proto-oncogene BCL6 is preferred to be deaminated by AID. BCL6 is a master regulator of the GC response to transcriptionally repress DNA

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damage response, cell cycle arrest and B cell maturation [20,21]. In the development of GCs, BCL6 positively regulates AID expression to mediate SHM in centroblasts formed dark zone or CSR in centrocytes formed light zone [20,21]. The accumulation of DNA lesions originated from high level of AID indirectly involves in BCL6 degradation, which is a feedback to decrease AID expression [22–24]. As a consequence, B cells with the highest affinity antibodies for antigens exit light zone of GC and mature to be plasma cells or memory B cells [25]. Genomic aberrations of BCL6 or alterations of genes that modulate BCL6 expression during the GC reaction lead to sustained BCL6 activation, which promotes the development of GC-derived lymphomas [26]. BCL6 overexpression is achieved through AID induced translocations in the first intron (~40%) or mutations of its promoter (~15%) in DLBCL patients [27]. However, in other DLBCLs (~45%) without BCL6 mutations or translocations [27], whether AID involved in modulating BCL6 expression is yet to be confirmed.

Here, we used the AID-deficient DLBCL cells to identify that AID and DNMT1 formed a complex to maintain the methylation of BCL6 promoter, thus negatively regulated BCL6 transcription by binding to its −0.4 kb region of the promoter. Moreover, the proteasome inhibitor MG132 blocked degradation of AID and DNMT1, and resulted in accumulation of AID and DNMT1, manifesting apparent cell apoptosis and tumor growth inhibition. Our results provide a mechanistic insight into the maintenance function of AID to subcutaneous DLBCL cell xenograft tumor, and identify undeveloped effect of MG132 in the repression of BCL6 expression and DLBCL treatment through inhibiting AID and DNMT1 degradation.

### Materials and methods

**Constructs and cells**

The pCas9-AID and pCas9-DNMT1 recombinant transgenes with gRNAs for AID and DNMT1 were constructed by ligating gRNA for AICDA or DNMT1 to pL-CRISPR.EFS.PAC plasmids, respectively. The sequences of gRNAs for AICDA or DNMT1 were listed in the Supplementary Table S1. The gRNA sequences were commercially confirmed (Sunny). pWPI-AID-GFP and pWPI-BCL6-GFP lentivirus constructs were ligated AID and BCL6 cDNA to pWPI-GFP plasmids, respectively. The sequences of primers for amplifying AID and BCL6 cDNA as follows: **AID F** (5'-CTGGGACACCCATATGGACAGCCTCTTGATG-3'), **AID R** (5'-CATTCCCTGAAGTTGCTATTAAAGTCCC-3'), **BCL6 F** (5'-GGGTGTAAAAATCGGCCCTCG-3') and **BCL6 R** (5'-GGGTGTAAAAATCGGCCCTCG-3').

**Genomic DNA isolation and bisulfite sequencing**

Genomic DNA was extracted from 1 to 5 × 10⁶ cells using the Genomic DNA Extraction Kit (TaKaRa, #D824A) according to the manufacturer’s instructions. cDNA was then synthesized with PrimeScript™ RT reagent Kit (TaKaRa, #RR037A), according to the manufacturer’s protocol. Quantitative PCR was performed with real-time PCR using Mx3000P (Agilent Technologies). Primers were listed in Supplementary Table S2. The relative mRNA level of genes were calculated according to the formula 2^ΔΔCt using β-actin as an internal control.

**Flow cytometry and antibodies**

To measure the proliferation and apoptosis ability of DLBCL cells, cells were prepared and stained according to standard procedures.
Measuring the ability of cells to proliferate involved the use of anti-CFSE (carboxyfluorescein succinimidyl ester) (BD eBioscience, #C34554) to stain the cells in advance for 6 hours. The cells were then washed twice with 1× PBS at 4 °C and analyzed by flow cytometry. Nutrition deficiency was performed to measure apoptosis. Cells were collected and washed twice with 1× PBS at 4 °C, incubated with anti-Annexin V and anti-7-ADD (BD eBioscience, #559763) for 15 minutes at room temperature. Finally, the cells were resuspended in flow cytometry buffer and analyzed by flow cytometry. All data were collected using a CytoFLEX Flow Cytometer (Beckman Coulter).

Immunoblot analysis

Cell pellet was lysed in RIPA buffer [50 mm Tris-HCl (pH 8.0), 0.15 m NaCl, 1% Triton X-100, 0.5% NaDeo, 0.1% SDS, 1 mm EDTA, 1 mm EGTA, 1 mm PMSF (Ameresco) and 1 µg/ml Peptatin A (Sigma) protease inhibitors]. The cells were then sonicated using BioruptorUMUCD-200 (Diagenode) for 15 min at a low speed. Cell lysates were centrifuged for 20 min at 15 000g at 4 °C, and the protein supernatant was collected. Protein samples were loaded on a 10% (w/v) Tris-HCl SDS-PAGE gels for electrophoresis, transferred to PVDF membrane (Millipore), blotted, and then probed with anti-AID (CST, #4959), anti-bcl-2 (Abcam, #ab33901), anti-DNMT1 (Abcam, #ab13537), anti-DNMT3A (Abcam, #ab2850), anti-DNMT3B (Abcam, #b2850), anti-Caspase 3 (Abcam, #ab13586) antibodies. Anti-GAPDH (Abcam, #ab9485) was used as a loading control. The signal was further detected using the secondary antibody of goat anti-Rabbit, goat anti-Rat, goat anti-Mouse IgG conjugated with horseradish peroxidase (Thermo Fisher). Band signal was visualized by Immobilon™ Western Chemiluminescent™ (Amersco). Integrating optical density was analyzed using gel-pro analyzer software (Media Cybernetics) and ratios of bands were all referenced to the loading control.

Immunoprecipitation

Briefly, Total protein was extracted as described in the part of immuno blot analysis. After pre-clearing the chromatin with Dynabeads Protein G beads (2×) (Invitrogen, #10003D), set a part of the aliquot aside as the input sample. Proteins from 5×10⁶ cells were then incubated with 5 µg specific antibody or normal IgG overnight at 4 °C. Anti-AID (Abcam, #ab59361), anti-DNMT1 (Abcam, #ab13537), immune complexes were pulled down through incubation with Dynabeads Protein G beads (2×) (Invitrogen, #10003D) for 3 hours. The beads were washed at 4 °C for ten times with RIPA buffer containing different concentration of NaCl. The pulldown proteins were denatured at 100 °C, and loaded on SDS-PAGE gels to perform immunoblot.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) was carried out according to the methods reported by Ji Y [28,29]. Briefly, 30×10⁶ cells were cross-linked with 1% HCHO (Sigma) for 15 min at room temperature, and the reaction was terminated with 0.125 m glycine. The precipitate was washed, resuspended in RIPA buffer and sonicated to fragment DNA of 0.3–0.5 kb. After pre-clearing the chromatin with Dynabeads Protein G beads (2×) (Invitrogen, #10003D), about one tenth of the aliquot was stored up as the input sample. The remnants were incubated with 5 µg specific antibody or normal IgG overnight at 4 °C. Immune complexes were pulled down with Dynabeads Protein G beads (2×) (Invitrogen, #10003D). After reversal of cross-links and purification of the DNA, qPCR was performed using SYBR Premix Ex Taq™ II (TaKaRa, #DRR081A) with Mx3000 thermocycler (Agilent Technologies). The primer sequences used for qPCR were ChIP-BCL6_F (CCTTCGCTGTAG-CAAAGCCT) and ChIP-BCL6_R (AACCTCTCGCTCCCTTTTGT). Input samples were diluted so that each IP and input sample would give approximately equal qPCR signals. Using standard curves generated for each region analyzed in each experiment, the amount of DNA obtained from immunoprecipitates and the input chromatin was calculated. ChIP-qPCR signals were expressed according to the following equation: (IP/Inputcorr)/positive control = (((IPspecific antibody – IPIgG)/ Input) × 1000)/positive control. ChIP experiments were performed with antibodies for anti-AID (Abcam, #ab59361), anti-DNMT1 (Abcam, #ab13537) and normal goat IgG (Santa Cruz, #sc2346).

Construction of luciferase plasmids and transient reporter assay

The fragments of promoter (p) and the sub-regions 1–4 (p1, p2, p3 and p4) of BCL6 gene were amplified by PCR from genomic DNA. Primers used were listed in Supplementary Table S3. The amplified fragments were digested with MluI and XhoI and were cloned into the pGL3-basic vector upstream of the firefly luciferase–encoding region. The constructed vector sequences were commercially confirmed (Sunny, China). SU-DHL-4, OCI-LY10 and OCI-LY19 cells were transiently transfected by equimolar amounts of reporter constructs with BCL6 promoter segments and the pGL3-basic vectors together with the X-tremeGENE HP DNA transfection reagent (Roche, #06366236001). Cells were cultured for 72 hours prior to collection. Luciferase activity was measured by the Luciferase Assay kit (Promega, #E1500) with an EnVision 2103 Multilabel Reader (PerkinElmer). Luciferase activity was presented as the ‘fold change’ relative to that obtained with pGL3-basic.

In vivo tumor cell engraftment and treatment of mice

NOD/SCID (Non-obese diabetic/severe combined immunodeficiency) mice were maintained in specific pathogen-free facilities at the Xi’an Jiaotong University Laboratory Animal Center. A murine model of human DLBCL was established by subcutaneous injection of 2×10⁶ DLBCL cells into the right flank of NOD/SCID mice. The tumor growth was monitored by measuring tumor size in two orthogonal dimensions. Tumor volume was calculated using the formula $V = \frac{1}{2}abl$, where a, b, and l are the diameters of the tumor. The tumor volume was continuously monitored twice a week (on Mondays and Wednesdays). All of the mice were euthanized 24 days post MG132 therapy. The tumors in the control and treatment groups were excised and weighed. All animal procedures were approved by the Institutional Animal Care and Use Committee of Xi’an Jiaotong University.

Statistical analysis

Unpaired t-test and ANOVA multiple tests were performed with GraphPad Prism 6.0 (GraphPad Software, LaJolla, CA, USA). Data were considered statistically significant if p values were less than 0.05, as indicated.
Results

AID loss impairs DLBCL cell apoptosis by elevating BCL6 level

To explore the action of AID in DLBCL cells without BCL6 translocations, genomic DNA derived from SU-DHL-4, OCI-LY10 and OCI-LY19 DLBCL cell lines were amplified for 12.7 kb ‘major breakpoint region’ spanning BCL6 intron 1 by using long-range PCR, respectively [30] (Fig. S1A). SU-DHL-4 cells were used as a negative control because previous studies have been reported that they were lack of rearrangement for BCL6 locus, while OCI-LY7 cells carrying BCL6 rearrangement were taken as positive control [31]. The relative density of the PCR products for BCL6 intron 1 showed insignificant difference in OCI-LY10 and OCI-LY19 compared to that in SU-DHL-4, indicating the selected DLBCL cells lacked BCL6 translocations (Fig. S1B). To further exclude the absence of rearrangement in both alleles of BCL6 gene, semi-quantitative PCR for amplifying BCL6 intron 1 products was performed to SU-DHL-4, OCI-LY10 and OCI-LY19 cells (Fig. S1C). We observed that the relative density of PCR fragments derived from OCI-LY10 and OCI-LY19 cells were equal to that from SU-DHL-4 cells (Fig. S1D). These results demonstrate that the BCL6 translocations are absent in the three selected SU-DHL-4, OCI-LY10 and OCI-LY19 DLBCL cell lines.

The DLBCL cells were transduced using CRISPR/Cas9 with gRNAs for AID to generate AID knockout SU-DHL-4 (4AIDKO), OCI-LY10 (10AIDKO) and OCI-LY19 (19AIDKO) cell lines, respectively (Fig. S2A). The levels of mRNA and protein for AID were significantly depleted in the three AIDKO DLBCL cells compared to their wild-type (WT) counterparts (Fig. 1A to D). To determine whether AID loss had an impact on cellular function, apoptosis of AIDKO DLBCL cells were rigorously examined using Annexin V staining in absence of nutrition. The three AIDKO cells presented approximately 30% less of Annexin V+ populations compared to that derived from WT ones (p < 0.001) (Fig. 1E), indicating reduced apoptosis of DLBCL cells after AID deficiency. The cells labeled with CFSE were monitored and dye dilution was tracked by flow cytometry, the collected data showed that the cell division rates of 4AIDKO, 10AIDKO and 19AIDKO cells were faster than those of WT cells (Fig. S2B). In addition, the transcripts of pro-apoptosis genes (FAS, BIMEI, BMF, BAD and BAX) obviously dropped in AIDKO cells (all p < 0.05) (Fig. S2C). Meanwhile, the transcription levels of anti-apoptotic genes (BCL2, A1, BCLW, CLAP1, CFLIP, MCL1) apparently increased in AIDKO cells (all p < 0.05) (Fig. S2D).

Fig. 1. AID deficiency impairs apoptosis of DLBCL cells. (A, B) AID depletion by three guide RNAs (AIDKO1-AIDKO3) of CRISPR/Cas9 in SU-DHL-4 cells was confirmed by qRT-PCR (A) and immunoblots (B). Data shown are representative of 3 technical replicates. GAPDH protein was used as an internal control for immunoblots. AIDKO2 was the gRNA with the best efficiency in AICDA knock out. SU-DHL-4 cells were represented by 4 in abbreviations. (C, D) The depleting efficiency of AID by CRISPR/Cas9 vector with AIDKO2 gRNA was also confirmed in OCI-LY10 and OCI-LY19 cells by qRT-PCR (C) and immunoblot (D). Data shown are representative of 3 technical replicates. GAPDH protein was used as an internal control. OCI-LY10 and OCI-LY19 cells were represented by 10 and 19 in abbreviations, respectively. (E) Flow cytometry was performed for apoptosis maker Annexin V and 7-AAD stained WT and AIDKO DLBCL cells (4, 10 and 19) after nutrition deficiency treatment. Histograms indicate the percentages of apoptosis cells. Data shown are representative of 3 independent experiments. Data are represented as mean ± SD. *,**, and *** represent p < 0.05, p < 0.01 and p < 0.001, respectively.
Taken together, these results suggest that AID deficiency impairs the DLBCL cell proliferation.

To investigate whether AID was involved in BCL6 expression in the DLBCL cells, the mRNA and protein levels of BCL6 were analyzed in WT and AIDKO DLBCL cells, respectively. We observed that the expression levels of BCL6 increased 5 to 15 folds in AIDKO cells in comparison to that in WT cells (Fig. 2A). The specific elevated BCL6 level in three AIDKO DLBCL cells was confirmed by immunoblots (Fig. 2B). The data suggest that AID deficiency potentiates to up-regulate oncogenic BCL6 expression in DLBCL cells with higher proliferative rate.

The deamination of AID has no role for DNA demethylation on BCL6 gene

To evaluate whether AID deamination contributed to BCL6 expression by mutations, genomic DNA was purified from WT and AIDKO DLBCL cells, respectively. It has been reported that ±1-kilobase (kb) segment of the major transcription start site (TSS) for each gene is the preferred region for AID targeting [19]. A 0.92 kb BCL6 segment, located upstream of TSS, was selected to amplify, sequenced and analyzed for point mutations with 34,960 nucleotides of sequence (Fig. 2C). The mutation frequency for

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**Fig. 2.** The deamination of AID has no role for DNA demethylation on BCL6 gene. (A) BCL6 transcripts were detected in 4WT and 4AIDKO, 10WT and 10AIDKO, 19WT and 19AIDKO by qRT-PCR. Data shown are representative of 3 technical replicates. (B) BCL6 protein levels were measured in 4WT and 4AIDKO, 10AIDKO and 19AIDKO cells by immunoblots, and GAPDH protein was taken as an internal control. (C) Schematic diagram shows AID mediated BCL6 mutation region. Not drawn to scale. (D) Histograms represent point mutation frequency of BCL6 promoter in 4WT and 4AIDKO cells. Mutation was measured in DNA from 2 independent preparations. (E) Schematic diagram shows CpG sites in the promoter of BCL6 gene. The hollow box represents BCL6 promoter. The shadow boxes represent CpG sites. The segment for 17 CpG sites locates in −1.07 kb to −0.84 kb of BCL6 promoter. The segment for 4 CpG sites is in the −0.51 kb to −0.24 kb of BCL6 promoter. Not drawn to scale. The bisulfite sequencing was used to examine methylation status of these CpG sites in BCL6 promoter. Black circles signify methylated CpGs, and white circles indicate demethylated CpGs. (F) Histograms indicate results for demethylation frequency of CpG sites in BCL6 promoter as detecting in E. Methylation was measured in DNA from 2 independent preparations. Data are presented as mean ± SD. *, ** and *** represent p < 0.05, p < 0.01 and p < 0.001, respectively. n.s. indicates no significance.
BCL6 analyzed from WT cells displayed 1.9 × 10⁻² mutations per base pair (bp). AIDKO cells yielded about 1.34 × 10⁻² mutations per bp (Fig. 2D). Comparing to the data, we conclude that BCL6 mutation frequency shows insignificant difference between WT cells and AIDKO cells.

AID involves in DNA demethylation process and usually targets promoters in B or non-B cells [5,10]. To verify the epigenetic role of AID related to BCL6 expression in AIDKO cells, the bisulfite sequencing was used to examine methylation status of BCL6 promoter region (Fig. 2E). The methylation level of the 4 CpG sites located in the −0.51 kb to −0.24 kb did not increase along with AID deficiency. Instead, it slightly decreased in AID-deficient DLBCL cells (Fig. 2F). However, the methylated levels of 17 CpG sites located in the −1.07 kb to −0.84 kb did not show significant difference in SU-DHL-4 cells with or without AID expression (Fig. S3A and B), suggesting an indirect role of AID in methylation on CpG sites closed to TSS in BCL6 promoter. There is no tight link between AID deamination activity and BCL6 demethylation.

AID assists DNMT1 to attenuate BCL6 expression via maintaining DNA methylation

To search the factors assisted by AID involved in BCL6 methylation, we focused on conserved DNA methyltransferases, including DNMT1, DNMT3A and DNMT3B, which play the most important roles in de novo synthesis and maintenance of DNA methylation in mammal cells [32–34]. To gain more insights into the mechanism of DNA methylation controlling BCL6 expression in DLBCL cells, we demonstrated that DNMT1 but not DNMT3A or DNMT3B dramatically reduced in AIDKO DLBCL cells compared to WT counterparts (Fig. 3A and B; Fig. S4A). To identify the involvement of endogenous DNMT1 in BCL6 methylation, we created DNMT1 knockout SU-DHL-4 (4DNMT1KO), OCI-LY10 (10DNMT1KO) and OCI-LY19 (19DNMT1KO) cell lines using CRISPR/Cas9 technique (Fig. 3C and D; Fig. S4B). The BCL6 expression was up-regulated in contrast to decreased DNMT1 level in the three DNMT1KO DLBCL cells (Fig. 3C and D; Fig. S4C). To test whether AID synergized DNMT1 to inhibit BCL6 expression, 4WT and 4AIDKO DLBCL cells were treated with a DNMT1 inhibitor 5-Azacytidine [35], we showed that BCL6 mRNA level was at the peak following the repression by both AID and DNMT1 (Fig. 3E). The immunoblot analysis also confirmed a promoting effect to BCL6 expression when both AID and DNMT1 were absent in DLBCL cells (Fig. 3F, lane 4, 8 12). These findings demonstrate that AID and DNMT1 collaborate to inhibit BCL6 expression in DLBCL cells.

Following the revelation of a reduced DNMT1 level in AIDKO cells (Fig. 3A), we hypothesized that AID regulated DNMT1 expression at either a transcription or a post-transcription level. The transcripts of DNMT1 exhibited conspicuous change in three DLBCL cells in presence or absence of AID (Fig. 3G), arguing that AID modulated DNMT1 expression at a post-transcription level. We used the proteasome inhibitor, MG132, which is used to inhibit protein degradation. The AID and DNMT1 expression increased in the three DLBCL cells after MG132 treatment (Fig. 3H, lane 3, 7 and 11). Meanwhile, both AID and DNMT1 were disappeared following 5-Azacytidine treatment in DLBCL cells (Fig. 3H, lane 2, 6 and 10). However, the 5-Azacytidine induced deletion was rescued by MG132 treatment (Fig. 3H, lane 4, 8 and 12). We interpreted these findings that AID and DNMT1 stabilized each other to avoid their degradation. To confirm the existence of AID-DNMT1 complex in DLBCL cells, we performed immunoprecipitation (IP) experiments to reveal the association of AID and DNMT1. Pre-clearing of nuclear extracts using anti-AID or anti-DNMT1 removed the complexes without AID or DNMT1. The complexes hosted by anti-AID showed the existence of DNMT1 (Fig. 3I). The DNMT1-containing complexes hosted by anti-DNMT1 revealed AID existence (Fig. 3J). The results suggest that AID and DNMT1 co-reside in DLBCL cells to form complexes. Taken together, the data demonstrate that AID assists DNMT1 as a cofactor to attenuate BCL6 by maintaining DNA methylation.

AID-DNMT1 complex binds to −0.4 kb to 0 kb BCL6 promoter region

To investigate whether AID and DNMT1 bound to BCL6 promoter directly, Chromatin Immunoprecipitation (ChiP) assays were performed using antibodies against AID and DNMT1. Immunoprecipitated DNA was analyzed by qPCR with primers that amplify two fragments which locate in −0.43 kb to −0.23 kb (site 1) and −0.21 kb to −0.03 kb (site 2) regions (Figure S5A). AID and DNMT1 binding to the two sites were extremely higher in SU-DHL-4, OCI-LY10 and OCI-LY19 cells than that in their KO counterparts, respectively (both p < 0.05) (Fig. 4A and B). We additionally revealed that enrichment of H3k4me3, which marked active promoter, largely presented in both WT and AIDKO cells (Figure S5B), suggesting that AID-DNMT1 complex binds to H3k4me3 enriched BCL6 promoter directly.

To identify whether the distinct recruitment of AID contributed to the repression of BCL6 transcription, a 1.8 kb BCL6 promoter segment (p) (positions −1.8 kb to −0 kb) was amplified and ligated to constructs with a firefly luciferase–encoding region (Fig. 4C). We observed that the construct with p induced more than 3 folds higher relative luciferase activity in AIDKO DLBCL cells than that in WT DLBCL cells (p < 0.05) (Fig. 4D to F), indicating AID loss induced BCL6 promoter-driven transcription initiation. To further test the potential AID targeted minimum sequence needed for repressing BCL6 transcription, we generated four luciferase reporter assay systems containing four small segments derived from the 1.8 kb region, including p1 (positions −0.4 kb to 0 kb), p2 (positions −0.9 kb to −0.4 kb), p3 (positions −1.4 kb to −0.9 kb) and p4 (positions −1.8 kb to −1.4 kb) (Fig. 4C). The construct with p induced approximately 2.5 to 3 folds more relative luciferase activity in AIDKO DLBCL cells than that in WT DLBCL cells (all p < 0.05), while other three segments (p2, p3 and p4) had no function in arousing luciferase activity (Fig. 4D to F), suggesting p1 was the specific region that AID functionally repressed BCL6 expression. Similarly, we tested p and p1 regions mediated nearly 3 to 5 folds more relative luciferase activity in DNMT1KO cells than WT cells (Fig. 4G to I), indicating the minimal sequence of AID and DNMT1 targeting is −0.4 kb to 0 kb in BCL6 promoter. Together, these results suggest AID-DNMT1 complex abrogates BCL6 transcription level by binding to the −0.4 kb −0 kb region of BCL6 promoter in DLBCL cells.

MG132 suppresses DLBCL growth in vitro and in vivo

On the basis that AID-DNMT1 complex inhibited BCL6 transcription in DLBCL cells, we asked whether MG132 suppressed transcription level of BCL6 was capable of antilymphoma activity in DLBCL. After the treatment of WT, AIDKO2 and DNMT1KO3 cells derived from SU-DHL-4, OCI-LY10 and OCI-LY19 cell lines with MG132, the trend of cell apoptosis was observed by analyzing Annexin V staining. The apoptosis ratios of MG132 treated WT, AIDKO2 and DNMT1KO3 DLBCL cells apparently increased (Fig. 5A). The cytotoxicity of MG132 to WT DLBCL cells were superior to either AIDKO2 or DNMT1KO3 cells, indicating that MG132 killing DLBCL cells through accumulating AID and DNMT1 (Fig. 5A). The active caspase 3 level rose after MG132 treatment (Fig. S6A). In addition, the anti-apoptotic gene expression including BCL2 as well as BCL2 also dropped (p < 0.05) (Fig. S6B). Several apo-
Fig. 3. The stabilized AID-DNMT1 complex suppresses BCL6 expression in DLBCL cells. (A) Immunoblot measurement of DNMT1 and AID protein level was performed in 4WT and 4AIDKO, 10WT and 10AIDKO, 19WT and 19AIDKO cells, respectively. GAPDH protein was used as an internal control. (B) DNMT3A, DNMT3B and AID proteins were measured in WT and AIDKO DLBCL cells by immunoblots. GAPDH protein was taken as an internal control. (C) Immunoblot detection of DNMT1, AID and BCL6 proteins was performed in SU-DHL-4 cells with CRISPR/Cas9 including three gRNAs for depleting DNMT1. DNMT1KO3 was the gRNA with the best efficiency in DNMT1 knock out. GAPDH protein was used as an internal control. (D) DNMT1, AID and BCL6 protein levels were detected in 10WT, 19WT and 10DNMT1KO, 19DNMT1KO cells by immunoblots, and GAPDH protein was used as an internal control. (E) BCL6 transcripts were detected in 4WT and 4AIDKO cells after 5-Azacytidine (10 μM) (5-Aza) treatment for 24 hours in vitro by qRT-PCR. Data shown are representative of 3 technical replicates. (F) DNMT1, BCL6 and AID protein levels were detected in 4WT and 4AIDKO, 10WT and 10AIDKO, 19WT and 19AIDKO cells treated with 5-Azacytidine as in E. GAPDH protein was used as an internal control. (G) DNMT1 transcripts were detected in 4WT and 4AIDKO, 10WT and 10AIDKO, 19WT and 19AIDKO cells by qRT-PCR. Data shown are representative of 3 technical replicates. (H) Immunoblots of DNMT1 and AID protein levels were performed in 4, 10 and 19 cells treated with MG132 (10 μM) and/or 5-Azacytidine (10 μM). GAPDH protein was taken as an internal control. The 5-Azacytidine treatment was 24 hours, and MG132 treatment was 8 hours, the combined treatment of DLBCL cells (4, 10 and 19) was performed by adding MG132 following 5-Azacytidine treatment for 16 hours to continuously treat for 8 hours. (I, J) DNMT1 and AID proteins were detected by immunoblots after immunoprecipitation (IP) by anti-AID pulldown (I) and anti-DNMT1 (J) in 4, 10 and 19 cells. Data shown are representative of 3 independent experiments. Data are presented as mean ± SD. *,**, and *** represent p < 0.05, p < 0.01 and p < 0.001, respectively. n.s. indicates no significance.
sis associated genes (for example, BIMEI, BMF, BOK, BAD, PUMA, BAK) level apparently increased (all p < 0.05) (Fig. S6C). In general, MG132 has the ability to induce DLBCL cell apoptosis by inhibiting DNMT1 and AID degradation through proteasome pathway.

To determine whether MG132 could affect lymphoma growth in vivo, the murine WT, AIDKO2 and DNMT1KO3 DLBCL cells xenogeneic tumor model were generated. When the tumor volume reached to 80 mm³–100 mm³, the intraperitoneal administration of solvent or MG132 to mice was performed (Fig. S6D). The administration of MG132 strongly impaired the expansion of WT DLBCL cells, manifesting reduced tumor volume and tumor weight. In contrast, solvent treatment completely failed to exert any inhibitory effects on the progression.
of WT DLBCL cells, showing big tumor volume and much tumor weight (Fig. 5B and C). However, the tumor volume and tumor weight from AIDKO2 and DNMT1KO3 cells xenogeneic tumor models showed relatively inefficient inhibition effect after MG132 treatment, indicating that MG132 inhibited DLBCL xenogeneic tumor growth via increasing AID and DNMT1 activity at the BCL6 promoter region in vivo. These findings indicate that MG132 has a significant therapy function in the treatment of DLBCL disease in vitro and in vivo.

Discussion

Many experiments in a variety of systems have confirmed the role of AID in plastic diversity of DNA methylation and corresponding gene regulation in normal GCs. DLBCLs originate from GCs and are characterized by BCL6 dysregulation [5,11,36]. However, until now, the connections are limited between AID’s epigenetic role or cofactor role and BCL6 expression in DLBCL. The present results here demonstrate...
that AID assists DNMT1 to bind to $-0.4$ kb $-0$ kb segment of *BCL6* promoter and mediates BCL6 to undergo DNA methylation. The process consequently inhibits BCL6 expression in DLBCLs without BCL6 mutations or translocations, thereby providing a mechanism for the therapy of proteasome inhibitor MG132 to DLBCL by inducing accumulation of AID and DNMT1. The data here reveal that an alternative cofactor role of AID to DNMT1 in maintaining BCL6 methylation in DLBCL.

Deregulated BCL6 expression is commonly associated with DLBCLs, due to its promoter mutations, chromosomal translocations or epigenetic modifications or cofactor role in gene expression [22,37–38]. Therefore, understanding BCL6 deregulation from AID mediated gene expression regulation by epigenetic alteration or alternative cofactor role could help in revealing molecular pathogenesis of DLBCLs beyond AID associated mutations or translocations [27]. Studies on AID targeting in the genome have been demonstrated that AID occupies at a large number of promoters beyond Ig loci, implying AID might be involved in controlling gene expression [39]. Here, we show that AID and DNMT1 formed complex to bind to BCL6 promoter (Fig. 3 and Fig. 4). This indicates that the occupancy of AID-DNMT1 complex on BCL6 promoter efficiently impedes the access of transcription initiation factors to BCL6 locus, thus suppresses BCL6 transcription in DLBCL. Notably, the data provided here indicate that the methylated CpG sites ($-0.51$ kb to $-0.24$ kb) are located in the AID-DNMT1 complex binding region in BCL6 promoter ($-0.4$ kb $-0$ kb) (Fig. 2 and Fig. 4). On the basis of our findings and those of others [33], we suggest that DNMT1 instead of AID is a key factor to maintain methylation of BCL6 promoter in DLBCL (Fig. 2, Fig. 5 and Fig. S3). AID recruits DNMT1 to the $-0.4$ kb $-0$ kb region of BCL6 and assists DNMT1 as a cofactor to maintain the methylation of BCL6 promoter, thus represses BCL6 expression in DLBCL.

The role of AID to BCL6 in DLBCL is depicted as AID mediated BCL6 mutations or translocations inducing uncontrolled BCL6 expression [27,37–39]. We excluded structural variants of BCL6 locus in the DLBCL cells used in our experiments (Fig. 2). Our results demonstrate that AID assisted DNMT1 to inhibit BCL6 transcription through maintaining DNA methylation on its promoter (Fig. 3, Fig. 4 and Fig. S3). It seems that one contradiction between the common deamination function of AID to BCL6 and our results appeared. One likely explanation for this is that the reported high expression of AID in DLBCL is the effect of its antagonism to BCL6 high expression. Our hypothesis was confirmed by constructing BCL6 and AID over-expressing DLBCL cell lines. The data indicate that overexpressed BCL6 increases AID levels (Fig. S7A and B), while increased AID forces to reduce BCL6 expression (Fig. S7C and Fig. 2).

For the initiator of DLBCL pathogenesis, we propose that AID off-target might be the main driver of DLBCL with BCL6 translocations, while BCL6 deregulation may be the main inducer of DLBCL without BCL6 translocations. It seems that AID has two edges in DLBCL. For DLBCL without translocations, up-regulation of AID might be a positive way to perform antagonism to BCL6 induced DLBCL deterioration. Therefore, we suggest a AID-BCL6 regulation loop in DLBCL. Firstly, BCL6 deregulation drives the deterioration of DLBCL, high expression of BCL6 mediate up-regulation of AID. Secondly, to antagonize BCL6 driven DLBCL, highly expressed AID consecutively down-regulates BCL6 level through forming complex with DNMT1 (Fig. S7D).

In the last decade, many novel therapeutic regimens have been developed to treat DLBCL, such as radio- and/or immuno-, multi-agent chemotherapy, and even R-CHOP [40–43]. Clinical therapeutic strategies for DLBCL by targeting BCL6 through BCL6 inhibition or degradation are well appreciated [44–47]. However, these conventional therapeutic methods have less migration effect in cancer therapy, there are still more than 30% of patients, who are non-responsive to the available treatment or will develop relapsed/refractory disease with resistance [48]. Based on the data shown here, we attempted to find another effective therapeutic approach for DLBCL. Therefore, a proteasome inhibitor, MG132, was used here to treat DLBCL. Indeed, it is found from the treatment data in vitro and in vivo that AID and DNMT1 accumulation by MG132 treatment can slow down the disease progression or even treat DLBCL (Fig. 5), suggesting a new selective therapy strategy in clinical DLBCL treatment. The MG132 treatment suppressed BCL6 transcription in DLBCL, which is superior to and eliminated the post-transcription degradation of BCL6 protein.

Here, our data support a possible model by which the interaction of AID and DNMT1 controls BCL6 transcription in DLBCL: (1) the loss of AID or DNMT1 leads AID-DNMT1 complex to disassociate from BCL6 promoter. As a consequence, the methylated BCL6 promoter undergoes demethylation and the expression of BCL6 increases in DLBCL (Fig. 6A). (2) AID-DNMT1 complex is recruited to the $-0.4$ kb $-0$ kb region of BCL6 promoter, AID assists DNMT1 to maintain methylation of BCL6 promoter and inhibits BCL6 expression in DLBCL (Fig. 6B). (3)
MG132 mediates BCL6 repression by avoiding AID and DNMT1 degradation. AID-DNMT1 complex has more opportunity to be recruited to BCL6 promoter, and drives dynamic methylation (Fig. 6C).

In conclusion, our work provides a novel mechanism of AID and DNMT1 cooperation maintains the methylation of BCL6 promoter, which in turn suppresses BCL6 expression in DLBCL. Our model might be useful for clinical staging of DLBCL by using AID and BCL6 proteins. In addition, blockade of AID and DNMT1 degradation by proteasome inhibitor MG132 could induce DLBCL cell apoptosis and would develop an effective therapeutic strategy for DLBCL in future.

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Conflict of Interest

The authors do not have any conflict of interest.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper or in the Supplementary Materials.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2020.01.002.

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