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Activation-induced cytidine deaminase displays an alternative co-factor for modulating *PIM1* expression in diffuse large B cell lymphoma cell lines

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ABSTRACT

Diffuse large B cell lymphoma (DLBCL) is a B cell neoplasm characterized by high *PIM1* expression, which is responsible for poor prognosis. Activation-induced cytidine deaminase (AID) is closely linked to *PIM1* hypermutation in DLBCL. Here, we found that the DNA methyltransferase 1 (DNMT1) level decreased with AID depletion in the DLBCL cell line SU-DHL-4, and increased significantly when AID was highly expressed. The double ablation of AID and DNMT1 contributed to increased *PIM1* expression, which initiated faster DLBCL cell proliferation, whereas ten-eleven translocation family member 2 (TET2) decreased with AID deficiency and increased with AID overexpression in DLBCL cell line OCI-LY7. The double depletion of AID and TET2 was associated with decreased *PIM1* levels and showed slower cell division. We suggest an alternative role of AID as a co-factor of DNA methylation cooperated with DNMT1, or of DNA demethylation associated with TET2 in modulating *PIM1* expression. Our findings demonstrate that AID interacts with either DNMT1 or TET2 to form a complex to bind with a *PIM1* promoter and thus is responsible for the modulation of *PIM1* expression. These results provide insights into an alternative role of AID to DLBCL-associated genes.

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Introduction

Diffuse large B cell lymphoma (DLBCL) is a hematological malignancy characterized by a large degree of heterogeneous gene expression (1-3). One such gene is proto-oncogene *PIM1* (the proviral insertion site of Moloney murine leukemia virus) which is highly expressed in up to 50% of DLBCL patients (4,5) and promotes survival and proliferation of DLBCL cells (6-8) . *PIM1*, located on chromosome 6p21, produces a transcript that contains a G/C-rich sequence in the 5′ untranslated region (UTR) and five copies of AUUUA destabilizing motifs in the 3′ UTR. The use of alternative translation initiation sites (AUG or CUG) results in the synthesis of 34 KD and 44 KD, two different protein isoforms that both retain their serine/threonine kinase activity. Moreover, PIM1 kinases have no regulatory domains (5). The crystal structure of PIM1 reveals the presence of a unique hinge region that connects the two lobes of the protein kinase domain. As a result, the manner of ATP binding to PIM1 kinases fundamentally differs from the manner in which it binds to other protein kinases, which develop several small-molecule inhibitors for PIM1 kinases. These compounds either interact with the hinge backbone of PIM1 through hydrogen bond formation or form polar interactions with the active site lysine residue (9). However, PIM1 inhibitors only modestly impair DLBCL cell survival, suggesting that PIM1 kinases represent molecular progression markers rather than primary therapeutic targets in DLBCL (10). Thus, investigating the mechanisms of *PIM1* expression in DLBCL beyond the direct inhibition of PIM1 would be an effective approach for improved treatment of DLBCL with clinical heterogeneity.

Previous studies indicate that aberrantly high *PIM1* expression occurs primarily due to activation-induced cytidine deaminase (AID)-mediated chromosomal translocations or abnormal *PIM1* hypermutation, leading to DLBCL lymphomagenesis (10-12). A genome-wide translocation sequencing (HTGTS) study demonstrated an important role of AID in controlling methylation diversity in germinal center B cells (GCB) (13), indicating the possible involvement of AID in the regulation of gene expression through its epigenetic functions (8,13-15). Elucidating the mechanism underlying the alternative epigenetic modulation of AID to *PIM1* might provide a good modality for DLBCL treatment.

Here, we used AID-deficient and overexpressed DLBCL cells to identify that AID positively or negatively regulates *PIM1* by binding to the promoter together with DNA methyltransferase 1 (DNMT1) or ten-eleven translocation family member (TET2) respectively(16,17). We observed that *PIM1* expression is regulated either by treatment with

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5-azacytidine (an inhibitor of DNMT1) or with DMOG (an inhibitor of TET2). Our results provide a novel concept for an alternative co-factor role of AID in modulating gene expression by forming different complexes with TET2 or DNMT1.

Materials and Methods

Cell lines and cell treatment

BeNa Culture Collection provided SU-DHL-4 and OCI-LY7 DLBCL cell lines. The cells were cultured in IMDM with 10% FBS, non-essential amino acids, 1% penicillin-streptomycin, and 50 μm β-mercaptoethanol. The culture was maintained at 37 °C with 5% CO2.

SU-DHL-4 cells were treated with 5-Azacytidine (10 μM) (S1782, Selleckchem, Houston, TX, USA) for 24 hours. The OCI-LY7 cells were treated with DMOG (1.5 mM) (S7483, Selleckchem, Houston, USA) for 96 h.

Establishment of AID-deficient and AID-overexpressed DLBCL cell lines

Dr. Junjie Zhang from the University of Southern California (Los Angeles, USA) gifted the pL-CRISPR.EFS. PAC. The *AICDA* sgRNA was designed at Zhang laboratory's CRISPR design website (http://crispr.mit.edu/), then sequenced by Sunny Biotech Co., Ltd. A non-genome targeting sgRNA was utilized as a control. The gRNA sequences are listed in Table 1. The Pwpi-GFP plasmids were used to create Pwpi-AID-GFP lentivirus constructs by inserting AID cDNA(10). The sequences of primers for amplifying AID cDNA were as follows: AID_F (5' -CTGGACACCACTATGGACAGCCTCTTGATG-3'), AID_R (5'-CATTCCTGGAAGTTGCTATTAAAGTCCC-3'). To generate stable DLBCL cell lines with integrated pCas9-AID or Pwpi-AID-GFP transgenes, the plasmids pCas9-AID or Pwpi-AID-GFP were transfected into 293 T cells which had been seeded 24 h before transfection at a density of 1 × 10^6 cells per 5 cm plate. Using the X-treme GENE HP DNA transfection reagent (Roche, Mannheim, Germany), the ΔR9 and pVSVG helper plasmids were co-transfected into cells. After 72 hours, supernatants were collected. To infect DLBCL cells (1 × 10^6), a freshly prepared lentivirus with either AID knock-out or AID-overexpression was used. The infection was done with a 1000 × g spin inoculation for 90 minutes at room temperature, along with 10 μg/mL polybrene. Puromycin (0.6 μg/mL) was used to select stably integrated DLBCL cells for 5 days.

RNA extraction and quantitative RT-PCR

Isolation of total RNA from cell pellets was carried out using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's guidelines. Synthesis of cDNA for use in quantitative PCR studies was performed using the PrimeScript™ RT reagent Kit (TaKaRa Bio, Inc.). Real-time PCR reactions were carried out on an Mx3000P qPCR system (Agilent Technologies, Inc.) using SYBR-Green dye (TaKaRa Bio, Inc.) in triplicate. An endogenous control was established by measuring the expression of the ACTB gene product. The fold change in each group was calculated and compared (18,19). Table 2 provides the list of primers used.

In vitro cell proliferation

To assess proliferation rates, 1× 10^6 cells were cultured in six-well plates with 3 mL of FBS-free Dulbecco's Modified Eagle Medium (Hyclone) supplemented with non-essential amino acids and penicillin-streptomycin for 36 h. The cells were stained with trypan blue, and the absolute number was determined using the Nexcelom chamber.

Immunoblot analysis

To extract the proteins, cell pellets were dissolved in RIPA buffer with various protease inhibitors (Sigma, Shanghai,China). After sonication and centrifugation, the protein supernatant was collected and loaded onto SDS-PAGE gels. The blots were then probed with specific antibodies including anti-AID, anti-TET2, anti-DNMT1, and anti-PIM1(CST, Danvers, MA, USA). GAPDH was used as a loading control. The protein signal was detected using secondary antibodies conjugated with horseradish peroxidase and visualized with chemiluminescence.

Chromatin immunoprecipitation

Previously published protocols were followed for chromatin immunoprecipitation (ChIP) experiments (20). Briefly, 30 million cells were fixed with 1% HCHO (Sigma) for 15 minutes at room temperature and then quenched with 0.125 M glycine. Chromatin was isolated, sonicated to obtain fragments of 300-500 bp, and pre-cleared with Dynabeads Protein G beads (Invitrogen). Approximately half million cell equivalents were taken as input, and the remaining chromatin was incubated overnight at 4°C with 5 μg of specific antibody or normal IgG. Immunocomplexes were captured with Dynabeads Protein G beads (Invitrogen), cross-links were reversed, and DNA was purified for qPCR using SYBR Premix Ex TaqTM

Table 1. The sequence of gRNAs for Crispr/Cas9 targeting AID.

gRNA	Oligo 1 (5'-3')	Oligo 2 (5'-3')
AICDA-gRNA-1	CACCGGCCTCTTCACTACGTAGCACAGG	AAACCCtGTGCTACGTAGTGAAGAGGCC
AICDA -gRNA-2	CACCGGTAAGTCATCAACCTCATACAGG	AAACCCtGTATGAGGTTGATGACTTACC
AICDA-gRNA-3	CACCGGGACTTTGATAGCAACTTCCAGG	AAACCCtGGAAGTTGCTATCAAAGTCCC
Non-targeting control	CGCTTCCGCGGCCCGTTCAA	ACGGAGGCTAAGCGTCGCAA

Table 2. Sequences of primers used for quantitative real-time PCR

ID	Forward strand (5'-3')	Reverse strand (5'-3')
AICDA	CTACATCTCGGACTGGGACC	TCAGACTGAGGTTGGGGTTC
PIM1	AAAATCAACTCGCTTGCCCA	GGATGCCTGAGTAGACCGAG
ACTB	ACTCTTCCAGCCTTCCTTCC	CGTACAGGTCTTTGCGGATG

Table 3. Primer sequences used in qPCR and PCR for ChIP.

ID	Forward strand (5'-3')	Reverse strand (5'-3')
ChIP-PIM1	GATCAATCGGCCTCTGGTTG	CAGGAGTAAAGGGGAGGAGC

(TaKaRa) with an Mx3000 thermocycler (Agilent Technologies). The primer sequences used for qPCR are available in Supplementary Table 3. The ChIP-qPCR signals were normalized as follows:

(IP/Inputcorr)/positive control = (((IPspecific antibody − IPIgG)/Input) × 1000)/positive control.

ChIP experiments were performed with the following antibodies: anti-AID (ab59361, Abcam), anti-TET2 (#18950, CST), anti-DNMT1 (Abcam, ab13537), and normal goat IgG (#sc2346; Santa Cruz). PCR of the DNA from ChIP was performed for 25 cycles. The bands were observed on 1.5% agarose gels and images were acquired.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6.0 (GraphPad Software, LaJolla, CA, USA) with unpaired t-tests and ANOVA multiple tests. A p-value of less than 0.05 was considered statistically significant, as indicated.

Results

AID results in the divergent *PIM1* expression in distinct DLBCL cell lines

PIM1-related B cell tumorigenesis is linked to AID-mediated hypermutation. To explore the roles of AID in the regulation of *PIM1* expression in DLBCL, CRISPR/Cas9 technology was used to establish AID-depleted DLBCL cell lines (AID-KO SU-DHL-4 and AID-KO OCI-LY7) as described in our previous study (11), and lentivirus of Pwpi-AID-GFP was used to construct stable cell lines with overexpression of AID. *PIM1* mRNA levels of AID-KO SU-DHL-4 cells were elevated by almost 9-fold compared with those in AID-WT SU-DHL-4 cells ($p < 0.05$) (Fig. 1A). Meanwhile, *PIM1* expression in AID-KO OCI-LY7 cells dropped to about 70% in comparison with AID-WT OCI-LY7 cells ($p < 0.05$) (Fig. 1B). Immunoblot analysis also showed an increased PIM1 protein level in AID-KO SU-DHL-4 (Fig. 1C) but a decreased amount in AID-KO OCI-LY7 cells (Fig. 1D). In addition, the grayscale analysis results also identified an increase and decrease of PIM1 protein level in AID-KO and AID-OE SU-DHL-, and AID-WT and AID-OE OCI-LY7 cells, respectively (all $p < 0.01$) (Fig. 1C and D). These results show that AID deficiency induces divergent *PIM1* expression in distinct DLBCL cells.

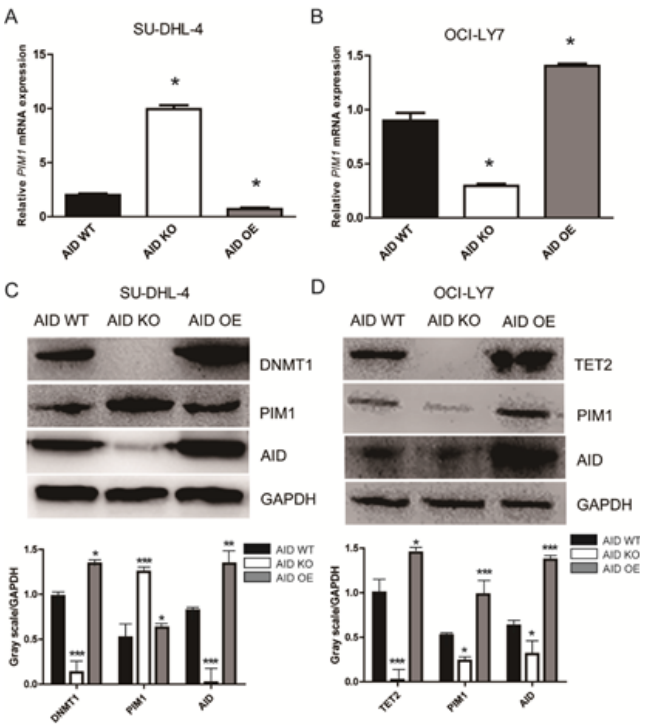

Figure 1. AID mediates divergent PIM1 levels in distinct DLBCL cells. (A) *PIM1* mRNA levels in 4AID-WT, 4AID-KO, and 4AID-OE were detected by Real-Time PCR. (B) *PIM1* mRNA levels in 7AID-WT, 7AID-KO, and 7AID-OE were detected by Real-Time PCR. β-actin was taken as an internal gene control. (C) DNMT1, PIM1, and AID protein levels in 4AID-WT, 4AID-KO, and 4AID-OE were detected by immunoblots. GAPDH protein was taken as an internal control. The immunoblots were performed by grayscale analysis, the relative grayscale of PIM1 bands in immunoblots relative to GAPDH was performed, analyzed statistically, and presented as histograms. (D) TET2, PIM1, and AID protein levels in 7AID-WT, 7AID-KO, and 7AID-OE were detected by immunoblots. GAPDH protein was taken as an internal control. The immunoblots were performed by grayscale analysis, the relative grayscale of PIM1 bands in immunoblots relative to GAPDH was performed. Statistical analysis was done and presented as histograms. Data are presented as mean ±SD. *, ** and *** represent P<0.05, P<0.01 and P<0.001, respectively. OE, overexpression.

AID binds to the promoter of *PIM1* as a co-factor

AID has also been reported to interact with DNMT1 in AID-positive hematopoietic cancers (16,21) and form a complex with TET2 in DLBCL (11,22). We investigated whether epigenetic modification of AID to *PIM1* expression beyond the mutation function of AID might occur in DLBCL cell lines. Data derived from DNMT1 and TET2 immunoblots indicated that along with AID depletion, DNMT1 protein level decreased in AID-KO SU-DHL-4 cells ($p < 0.001$), while TET2 protein level dropped in AID-KO OCI-LY7 cells ($p < 0.01$) (Fig. 1C and D). To verify whether the AID/DNMT1 complex and AID/TET2 complex directly binds to *PIM1* to modulate *PIM1* expression, we performed a ChIP experiment using antibodies against H3K4me3, AID, DNMT1, and TET2. Immunoprecipitated and input DNA were analyzed by qPCR using primers that amplify fragments in the *PIM1* promoter. Abundant AID, DNMT1, and TET2 bound to the *PIM1* promoter along with H3K4me3 enrichment (Fig. 2A-2D). DNMT1 binding dropped nearly 2-fold in AID-KO SU-DHL-4 cells ($p < 0.05$), while TET2 dropped 26-fold in AID-KO OCI-LY7 cells ($p < 0.01$) (Fig. 2A and 2C). In addition, the *PIM1* PCR fragments visualized on 1.5% agarose gels showed an absence of bands in AID-KO DLBCL cells (Fig. 2B, Fig. 2D). These results suggest that

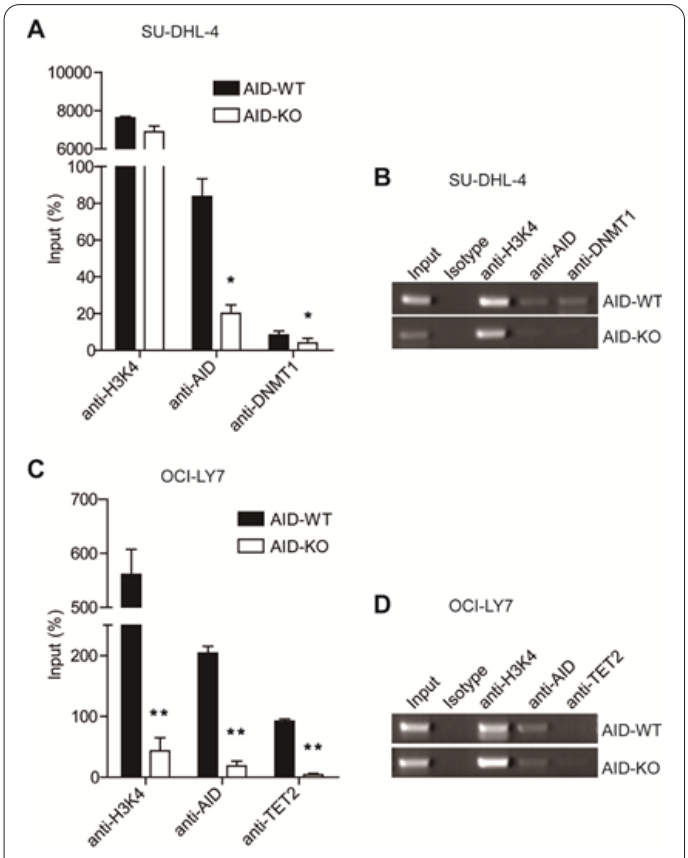

Figure 2. AID binds to the promoter of *PIM1* as a co-factor. (A, B) Genome DNA from Chromatin Immunoprecipitation (ChIP) of *PIM1* promoter by anti-H3K4me3, anti-AID, and anti-DNMT1 pull down in AID-WT, and AID-KO SU-DHL-4 cells were detected by quantitative PCR (A) and PCR (B). (C, D) Genome DNA from Chromatin Immunoprecipitation (ChIP) of *PIM1* promoter by anti-H3K4me3, anti-AID, and anti-TET2 pull down in AID-WT, and AID-KO OCI-LY7 cells were detected by quantitative PCR (C) and PCR (D). Data are presented as mean ±SD. * and ** represent P<0.05 and P<0.01, respectively.

AID-DNMT1 and AID-TET2 complexes recruit *PIM1* promoters in distinct DLBCL cell lines.

Combined AID/DNMT1 or AID/TET2 depletion leads to reduced or increased PIM1 levels in DLBCL cells

To further elucidate the combined role of AID and DNMT1 or AID and TET2 in DLBCL cells, we introduced the DNMT1 inhibitor 5-Azacytidine or TET2 inhibitor DMOG(23,24). Administration of 5-Azacytidine caused a nearly 13-fold increase of *PIM1* transcripts in AID-KO SU-DHL-4 cells ($p < 0.01$) (Fig. 3A), and about 80% decrease of *PIM1* transcripts in AID-KO OCI-LY7 cells ($p < 0.001$) (Fig. 3C). According to the immunoblot results, the 5-Azacytidine or DMOG treatments caused a decrease in DNMT1 or TET2 and AID (Fig. 3B, D, lanes 2 and 4). In addition, the absence of both AID and DNMT1 caused apparent elevated PIM1 levels in AID-KO SU-DHL-4 ($p < 0.001$) (Fig. 3B, lane 4), while depletion of both AID and TET2 led to reduced PIM1 protein level in AID-KO OCI-LY7 ($p < 0.001$) (Fig. 3D, lane 4). The grayscale analysis also confirmed alteration of the PIM1 protein level caused by combined inhibition of AID-DNMT1 or AID-TET2 (Fig. 3B, D). The data identified the combined enhancement or suppression effect of AID/DNMT1 or AID/TET2 to PIM1 in DLBCL. Here, the data suggest that AID could play different roles beyond its mutation function when forming complexes with different proteins, such as DNMT1 and TET2.

AID/DNMT1-mediated PIM1 inhibition or AID/TET2-mediated PIM1 activation show divergent DLBCL cell proliferation

To elucidate the impact of AID/DNMT1 or AID/TET2 mediated *PIM1* inhibition or activation on the cellular function of DLBCL, we kinetically measured cell numbers in the absence of nutrition for up to 36 h. Interestingly, we observed elevated cell division in AID-KO SU-DHL-4 and AID-WT OCI-LY7 cells. Contrastingly, proliferation was not reported in AID-WT SU-DHL-4 and AID-KO OCI-LY7 cells (Fig. 4). The results suggest a divergent proliferation in DLBCL cell lines, in which *PIM1* expression is inhibited by the AID/DNMT1 complex in SU-DHL-4 cells, while *PIM1* expression is increased by the AID/TET2 complex in OCI-LY7 cells.

Discussion

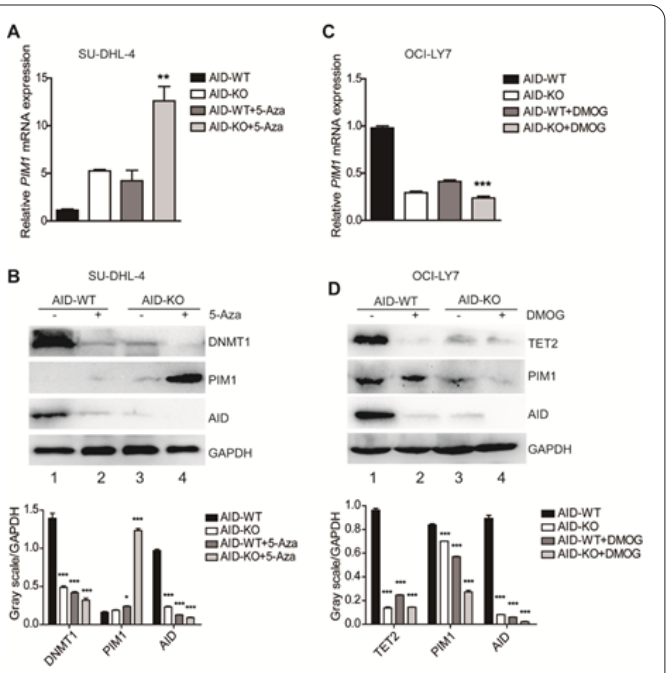

Figure 3. Combined depletion of AID/DNMT1 or AID/TET2 leads to elevated or dropped PIM1 levels in DLBCL cells. (A) the transcript levels of *PIM1* in 4AID-WT and 4AID-KO after being treated with 5-Azacytidine (10μM) for 24 hours were detected by Real-Time PCR. β-actin was used as an internal gene control. (B) the transcript levels of *PIM1* in 7AID-WT and 7AID-KO after being treated with DMOG (1.5 mM) for 96 hours were detected by Real-Time PCR. β-actin was taken as an internal gene control. (C) DNMT1, PIM1, and AID protein levels in 4AID-WT and 4AID-KO after being treated with 5-Azacytidine (10μM) for 24 hours were detected by immunoblots. GAPDH protein was used as an internal control. The immunoblots were performed using grayscale analysis; the relative grayscale of PIM1 bands in immunoblots relative to GAPDH was statistically analyzed and shown as histograms. (D) TET2, PIM1, and AID protein levels in 7AID-WT and 7AID-KO after being treated with DMOG (1.5 mM) for 96 hours were detected through immunoblots. GAPDH protein was taken as an internal control. The immunoblots were performed by grayscale analysis, the relative grayscale of PIM1 bands in immunoblots relative to GAPDH was performed, statistically analyzed, and presented as histograms. Data are presented as mean ±SD. *,** and *** represent P<0.05, P<0.01 and P<0.001, respectively.

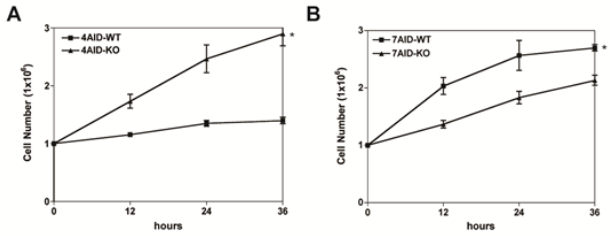

Figure 4. AID/DNMT1 mediated PIM1 inhibition or AID/TET2 mediated PIM1 activation show divergent proliferation of DLBCL cells. The proliferation rates of the 4AID-WT/KO cells (A) and 7AID-WT /KO cells (B) in the absence of nutrition for 36 h, respectively. Five independent experiments were repeated. Data represent the average ± SEM. * represents P<0.05.

Previous studies suggest that the deregulation of *PIM1* is linked to DLBCL pathogenesis(3,4). The complex mechanisms involved in *PIM1* regulation are reported in DLBCL and include a series of gene networks involved in transcription and post-transcription regulation(25,26). However, the AID/DNMT1 or AID/TET2 cooperation would be another possible method of *PIM1* expression modulation. This alternative *PIM1* regulatory mechanism is yet to be elucidated. According to this study, *PIM1* was either silenced or activated in different DLBCL partially due to the effects of AID/DNMT1 or AID/TET2 complex binding to the *PIM1* promoter. These results suggest an alternative co-factor role of AID to *PIM1* in DLBCL and enable cancer to be classified based on the presence or absence of *PIM1*.

AID tends to cooperate with other proteins (15,27), which recruit it to DNA transcription regions (28,29), with the aim of regulating gene expression. However, whether gene expression is modulated by AID and other protein cooperation is yet to be elucidated. Here, we identified that the AID/DNMT1 or AID/TET2 complex is involved in the silencing or activation of *PIM1* in DLBCL (Fig. 4). The results partially explain the findings that AID has a role in DNA epigenetic regulation, mainly methylation or demethylation modifications, which manifest through the promotion or inhibition of gene expression (Fig. 2). As a co-factor, AID assists DNMT1 in inhibiting *PIM1* expression or enables TET2 to induce *PIM1* expression in DLBCL (Fig. 4). This concept provides a new approach to understanding AID's function in cancers.

Some patients with DLBCL develop drug resistance to traditional therapeutic regimens, such as chemotherapy or R-CHOP (anti-CD20 monoclonal antibody-rituximab, combined with cyclophosphamide, vincristine doxorubicin, and prednisone) (30-32). Targeting *PIM1* inhibitors could also be a therapeutic alternative (33), however, the effect is unsatisfactory (34). Our results revealed weak *PIM1* expression in SU-DHL-4 DLBCL cells while strong *PIM1* expression in OCI-LY7 DLBCL cells (Fig. 1 and Fig. 4). This suggests a way of classifying DLBCL through negative or positive *PIM1* expression. For the DLBCL with weak *PIM1* expression, alternative treatment targets would be selected instead of *PIM1* inhibition (Fig. 3). In addition, selecting checkpoints identified in this study (AID, DNMT1, and TET2) would be an effective therapy for DLBCL (Fig. 4). Identifying the specific pathogenesis of heterogeneous DLBCL has great potential for the development of personalized treatment.

However, the study has limitations. First, we checked the existence of AID/DNMT1 in OCI-LY7 and AID/TET2 in SU-DHL-4, but the AID/DNMT1 had weaker enrichments to *PIM1* in OCI-LY7, and AID/TET2 had lower binding to *PIM1* in SU-DHL4. The latent mechanisms need to be further explored. Second, more investigations into the alternative role of AID in DLBCL should be performed to the abundance of cancer-associated genes to confirm our proposed new role of AID beyond the deamination function. Third, all the hypotheses and results need to be verified in clinical DLBCL samples.

In conclusion, our data suggest an alternative co-factor role of AID to *PIM1* in DLBCL. The AID-DNMT1 or AID-TET2 complex directly binds to the promoter of *PIM1*, thus inhibiting or enhancing *PIM1* expression in the progression of DLBCL. Differential treatment of DLBCL based on *PIM1* expression would be an effective method of individualizing therapy. In the future, our findings may be useful for the in-depth exploration of AID's alternative role beyond its deamination in tumor-related genes in DLBCL.

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Interest conflict
The authors declare that they have no competing interests.

Consent for publications
All the authors read and proved the final manuscript for publication.

Availability of data and material
All data generated during this study are included in this published article.

Author Contributions
Y. W and YS M performed the majority of experiments. W.Z and Y.B generated the constructs. Y. Ji and Y. Ma designed the experiments and wrote the manuscript.

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Ethics approval and consent to participate
No human or animals were used in the present research. The study protocol was approved by the Ethics Committee of Xi'an Jiaotong University.

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