

EZH2 inhibitors restore epigenetically silenced CD58 expression in B-cell lymphomas

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ABSTRACT

Loss of CD58 is a common mechanism for tumor immune evasion in lymphoid malignancies. CD58 loss is known to occur due to both genetic and non-genetic causes; therefore, we hypothesized that restoring CD58 expression in lymphoma cells may be an effective treatment approach. To explore the potential for restoring CD58 expression, we first screened 11 B-cell lymphoma lines and found that 3 had decreased CD58 expression. Among these, CD58 was genetically damaged in two lines but not in the third line. Using the cell line with down-regulated CD58 without a genetic abnormality, we performed epigenetic library screening and found that two EZH2 inhibitors, EPZ6438 and GSK126, specifically enhanced CD58 expression. By examining the effect of three EZH2 inhibitors with different selectivity profiles in different B-cell lines, EZH2 inhibition was shown to have a common activity in upregulating CD58 expression. Restoring the expression of CD58 in lymphoma cells using an EZH2 inhibitor was shown to enhance interferon- γ production of T and NK cells against lymphoma cells. H3K27 was shown to be highly trimethylated in the CD58 promoter region, and EZH2 inhibition induced its demethylation and activated transcription of the CD58 gene. These results indicated that EZH2 is involved in the epigenetic silencing of CD58 in lymphoma cells as a mechanism for tumor immune escape, and EZH2 inhibitors are able to restore epigenetically suppressed CD58 expression. Our findings provide a molecular basis for the combination of an EZH2 inhibitor and immunotherapy for lymphoma treatment.

1. Introduction

The tumor microenvironment plays a critical role in the development and progression of lymphoid malignancies. Lymphoma cell survival and proliferation *in vivo* are often dependent on interactions with the non-tumor cell components of the microenvironment to some degree. On the other hand, lymphoma cells also modulate these cellular interactions to escape from immune surveillance.

Understanding of these interactions has contributed to the development of novel immunotherapies. For instance, immune checkpoint inhibitors have been highly effective for the treatment of Hodgkin lymphoma (Ansell et al., 2015); Chen et al., 2017; Armand et al., 2018. Hodgkin lymphoma is characterized by malignant Hodgkin/Reed-Sternberg (H/RS) cells dispersed within the immune cell infiltrates, and a PD-1-blocking antibody inhibited the interaction between PD-1 and its ligands expressed on tumor-infiltrating T cells and H/RS cells, respectively (Ansell et al., 2015; Yamamoto et al., 2008; Carey et al., 2017). Although PD-1 signaling pathway is also suggested to be

involved in other lymphoma subtypes, PD-1 blockade therapy is not as effective in Hodgkin lymphoma, at least when it is used as a single agent (Ansell et al., 2019; Lesokhin et al., 2016). One possible reason for the limited efficacy of immune checkpoint inhibitors in non-Hodgkin lymphomas is the frequent involvement of additional immune escape mechanisms. In the comprehensive genomic analysis of diffuse large B-cell lymphoma (DLBCL), molecules involved in antigen presentation and costimulatory signaling, such as HLA class I/II, CD58, B2M, CD70, and 4-1BBL, were shown to be recurrently mutated (Challa-Malladi et al., 2011); Dubois et al., 2016; Pasqualucci et al., 2011; Karube et al., 2018; Schmitz et al., 2018; Reddy et al., 2017; Chapuy et al., 2018. These findings suggested that defective immune synapse formation between lymphoma cells and immune effector cells is an important underlying mechanism of immune evasion in DLBCL.

The CD58 gene is one of the recurrent targets of genetic abnormalities in DLBCL and in other lymphoid malignancies such as peripheral T-cell lymphoma (Palomero et al., 2014) and adult T-cell leukemia/lymphoma (Kataoka et al., 2015; Yoshida et al., 2014). CD58 gene

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abnormality is also suggested to appear in the process of transformation of follicular lymphoma (FL) into DLBCL (Pasqualucci et al., 2014). CD58 is a member of the immunoglobulin superfamily, which has a function to bind to CD2 expressed on T and NK cells (Wang et al., 1999; Grakoui et al., 1999; Moingeon et al., 1989, and the CD2-CD58 interaction is especially important for tumor recognition by T and NK cells. Loss of CD58 expression is indicated to be associated with worse overall and event-free survivals in patients with DLBCL (Cao et al., 2016) and acute lymphoblastic leukemia (Li et al., 2016). CD58 expression is reported to be lost through both genetic and non-genetic mechanisms (Challa-Malladi et al., 2011); therefore, we hypothesized that restored expression of CD58 may facilitate T and NK cell-immune recognition of lymphoma cells and increase the efficacy of immune-targeted therapy.

To explore the non-genetic mechanism of defective CD58 expression in lymphoma cells, we performed epigenetic compound library screening using a B-cell lymphoma line with decreased CD58 expression without any CD58 gene abnormality. We found that EZH2 inhibitors specifically restored CD58 expression in these cells, and upregulation of CD58 by an EZH2 inhibitor enabled lymphoma cells to strongly stimulate T and NK cells. H3K27 was shown to be highly trimethylated in the CD58 promoter region, and EZH2 inhibition induced its demethylation and increased CD58 gene transcription. These results indicated that EZH2 is involved in the epigenetic silencing of CD58 in lymphoma cells as a mechanism for tumor immune escape, and an EZH2 inhibitor was effective for the restoration of epigenetically downregulated CD58 expression. Our findings provide a molecular basis for the effectiveness of EZH2 inhibitors and a rationale for their combination with immune-targeted therapies for the treatment of lymphomas.

2. Materials and methods

2.1. Analysis of European Genome-phenome Archive (EGA) data sets

Whole exome sequencing and RNA-seq gene expression data derived from 1001 DLBCL samples and the core set of 624 DLBCL samples were obtained from EGA (dataset id: EGA00001003600) (Reddy et al., 2017). Gene expression was measured using terms of fragments per kilobase of exon per million fragments mapped and normalized using the Cufflinks package, version 2.2.1 (Trapnell et al., 2010). Quantile normalization was performed and the data were log2 normalized. To evaluate the correlation between tumor CD58 expression with T cell activation activity, T cell activation signature was calculated as the geometric mean (log-average) of the expression of genes, which was suggested as T cell activation previously (Feske et al., 2001).

2.2. Cell lines and culture conditions

The following cell lines used in this experiment were described previously (Maesako et al., 2003; Epstein et al., 1978; Nozawa et al., 1988; Pulvertaft, 1964; Klein et al., 1968; Jadayel et al., 1997: FL lines FL18, FL218, FL318, FL518, FL618; a germinal center B cell-like (GCB)-DLBCL line SU-DHL-6; activated B cell-like (ABC)-DLBCL lines DLBCL2 and HBL-1; Burkitt lymphoma (BL) lines Raji and Daudi; and a mantle cell lymphoma (MCL) line Granta-519. These cells were maintained in RPMI1640 (Nacalai Tesque, Kyoto, Japan) containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin/L-glutamine (PSG) and cultured at 37 °C in a humidified incubator in the presence of 5 % CO₂. HEK293 T cells were maintained with DMEM (Nacalai Tesque) containing 10 % FBS and 1 % PSG.

2.3. Flow cytometry

Flow cytometric analysis was performed using FACSCalibur and Accuri C6 flow cytometers (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software (version 10.1; Tree Star Inc, San

Table 1

Primers used in the study.

CD58-exon6-R	ACTTGCCTCGTACTGACAGC
RT-PCR primers	
CD58-RT-PCR-F	CTTGAACITTAGGGCTGCTTGTGGCT
CD58-RT-PCR-R	GTTCTTTGTTAGTGGGTATTCTTCTTAAGCAGC
qPCR primers	
ACTB-F	CTAAGTCATAGTCCGCCTAGAAGCA
ACTB-R	TGGCACCCAGCACAATGAA
CD58-qPCR-F	CAACCTGTATCCCAAGCAGC
CD58-qPCR-R	TGGAGTTGGTTCTGTCTGGT
HLA-B-F	CTACCCCTGCGGAGATCA
HLA-B-R	ACAGCCAGGCCAGCAACA
HLA-C-F	CACACCTCTCCTTTGTGACTTCAA
HLA-C-R	CCACCTCCTCACATTATGCTAACA
B2M-F	ACACTGAATTACCCCCCACTG
B2M-R	TGCTTACATGTCTCGATCCCAAC
ChIP-qPCR primers	
CD58-ChIP-qPCR-1F	CCAGGCATTGAGGGCTTCT
CD58-ChIP-qPCR-1R	GGCTTCCTTGGTTGGACTCT
CD58-ChIP-qPCR-2F	CAGACTGATGAAGAGCGGGG
CD58-ChIP-qPCR-2R	TCTCAACCGGGGACTTTCCTT

Carlos, CA, USA). Cell sorting was performed using a FACSaria II cell sorter (BD Biosciences). Antibodies used were as follows: CD58-APC (TS2/9; BioLegend, San Diego, CA, USA), HLA-class I-PE (G46-2.6, BD Pharmingen, San Diego, CA), and APC- and PE-conjugated mouse IgG1- κ isotype controls (BioLegend).

2.4. Genomic DNA sequencing

Genomic DNA was extracted from cells using a QIAamp DNA Mini kit (QIAGEN Ltd, Crawley, UK). Exons of the CD58 gene were amplified by polymerase chain reaction (PCR) using the primers listed (Table 1). The PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and subjected to Sanger sequencing by using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

2.5. Compounds

A chemical library for epigenetics research (containing 80 compounds) was purchased from Sigma-Aldrich (S990043-EPI1; St. Louis, MO, USA). Three EZH2 inhibitors, EPZ6438, UNC1999, and GSK126, were obtained from Apexbio (Boston, MA, USA). Cells were seeded in a 96-well plate at 0.1×10^6 (Carey et al., 2017) cells per well, and each compound was added at a concentration of 0.1 μ M. After incubation for 4 days, cells were collected and analyzed.

2.6. Reverse transcription (RT)-PCR

Total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany) and complementary DNA (cDNA) was synthesized using a SuperScript III First-Strand and Synthesis system (Life Technologies, Carlsbad, CA, USA). RT-PCR was performed using Tks Gflex DNA polymerase (Takara, Shiga, Japan). Quantitative RT-PCR was performed using TB green Premix Ex Taq II (Takara). Relative gene expression was normalized to ACTB expression. The primers used for RT-PCR and sequencing are listed in Table 1.

2.7. Microarray-based gene expression analysis

Total RNA extracted from FL218 cells using an RNeasy Mini kit (Qiagen) were reverse transcribed, and labeled with cyanin-5 and cyanin-3, respectively, using a Low Input Quick Amp labeling kit (Agilent

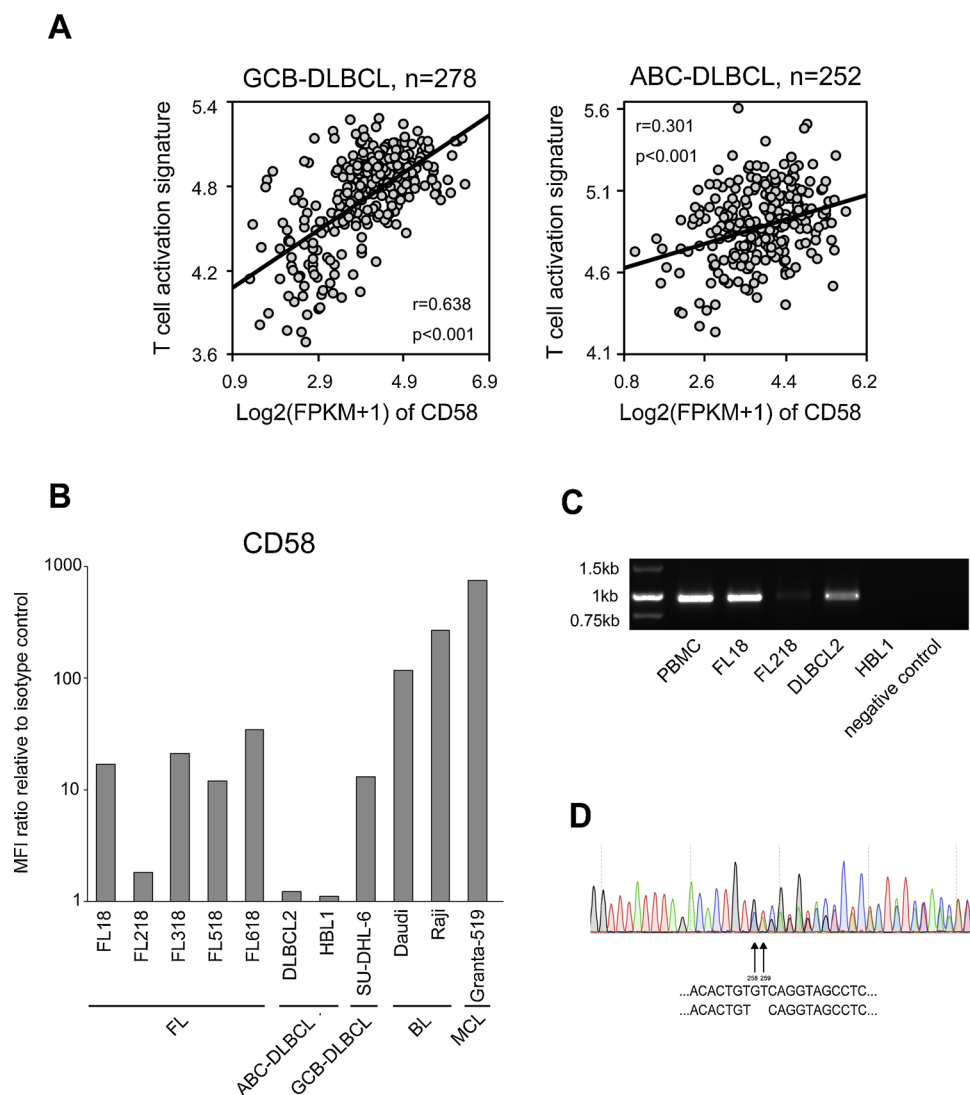


Fig. 1. (A) Gene expression data analysis of the RNA-seq DLBCL database. T-cell activation signature vs. CD58 expression in bulk tissue in GCB-DLBCL (n = 278) and ABC-DLBCL (n = 252) samples are shown (dataset id: EGA00001003600). T cell activation signature was calculated as the geometric mean of the expression of 89 genes, which was shown to be associated with T-cell activation.

(B) Surface CD58 expression on B-cell lymphoma lines analyzed by flow cytometry. Mean fluorescence intensity (MFI) ratio was calculated by dividing the MFI for CD58 by the MFI for the isotype control.

(C) CD58 mRNA expression levels evaluated by reverse transcription PCR (RT-PCR). Full length CD58 mRNA was amplified using cDNAs from the cell lines. Primers used for RT-PCR are listed in Table 1.

(D) Alteration of CD58 gene in DLBCL2. Monoallelic 2-base deletion (c.258_259del) that causes a premature stop codon were detected in DLBCL2 line by Sanger sequencing.

Technologies, Santa Clara, CA, USA). Labeled complementary RNA was applied to an Agilent SurePrint G3 Human 8 × 60 K ver. 3.0 microarray. The slide was scanned by an Agilent G2505C microarray scanner. Agilent Feature Extraction software (12.0.3.1) was used for background subtraction, LOWESS normalization, and calculation of the P value log ratio.

2.8. Lentiviral transduction of CD58 gene

The human CD58 transcript was PCR amplified using cDNA from peripheral blood mononuclear cells (PBMCs) derived from a healthy donor using a Ficoll-Paque density gradient (Cedarlane, Ontario, Canada). It was then subcloned into recombinant lentiviral vector, pCS-CAG-EGFP, which was constructed by replacing the CD19 promoter of a Eummar-L-CD19-EGFP vector with CAG promoter (Moreau et al., 2008; Sakai et al., 2009). The lentiviral vector was transduced into HEK293 T cells using X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany) with the packaging plasmid pCAG-HIVgp and the VSV-G- and Rev-expressing plasmids (pCMV-VSV-G-RSV-Rev). Supernatants containing virus particles were collected 72 h after transfection, and concentrated by ultracentrifugation at 20,000 × g for 2 h in Optima L-70K (Beckman Coulter, San Diego, CA, USA) and transduced into FL218 cells.

2.9. Cytokine production assay of T and NK cells

PBMCs were separated from the peripheral blood of a healthy donor using a Ficoll-Paque density gradient (Cedarlane), and total T cells and NK cells were collected by negative selection using MACS Cell Separation Technology (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were seeded in a 96-well round-bottom plate at 2×10^5 (Carey et al., 2017) cells/well and co-cultured with 4 Gy-irradiated 2×10^5 (Carey et al., 2017) FL218 cells/well at 37 °C in 200 µl of RPMI1640 medium supplemented with 10 % FBS, with or without anti-CD2 blocking antibody (RPA-2.10; Invitrogen, Carlsbad, CA, USA). After culturing for 72 h, supernatants were collected and examined for interferon (IFN)-γ production of T cells using an enzyme-linked immunosorbent assay (ELISA) (BioLegend). All measurements were made in triplicate and averaged. IFN-γ production of NK cells was analyzed likewise, with the exception that FL218 cells were pretreated with 10 µg/ml rituximab for 1 h before coculturing with NK cells.

2.10. Immunoblotting

Cells were washed with PBS and lysed in RIPA lysis buffer (Santa Cruz, CA, USA) supplemented with protease-inhibitor cocktail. Total cell lysates were subjected to SDS/polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene difluoride membrane

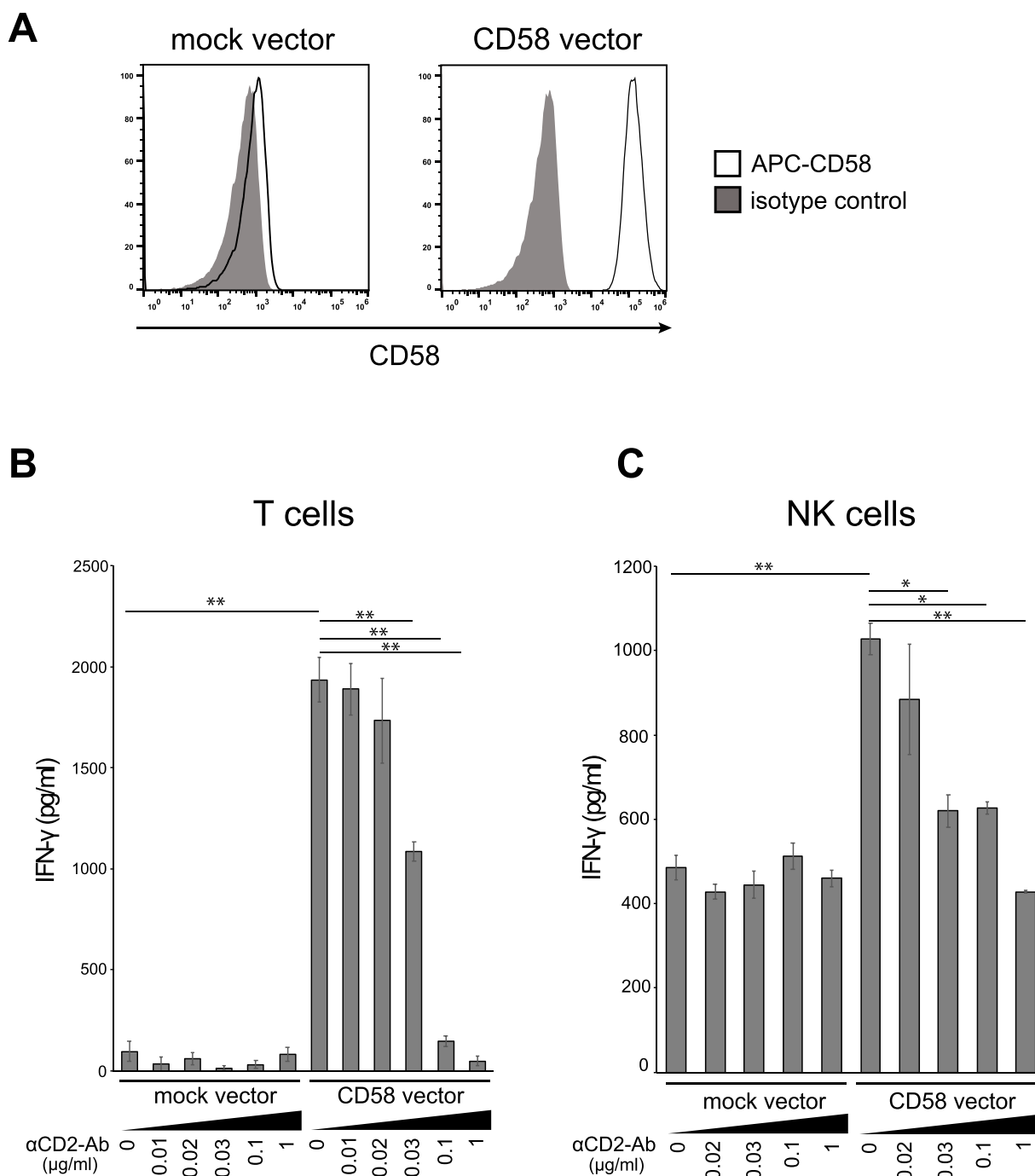


Fig. 2. (A) Forced CD58 expression in FL218 cells. Histograms of CD58 expression levels of FL218 cells transduced with mock vector (left) or CD58-expression vector (right).

(B) IFN- γ production by T cells cultured with FL218 cells transduced with CD58-expression vector. Peripheral blood T cells from a healthy donor were cocultured with FL218 cells transduced with a CD58-expression vector or mock vector for 3 days, and IFN- γ production levels were analyzed by ELISA. Anti-CD2 blocking antibody was added at the indicated concentrations. Data are the averages of triplicate results. Error bars represent standard error of the means of triplicates (* p < 0.05, ** p < 0.01; two-tailed Student's t -test).

(C) IFN- γ production of NK cells cultured with FL218 cells transduced with a CD58-expression vector. Peripheral blood NK cells from a healthy donor were analyzed as in (B). FL218 cells were treated with rituximab (10 μ g/ml) for 1 h before coculturing with NK cells (* p < 0.05, ** p < 0.01; two-tailed Student's t -test).

(Millipore, Bedford, MA, USA). Immunoblotting analysis was performed using an anti-trimethyl histone H3K27 monoclonal antibody (MABI 0323; MBL, Nagoya, Japan) or anti-histone H3 antibody (MABI 0301; MBL). The membranes were subsequently incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (GE Healthcare, Buckinghamshire, UK), and immunoreactive proteins were visualized by enhanced chemiluminescence reaction using SuperSignal West Pico

Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

2.11. Chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR)

FL218 cells were treated with either DMSO or EPZ6438 at concentrations of 1 or 5 μ M for 4 days. Cell were harvested and subjected to ChIP assays in accordance with a published method (Lee et al., 2006)

with the following modifications. FL218 cells were crosslinked with 1 % formaldehyde for 5 min at room temperature with gentle rotation, and then quenched with 0.125 M glycine. After washing, nuclei were sonicated on a Covaris S220 ultrasonicator (Covaris; Woburn, MA, USA), and the supernatants were used for immunoprecipitation with anti-trimethyl histone H3K27 monoclonal antibody (MABI 0323; MBL) or anti-histone H3 antibody (MABI 0301; MBL). The precipitated samples were analyzed by qPCR using the primers listed in Table 1.

3. Results

3.1. CD58 expression level correlates with T-cell activation signature in DLBCL

To examine the significance of CD58 expression in DLBCL, we analyzed a publicly available RNA-seq DLBCL database consisted of 278 cases of GCB-DLBCL and 252 cases of ABC-DLBCL (Fig. 1A). We found that the CD58 expression level significantly correlated with T-cell

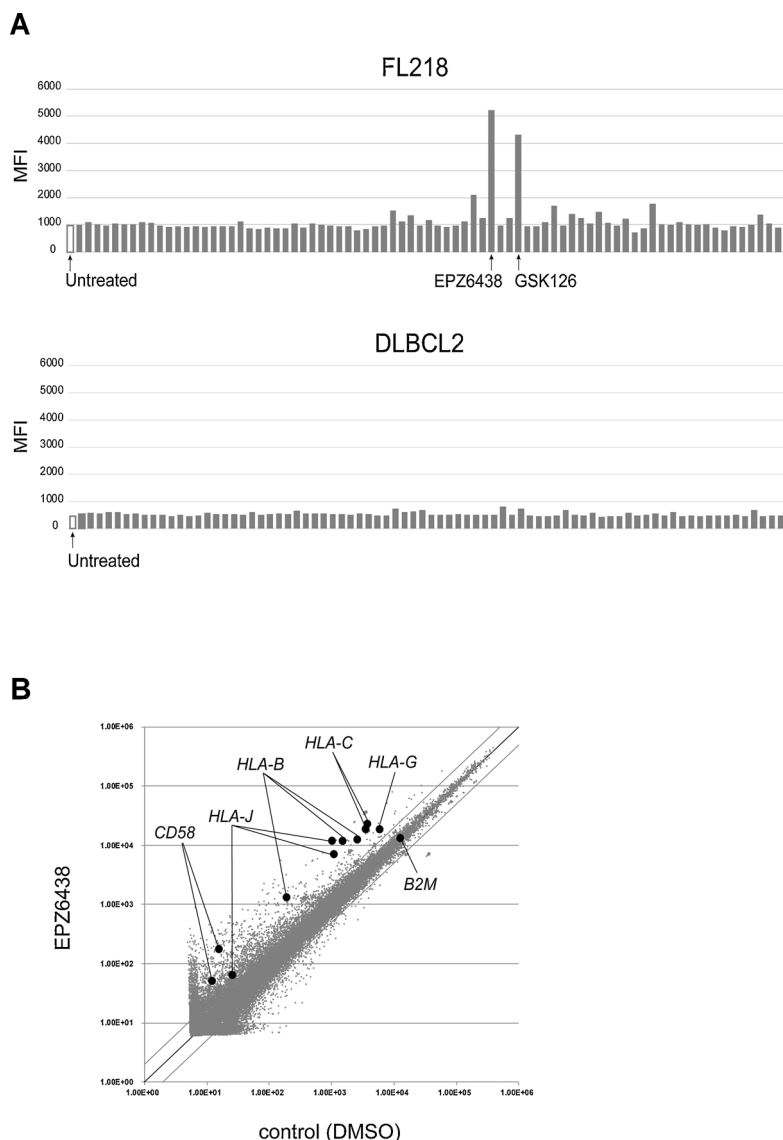


Fig. 3. (A) CD58 expression is upregulated by EZH2 inhibitors in FL218. The results of epigenetic compound library screening for CD58 upregulation in FL218 and DLBCL2. Cells were treated with 0.1 μ M of each compound for 4 days and analyzed for CD58 expression by flow cytometry. EPZ6438 and GSK126 were found to upregulate CD58 expression in FL218 cells.

(B) Microarray gene expression analysis of FL218 cells treated with EPZ6438. FL218 cells were treated with 5 μ M EPZ6438 or DMSO for 4 days and gene expression levels were compared. CD58, HLA-B, HLA-C, HLA-G, and HLA-J genes were upregulated more than 2-fold in FL218 cells treated with EPZ6438.

(C) Changes in gene expression levels by EPZ6438 treatment in B-cell lines. B-cell lymphoma lines were treated by EPZ6438 (5 μ M) for 4 days, and the expression levels of CD58, B2M, HLA-B, and HLA-C genes were examined by quantitative RT-PCR. Fold changes from those treated with vehicle control are shown. Means and standard errors of two technical replicates of qPCR are shown.

(D) Changes in surface CD58 and HLA class I expression by EPZ6438 treatment in B-cell lines. B-cell lymphoma lines were treated by EPZ6438 (5 μ M) for 4 days, and surface CD58 and HLA class I expression levels were analyzed by flow cytometry. The changes in mean fluorescence intensity relative to those treated with vehicle control are shown.

(E) EZH2 inhibitors upregulate CD58 expression in FL cell lines. FL cell lines were treated with two EZH2 inhibitors, UNC1999 and GSK126, at a concentration of 10 μ M for 4 days, and surface CD58 expression was analyzed by flow cytometry. The changes in mean fluorescence intensity of CD58 relative to those treated with vehicle control are shown.

(F) Mutation analysis of CD58 and EZH2 genes in DLBCL. The results based on the analysis of EGA database of the whole exome sequencing derived from 1001 DLBCL samples are shown. CD58 and EZH2 gene mutations are shown to be mutually exclusive.

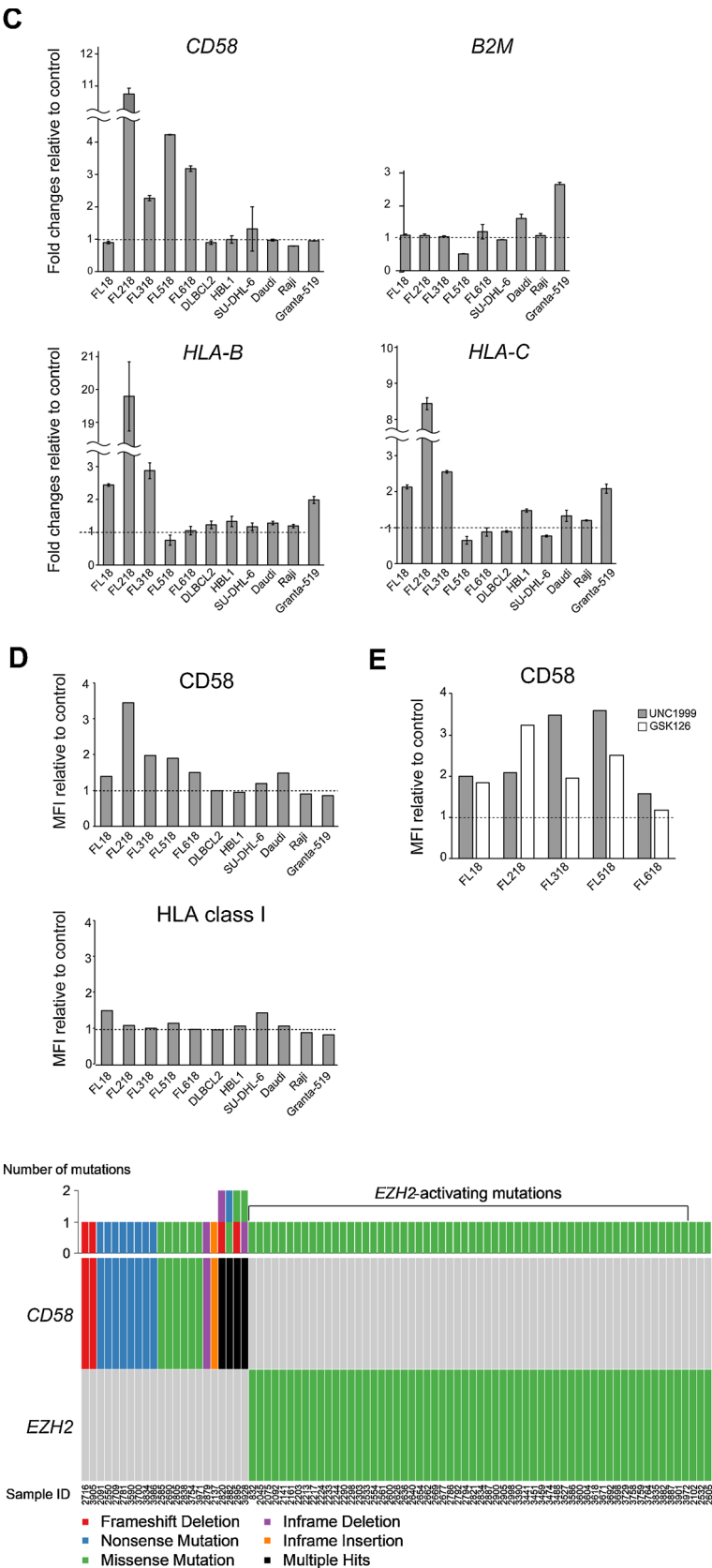


Fig. 3. (continued)

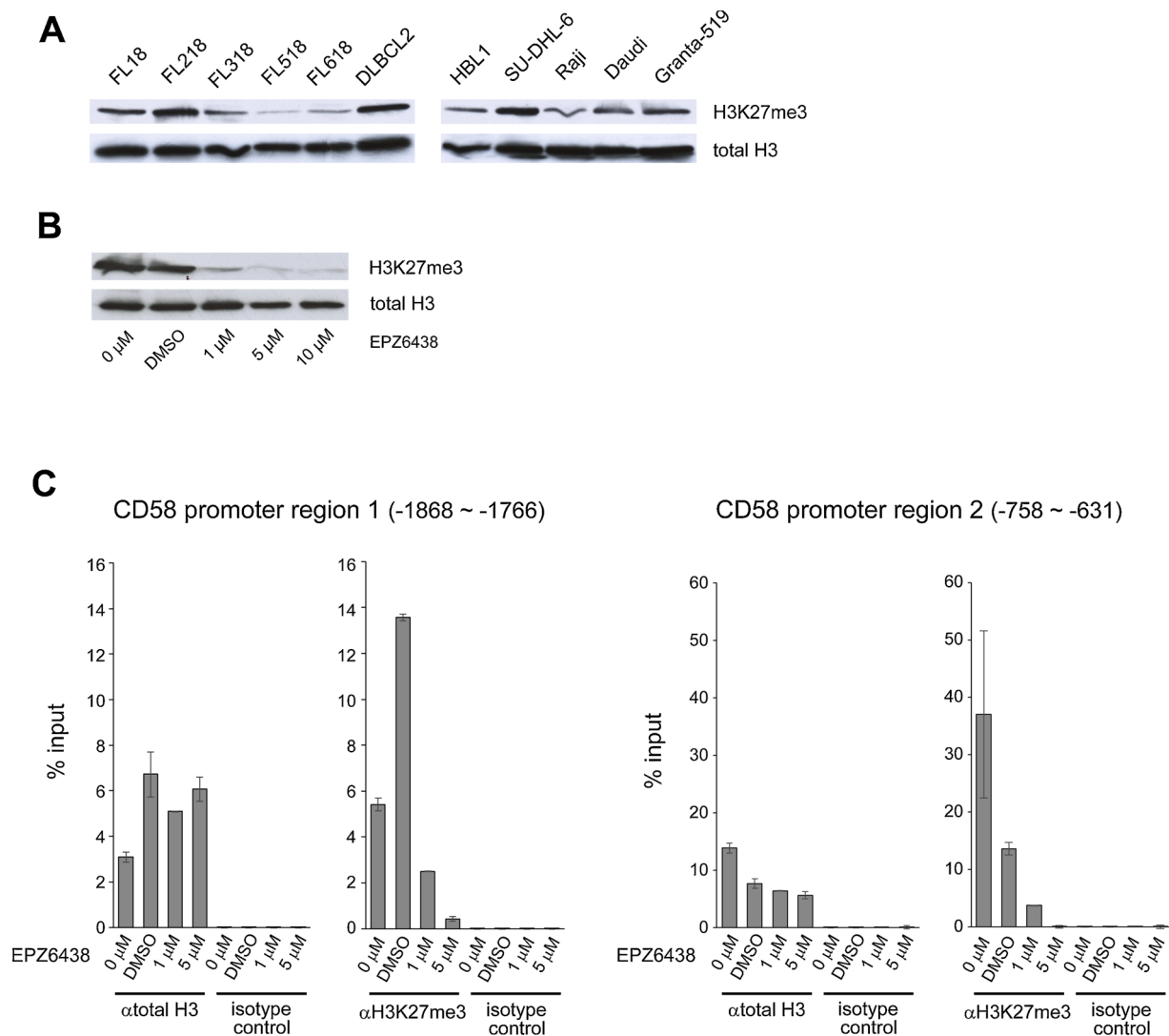


Fig. 4. (A) H3K27me3 and total H3 protein expression in B-cell lines. Whole cell lysates of B-cell lines were extracted and subjected to immunoblotting analysis for the detection of H3K27me3 and total H3 proteins.

(B) EPZ6438 treatment decreased H3K27me3 expression in FL218. FL218 cells were treated with EPZ6438 for 4 days at the concentrations indicated and were subjected to immunoblotting analysis.

(C) H3K27me3 is enriched in the CD58 promoter region and EPZ6438 treatment leads to its decrease. FL218 cells with or without EPZ6438 treatment were subjected to ChIP by anti-total H3 and anti-H3K27me3 antibodies, followed by qPCR of the CD58 promoter regions 1 and 2 (1868–1766 bp and 758–631 bp upstream of the CD58 coding sequence, respectively). The amounts of precipitated DNA relative to the input are demonstrated. Means and standard errors of two technical replicates of qPCR are shown.

activation signature in both GCB-DLBCL and ABC-DLBCL. These results suggested that CD58 expression level is actually associated with T-cell function in DLBCL tissues, regardless of the cell-of-origin subtype.

3.2. Screening for decreased CD58 expression in B-cell lymphoma lines

To investigate the mechanism of CD58 downregulation in B-cell lymphomas, we next sought lymphoma cell lines with low CD58 expression without genetic abnormalities. We examined surface CD58 expression by flow cytometry in 11 lymphoma cell lines: 5 FL lines (FL18, FL218, FL318, FL518, and FL618), 3 DLBCL lines (DLBCL2, HBL1, and SU-DHL-6), 2 BL lines (Daudi and Raji), and one MCL line (Granta-519). We found that 2 ABC-DLBCL lines, DLBCL2 and HBL1, almost totally lacked surface CD58 expression, and FL218 expressed at a very low level of CD58 (Fig. 1B). We examined CD58 mRNA expression in these cell lines by RT-PCR and found that it was severely suppressed (Fig. 1C). We analyzed their CD58 exonic regions by Sanger sequencing, and found a monoallelic 2-base deletion in exon 2 of

DLBCL2, which caused premature stop codon (Fig. 1D), and biallelic deletion of the exon 1–2 region in HBL1. On the other hand, no mutation was found in FL218, and involvement of a non-genetic mechanism of CD58 downregulation was suspected.

3.3. CD58 expression is important for immune recognition in lymphoma cells

CD58 is an adhesion molecule that is expressed broadly on the surface of antigen presenting cells, and it is important for the formation of stable immunological synapses with T and NK cells (Wang et al., 1999; Kanner et al., 1992; Altomonte et al., 1993; Sanchez-Madrid et al., 1982). To examine whether decreased CD58 expression in lymphoma cells actually hinders immune cell recognition, we compared the IFN- γ production of T and NK cells cocultured with lymphoma cells with different CD58 expression levels. Transduction of a CD58-expressing lentiviral vector into FL218 cells led to 100-times higher expression of surface CD58 compared with those transduced with mock vector

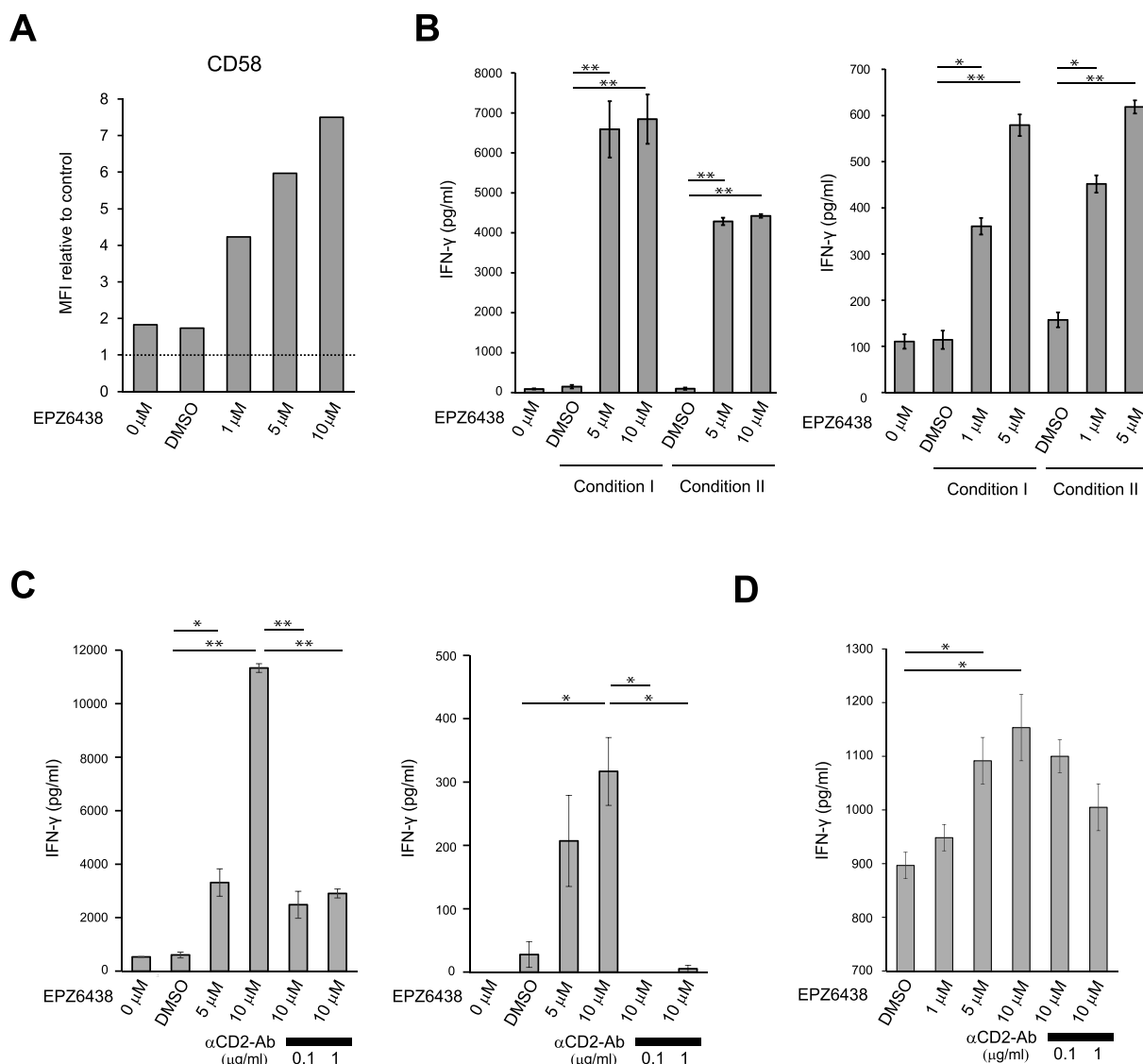


Fig. 5. (A) Changes in surface CD58 expression levels of FL218. Cells were treated with EPZ6438 for 4 days with indicated concentrations, and surface CD58 expression was analyzed by flow cytometry. The changes in mean fluorescence intensity of CD58 relative to those treated with vehicle control are shown.

(B) IFN- γ production by T and NK cells cocultured with EPZ6438-treated FL218 cells. FL218 cells were cultured with EPZ6438 for 4 days, and cocultured with peripheral blood T cells (left panel) or NK cells (right panel) in the absence (condition I) or presence (condition II) of EPZ6438 for a further 3 days. Before coculturing with NK cells, FL218 cells were treated with 10 μ M rituximab for 1 h. Then, IFN- γ production by T and NK cells was analyzed using ELISA. Data are the averages of triplicate results. Error bars represent standard error of the means of triplicates (* p < 0.05, ** p < 0.01; two-tailed Student's t -test).

(C) Increased IFN- γ production by T cells cocultured with EPZ6438-treated lymphoma cells is cancelled by CD2-CD58 blockade. FL318 (left panel) and FL618 (right panel) were cultured with EPZ6438 for 4 days, and then cocultured with peripheral blood T cells in the presence or absence of anti-CD2 blocking antibody for a further 3 days, and IFN- γ production by T cells was analyzed. Data are the averages of triplicate results. Error bars represent standard error of the means of triplicates (* p < 0.05, ** p < 0.01; two-tailed Student's t -test).

(D) Increased IFN- γ production by NK cells cocultured with EPZ6438-treated lymphoma cells is cancelled by CD2-CD58 blockade. FL218 cells were first cultured with EPZ6438 for 4 days, and after treated with 10 μ M rituximab for 1 h, they were cocultured with NK cells in the presence or absence of anti-CD2 blocking antibody for a further 3 days. Then, IFN- γ production by NK cells was analyzed. Data are the averages of triplicate results. Error bars represent standard error of the means of triplicates (* p < 0.05; two-tailed Student's t -test).

(Fig. 2A). After irradiation at a level of 4 Gy, the cells were cultured with peripheral blood T cells of a healthy volunteer for 3 days, and IFN- γ production of T cells was assessed by ELISA. The results showed that, although FL218 cells transduced with mock vector can hardly stimulate T cells, those transduced with the CD58-expressing vector had high capacity for inducing IFN- γ production by T cells (Fig. 2B). Enhanced IFN- γ production in T cells was cancelled by the addition of a CD2 blocking antibody in a concentration-dependent manner. Similarly, FL218 cells transduced with CD58-expressing vector were also shown to facilitate NK-cell response (Fig. 2C). These results suggested that the CD58 expression in lymphoma cells is critical to T and NK-cell immune

recognition, and induction of CD58 expression in lymphoma cells is able to intensify the anti-lymphoma immune response.

3.4. EZH2 inhibitors are able to restore CD58 expression

We considered that if we were able to restore the CD58 expression in lymphoma cells, it might be a promising approach for lymphoma immunotherapy. We hypothesized that CD58 may be able to be recovered pharmacologically in cases when CD58 is not genetically damaged. Using FL218 and DLBCL2, we performed epigenetic compound library screening to identify agents that can restore surface CD58

expression. Although there was no agent that can induce CD58 expression in DLBCL2, two EZH2 inhibitors, EPZ6438 and GSK126, were found to significantly upregulate CD58 expression in FL218 (Fig. 3A).

We examined gene expression alterations using an EZH2 inhibitor (EPZ6438) in FL218 and DLBCL2 with a microarray. In FL218 and DLBCL2, 1593 and 548 genes were upregulated 2-fold or higher, respectively. In addition to CD58, HLA-B, HLA-C, HLA-G, and HLA-J genes, but not B2M gene, were found to be significantly upregulated in FL218 by EZH2 inhibitor (Fig. 3B). In contrast, none of these genes were altered in DLBCL2.

We conducted quantitative RT-PCR to examine the changes in CD58, HLA-B, HLA-C, and B2M mRNA expression with EPZ6438 in B-cell lymphoma lines. We found that CD58 expression was significantly increased in 4 of 5 FL lines, and similarly, significant enhancement of HLA-B and HLA-C expression was observed in 3 of 5 FL lines including FL218. In contrast, B2M expression did not show a marked difference in cell lines other than Granta-519, a MCL line (Fig. 3C).

We next performed flow cytometric analysis to evaluate the alteration of CD58 and HLA class I protein expression levels by EPZ6438. We observed enhancement of surface CD58 expression in all 5 FL lines examined, although there was only a limited increase in HLA class I expression (Fig. 3D). Similarly, CD58 protein expression levels were upregulated by other EZH2 inhibitors with different selectivity profiles, UNC1999 and GSK126, in all FL lines examined (Fig. 3E). These results strongly indicated that EZH2 is directly involved in the regulatory mechanism of the CD58 gene.

In the analysis of the EGA database of the whole exome sequencing derived from 1001 DLBCL, mutations of CD58 were found in 22 samples, 13 of which are loss-of-function mutations (frameshift or stopgain mutations). On the other hand, EZH2 mutations were found in 61 samples, 58 of which are activating mutations (Y641 F/N/S/H/C, A677 G, or A687 V; Fig. 3F). Notably, these abnormalities are shown to be mutually exclusive, suggesting that genetic and epigenetic regulatory mechanisms of CD58 expression are independent from each other.

3.5. EZH2 inhibitor induces demethylation of H3K27me3 in the CD58 promoter region

EZH2 is a histone trimethylase that specifically catalyzes H3K27 trimethylation. We examined H3K27me3 and total H3 protein expression in B cell lines by immunoblotting, and found that H3K27me3 was expressed at variable levels (Fig. 4A). Among these cell lines, we detected a gain-of-function mutation of EZH2 Tyr646Asn in FL218 and SU-DHL6, both of which showed high H3K27me3 expression. Treatment of FL218 with EPZ6438 led to a decrease in H3K27me3 (Fig. 4B), and it was suggested that EPZ6438 can induce demethylation of H3K27.

We hypothesized that EZH2 is involved in epigenetic silencing of the CD58 gene, and EZH2 inhibitor induces CD58 transcription by reverting the epigenetic modification. We constructed two primer pairs to amplify the promoter region of CD58 gene (1868–1766 bp and 758–631 bp upstream of the CD58 coding sequence) and performed ChIP-qPCR analysis of FL218 cells. We found that H3K27me3 is enriched in the CD58 promoter region, and EPZ6438 treatment led to a decrease in H3K27me3 marker in this region (Fig. 4C). According to these results, it was indicated that CD58 gene promoter is inactivated by H3K27 trimethylation, and EZH2 inhibitor activates CD58 gene transcription by inducing demethylation of H3K27.

3.6. Increased CD58 expression in lymphoma cells by EZH2 inhibitor enhances T and NK-cell responses

We finally examined whether upregulation of CD58 in lymphoma cells by EZH2 inhibitor can actually enhance anti-lymphoma immune responses. FL218 cells were cultured with EPZ6438 or vehicle control for 4 days, and were washed and cocultured with allogeneic peripheral

blood T cells with or without EZH2 inhibitor for a further 3 days. Then IFN- γ secretion of the T cells was analyzed by ELISA of the supernatants.

EZH2 inhibitor pretreatment of FL218 cells increased CD58 expression in the cells (Fig. 5A) and led to enhanced IFN- γ production in cocultured T and NK cells, whether EZH2 inhibitor was present or not at the time of coculturing (Fig. 5B). We also found that EPZ6438 treatment of FL318 and FL618, but not FL518, enhanced IFN- γ production of cocultured T cells, and the addition of anti-CD2 blocking antibody cancelled the effect of EPZ6438 (Fig. 5C). Similarly, anti-CD2 blocking antibody inhibited the enhanced IFN- γ production of NK cells cocultured with EPZ6438-treated FL218 (Fig. 5D). According to these results, EZH2 inhibitor is suggested to have a function to facilitate T and NK-cell immune response via the upregulation of CD58 in lymphoma cells.

4. Discussion

CD2-CD58 is an important intercellular signaling pathway for the immune reactions in cytotoxic T and NK cells against tumor cells (Challa-Malladi et al., 2011; Altomonte et al., 1993; Gwin et al., 1996). Loss of CD58 is one of the most frequent mechanisms of immune escape in lymphoid malignancies, and CD58 loss can be caused by both genetic and non-genetic mechanisms. In this report, we demonstrated that EZH2 is involved in the epigenetic silencing of CD58 in lymphoma cells, and EZH2 inhibitors were effective for restoring epigenetically suppressed CD58 expression. H3K27 was shown to be highly trimethylated in the CD58 promoter region, and EZH2 inhibition induces its demethylation and activates transcription of the CD58 gene. Restoration of CD58 expression by EZH2 inhibition enhanced IFN- γ production of cocultured T and NK cells against lymphoma cells.

CD58 is a glycosylated adhesion molecule that is expressed on various cell types. It acts as a ligand for the CD2 receptor expressed on T cells and most NK cells, and it is required for their adhesion and activation (Wang et al., 1999; Kanner et al., 1992; Bolhuis et al., 1986). Among 11 B-cell lines analyzed, we found two ABC-DLBCL lines that lacked CD58 expression with inactivating gene alterations. In the report by Challa-Malladi et al. (2011), 67 % of DLBCL biopsies were shown to lack cell surface expression of CD58, and the frequencies were similar between ABC/nonclassified (NC)-DLBCL and GCB-DLBCL (68 % and 65 %, respectively). However, genetic lesions of CD58 were shown to be significantly more frequent in ABC/NC-DLBCL than in GCB-DLBCL (67.9 % and 32.1 % of the lesions found, respectively), suggesting that non-genetic mechanisms of CD58 downregulation are more frequently involved in GCB-derived lymphoma than in ABC-DLBCL. In our analysis of the DLBCL database, we found that CD58 and EZH2 mutations are mutually exclusive (Fig. 3F), which may imply that different DLBCL subtypes utilize different regulatory mechanisms of CD58 expression.

EZH2 is a catalytic subunit of the Polycomb Repression Complex 2 (PRC2), which confers transcriptional repression through H3K27 trimethylation (Bracken and Helin, 2009), and its role during B cell differentiation is well characterized. EZH2 is upregulated when B cells enter the germinal center reaction, and it suppresses anti-proliferative genes such as CDKN2A and pro-differentiation genes such as IRF4 and PRDM1, until B cells exit the germinal center (Velichutina et al., 2010; Beguelin et al., 2013; Caganova et al., 2013). Comprehensive genome sequencing analyses identified recurrent gain-of-function mutations of EZH2, mainly of Tyr641, in FL and GCB-DLBCL (Morin et al., 2010). In our analysis, FL218 and SU-DHL6 carried a gain-of-function mutation of EZH2 Tyr646Asn. CD58 expression levels were low in both cell lines and were upregulated by EPZ6438, although in varying degrees (Fig. 3C, D). Several EZH2 inhibitors have been developed for the treatment of lymphomas with EZH2 activation, and ongoing clinical studies have suggested the effectiveness of EZH2 inhibitors in a proportion of relapse/refractory FL and DLBCL (Italiano et al., 2018; Kim and Roberts, 2016; Lue and Amengual, 2018). However, their detailed

mechanism of action is still not well clarified, and it has been difficult to precisely predict their efficacy. According to our study, it can be speculated that one possible mechanism of action of EZH2 inhibitors is the restoration of epigenetically downregulated CD58, and lymphomas with high EZH2 activity, such as those carrying gain-of-function mutations of EZH2, are good targets for these agents.

In an experiment with a melanoma mouse model, EZH2 has been shown to epigenetically control molecules that are involved in the mechanisms of resistance to immunotherapy (Zingg et al., 2017). T cell accumulation induced by anti-CTLA-4 or IL-2 immunotherapy led to increased EZH2 expression in melanoma cells, and EZH2 silenced their immunogenicity and antigen presentation by downregulating molecules such as MHC. EZH2 inactivation was shown to reverse this resistance and synergize with anti-CTLA-4 and IL-2 immunotherapy to suppress melanoma growth. Although mice do not express CD58 molecules and the effect of EZH2 inhibition on CD58 expression was not evaluated in this melanoma experiment, it can be assumed that EZH2 activation may be commonly involved in the molecular mechanisms of immune escape in several different malignant tumors.

Our findings demonstrated one of the distinct molecular activities of EZH2 inhibitors on lymphoid malignancies. Comprehensive molecular profiling will become available for the diagnosis of lymphoma in the near future, and it is important to translate these results into clinical practice and utilize them for better treatment selection. Our findings suggested that EZH2 inhibitors may be effective for the treatment of lymphoma with epigenetic CD58 suppression. Because EZH2 inhibitors have been shown to potentially enhance T and NK cell responses against selected lymphoma cells by restoring its epigenetically downregulated CD58 expression, combination with immune-targeted therapies, such as immune checkpoint inhibitors, is expected to have synergistic effects for the treatment of lymphomas. Clinical studies will further clarify the activity of EZH2 inhibitors on lymphomas and their most suitable applications.

CRedit authorship contribution statement

Yasuyuki Otsuka: Investigation, Methodology, Writing - original draft. **Momoko Nishikori:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Funding acquisition. **Hiroshi Arima:** Resources, Formal analysis. **Kiyotaka Izumi:** Resources. **Toshio Kitawaki:** Resources, Methodology. **Masakatsu Hishizawa:** Resources, Funding acquisition. **Akifumi Takaori-Kondo:** Supervision.

Declaration of Competing Interest

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