The EZH2 inhibitor tazemetostat upregulates the expression of CCL17/TARC in B-cell lymphoma and enhances T-cell recruitment

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Abstract
An inhibitor of the histone methyltransferase enhancer of zeste homologue 2 (EZH2), tazemetostat, has been developed for the treatment of B-cell lymphoma, but its mechanisms of action are not fully elucidated. We screened for genes targeted by tazemetostat in eleven B-cell lymphoma cell lines and found that tazemetostat significantly increased the expression of chemokine (C-C motif) ligand 17 (CCL17)/thymus- and activation-regulated chemokine (TARC) in all, which codes for a chemokine that is a hallmark of Hodgkin/Reed-Sternberg (H/RS) cells in Hodgkin lymphoma. Notably, gene set enrichment analysis demonstrated a positive correlation between the genes upregulated by tazemetostat in five follicular lymphoma (FL) cell lines and those reported to be overexpressed in H/RS cells. The CCL17 promoter region was enriched in repressive histone modification H3K27me3, and tazemetostat induced H3K27 demethylation and activated gene transcription. CCL17 protein secretion was also induced by EZH2 inhibition, which was further enhanced by concurrent CpG stimulation. In vitro transwell migration assay demonstrated that CCL17 produced by tazemetostat-treated B cells enhanced the recruitment of T cells, which had the potential to exert antilymphoma response. Analysis of publicly available human lymphoma databases showed that CCL17 gene expression was inversely correlated with the EZH2 activation signature and significantly paralleled the CD4+ and CD8+ T-cell–rich signature in FL and germinal center B-cell–like diffuse large B-cell lymphoma. Our findings indicate that tazemetostat can potentially activate antilymphoma response by upregulating CCL17 expression in B-cell lymphoma cells and promote T-cell recruitment, which provides a rationale for its combination with immunotherapy.

KEYWORDS
B-cell lymphoma, CCL17, EZH2 inhibitor, Hodgkin lymphoma, tumor microenvironment
INTRODUCTION

The histone methyltransferase enhancer of zeste homologue 2 (EZH2) is a component of the polycomb group complex and is involved in repressing gene expression through trimethylation of histone H3 on lysine 27 (H3K27). It plays roles in the regulation of development, proliferation, and differentiation of various cell types, and its aberrant activity is involved in the pathogenesis of a number of malignancies, including B-cell lymphomas.

Activating mutations in the EZH2 gene are recurrently found in follicular lymphoma (FL) and germinal center B-cell-like diffuse large B-cell lymphoma (GCB-DLBCL). In addition, EZH2 overexpression is suggested to be involved in the pathogenesis of mantle cell lymphoma (MCL) and Burkitt lymphoma (BL). In light of these findings, an EZH2 inhibitor, tazemetostat, has been developed for the treatment of B-cell lymphoma and is approved for the treatment of FL with EZH2-activating mutations. However, clinical studies have shown that it is also effective to some extent for FL without EZH2 mutations and lymphomas of other histologic subtypes. Its usefulness seems to be limited as a single agent, and it is considered necessary to understand its mechanisms of action to maximize its efficacy and application.

In this study, we aimed to elucidate the biological effects of tazemetostat that are shared among B-cell lymphomas. We screened for genes upregulated by tazemetostat in B-cell lymphoma lines and found that chemokine (C-C motif) ligand 17 (CCL17)/thymus- and activation-regulated chemokine (TARC), a chemokine that is a hallmark of Hodgkin/Reed-Sternberg (H/RS) cells in Hodgkin lymphoma (HL), was significantly upregulated in all. Moreover, RNA sequencing (RNA-seq) and gene set enrichment analysis (GSEA) of five FL cell lines demonstrated a positive correlation between the genes upregulated by tazemetostat in FL cell lines and those reported to be overexpressed in H/RS cells.

CCL17 is a chemokine expressed in antigen-presenting cells such as dendritic cells, and as a ligand for CCR4, it induces trafficking of CCR4-positive T cells and facilitates the T-cell response. Although CCL17 receptor CCR4 is typically expressed on regulatory T (Treg) cells, increased CCL17 expression in lymphoma cell lines induced the recruitment of broader subsets of T cells, which had the potential to exert antilymphoma response.

According to these results, it is suggested that EZH2 inhibition in B-cell lymphomas promotes T-cell infiltration into the tumor microenvironment by upregulating CCL17. Our findings provide a rationale for the combination of an EZH2 inhibitor with immunotherapy for the treatment of B-cell lymphomas.

MATERIALS AND METHODS

2.1 Cell lines, primary lymphoma cells, and culture conditions

The following cell lines were used: transformed FL lines FL18, FL218, FL318, FL518, FL618; a GCB-DLBCL line SU-DHL-6; activated B-cell-like (ABC)-DLBCL lines DLBCL2 and HBL-1; BL lines Raji and Daudi; an MCL line Granta-519; and HL cell lines KM-H2, L428, and HDLM2. Cells were maintained in RPMI1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/L-glutamine (PSG). In the experiments of tazemetostat treatment, the cell lines were cultured at $2 \times 10^5$/mL in a 24-well plate for 4 days with indicated concentrations, or 5 µM if not otherwise noted, of tazemetostat (Apexbio).

Primary lymphoma cells were collected from pleural effusion of a patient with transformed indolent B-cell lymphoma after obtaining informed consent from the patient in accordance with the ethical standards of the Helsinki Declaration and the Institutional Review Board of Kyoto University Hospital. For the culture of primary lymphoma cells, CD40 ligand (CD40L)-transfected L cells22 irradiated with 50 Gy were first seeded at $5 \times 10^4$/mL in a 24-well plate and cultured overnight with DMEM containing 10% FBS and 1% PSG. After removing the medium, $1 \times 10^6$/mL primary lymphoma cells were cocultured with L cells in fresh RPMI1640 containing 10% FBS and 1% PSG for 3 days, with different concentrations of tazemetostat or carrier (0.1% dimethyl sulfoxide, DMSO).

2.2 Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using an RNaseasy Mini kit (Qiagen), and complementary DNA (cDNA) was synthesized using a SuperScript III First- Star and Synthesis system (Life Technologies). qRT-PCR was performed using TB Green Premix Ex Taq II (Takara) with primers listed in Table 1.

2.3 Transcriptome sequencing analysis, mutation analysis, and GSEA

Total RNA was extracted from cell lines using an RNaseasy Mini kit (Qiagen), and transcriptome sequencing and mutation analyses were performed at Riken Genesys (Kanagawa, Japan). Briefly, RNA-seq libraries were constructed using TruSeq Stranded mRNA Library Prep Kit (Illumina), and a multiplexed paired-end sequencing analysis was performed according to the manufacturer’s instructions. Single nucleotide variant (SNV) and insertion/deletion (Indel) calls were performed using samtools (0.1.19) and filtered by QUAL (≥10) and Variant Frequency (>0.3). The variants were annotated based on RefSeq (using in house program), and the genes of interest were picked up. GSEA was performed on the normalized RNA-Seq expression data, and the 83 genes that were reported to be upregulated in H/RS cells microdissected from HL tissues were using the Broad Institute desktop application (http://software.broadinstitute.org/gsea/downloads.jsp).

2.4 Chromatin immunoprecipitation (ChIP)-qPCR

FL318 cells were first cultured with indicated concentrations of tazemetostat or DMSO for 4 days. ChIP assay was performed in
accordance with a published method \(^2\) with following modifications: FL318 cells were crosslinked with 1% formaldehyde for 5 minutes at room temperature with gentle rotation and then quenched with 0.125 M glycine. After washing, nuclei were sonicated in a Covaris S220 ultrasonicator (Covaris), and the supernatants were used for immunoprecipitation with anti-H3K27me3 monoclonal antibody (clone MABI 0323; Active Motif), anti-histone H3 antibody (MABI 0301; Active Motif), or control IgG (#5415; Cell Signaling Technology). Using the precipitated samples, the CCL17 promoter region was analyzed by qPCR using the primers listed in Table 1.

### 2.5 Knockdown of KDM4C by shRNA

Two different KDM4C shRNAs were cloned into the retroviral vector pSicoR-mCherry. The sequence targeted by inserting synthetic double-stranded oligonucleotides (TRCN0000022054, sense oligo, 5′-TATACCTTTGGATTACGAAGATTTTTCAAGGAAGAAATCTTCTGTAATCCAGATTTTTTTCC-3′; antisense oligo, 5′-TCGAGAAAAAAAATACTTTGGATTACGAAGATTTTTCAAGGAAGAAATCTTCTGTAATCCAGATTTTTTTCC-3′; sense oligo, 5′-GGCGAGAGGATGGTATCTGTAATCCAGATTTTTTTCC-3′; and antisense oligo, 5′-GGCGAGAGGATGGTATCTGTAATCCAGATTTTTTTCC-3′). These vectors were cotransfected with packing plasmid mix (GE Dharmacon) into 293T-LentiX cell lines to produce lentivirus. Supernatant was collected 48 hours after transfection and concentrated by ultracentrifugation for 2 hours at 126000 g. Lentiviral particles were resuspended in RPMI1640 and transduced into HL cell lines HDLM2 and L428.

### 2.6 Enzyme-linked immunosorbent assay (ELISA) for the detection of CCL17

Cells were cultured with the indicated concentrations of tazemetostat, with or without 1 \(\mu\)M CpGODN-2006 (InvivoGen) for 1 day. Their supernatants were analyzed for CCL17 using a Human CCL17/TARC Quantikine ELISA Kit (R&D Systems).

### 2.7 Transwell T-cell migration assay

Peripheral blood mononuclear cells were separated from the peripheral blood of two healthy donors using a Ficoll-Paque density gradient (Cedarlane), and total T cells were collected by negative selection using MACS Cell Separation Technology (Miltenyi Biotec). Chemotaxis of T cells was evaluated using 24-well migration chambers with 5-\(\mu\)m-pore-size inserts (Corning). Lower chambers were filled with 600 \(\mu\)l of the supernatants of lymphoma cells cultured with the indicated concentrations of tazemetostat or DMSO for 4 days (FL318) or 3 days (primary lymphoma cells), and \(2 \times 10^5\) T cells were plated in the upper chambers and incubated at 37°C for 12 hours. In blocking experiments using the anti-CCR4 antibody, T cells were incubated for 1 hour with different concentrations of anti-CCR4 antibody (Cayman Chemical) before the cell migration assay. T cells that migrated from the upper to the lower chambers were collected, and the cell numbers were calculated using an MTT assay kit (Roche Diagnostics), and the T-cell subpopulation was analyzed by flow cytometry after migration.

### 2.8 Flow cytometry

Flow cytometry was performed using a FACSLyric flow cytometer (BD Biosciences). Antibodies used for flow cytometry were as follows: anti-CD4-APC or anti-CD4-FITC (A161A1; BioLegend), anti-CD8-PE/Cy7-A (RPA-T8; BioLegend), anti-CD45RA-FITC (H1100; BioLegend), CD25-PE/Cy7-A (BC96; BioLegend), and anti-Human FOXP3 Staining Set PE (236A/E7, eBioscience). The CD4+CD25+FoxP3+ population was classified into three subpopulations based on the expression levels of CD45RA and FoxP3, CD45RA/FoxP3low resting Treg cells, CD45RA/FoxP3high activated Treg cells, and CD45RA/FoxP3low cytokine-secreting nonsuppressive cells, and the sum of the former two cell groups was evaluated as total Treg cells.
2.9 | Interferon (IFN)-γ production assay of T cells

After the transwell migration assay, T cells were collected and seeded in a 96-well plate and cultured with 4 Gy-irradiated 1 × 10⁶ cells/well of FL318, whose supernatants were used for cell migration. In the indicated samples, 5 µg/mL of anti-PD-1 antibody (nivolumab; Ono Pharmaceutical) were added. After culturing for 3 days, supernatants were examined for IFN-γ production by T cells using a human IFN-γ ELISA kit (BioLegend).

2.10 | Analysis of human lymphoma databases

The core dataset of DLBCL samples (278 GCB-DLBCL and 252 ABC-DLBCL samples) was obtained from EGA (dataset id: EGA00001003600), and a dataset of 40 FL samples was obtained from International Cancer Genome Consortium (ICGC) Malignant Lymphoma-Germinal center B-cell-derived lymphomas project (https://icgc.org/icgc/cgp/64/345/53049). Gene expression was measured using terms of fragments per kilobase of exon per million fragments mapped and normalized using the Cufflinks package, version 2.2.124. Quantile normalization was performed, and the data were log2 normalized. To evaluate the correlation between CCL17 expression and the cell signatures of CD4⁺ T cells, CD8⁺ T cells, Treg cells, follicular dendritic cells, myeloid cells, and stromal cells, the geometric mean (log-average) of the expression of genes was calculated. The EZH2-activated signature was determined as a reciprocal of the signature calculated from the gene set upregulated by GSK343 in a GCB-DLBCL cell line. EZH2-activating mutations were defined as Y641F/N/S/H/C, A677G, and A687V.

3 | RESULTS

3.1 | CCR17 is upregulated by tazemetostat in B-cell lymphoma lines

In our previous analysis of microarray gene expression profiling of two human B-cell lymphoma lines, FL218 and DLBCL2, 89 genes were upregulated more than twofold in both cell lines after tazemetostat treatment. To explore the potential treatment targets of the agent, we selected seven candidate genes known to be associated with B-cell differentiation and/or intercellular interaction (CCL17, TNFRSF25, IL22RA1, ID2, BACH2, ITGAV, ID3), and their changes in gene expression after tazemetostat treatment were evaluated in 11 B-cell lymphoma lines by qRT-PCR. Among these genes, we found that CCL17 expression was significantly upregulated after tazemetostat treatment in all B-cell lymphoma lines tested, especially in those of germinal center B-cell origin (FL, GCB-DLBCL, BL) (Figure 1A, B).

CCL17 is a chemokine that is physiologically expressed in antigen-presenting cells such as dendritic cells, and is well known to be a hallmark of H/RS cells in HL. As H/RS cells are considered to be originated from germinal center B cells, we hypothesized that an EZH2 inhibitor may have a function of altering the gene expression pattern of B-cell lymphomas to those typical of H/RS cells. We compared gene expression profiling of five FL cell lines with and without tazemetostat treatment and three HL cell lines by RNA-seq, and found that tazemetostat treatment altered the gene expression of FL cell lines similar to that of HL cell lines (Figure 1C). Among 2589 genes expressed more than twofold in HL than in FL cell lines, 468 (18.1%) were upregulated more than twofold by tazemetostat in FL, whereas only 33 (1.3%) were downregulated less than 0.5-fold. On the other hand, among 1999 genes that were expressed less than 0.5-fold in HL than in FL cell lines, only six (0.3%) were upregulated more than twofold by tazemetostat in FL, whereas 76 genes (3.8%) were downregulated less than 0.5-fold.

We next performed GSEA for five FL cell lines with versus without tazemetostat treatment for 83 genes that were reported to be upregulated in H/RS cells microdissected from HL tissues. Notably, a positive correlation was demonstrated between genes upregulated by tazemetostat in FL cell lines and H/RS cells (Figure 1D, Table 2). To examine whether this effect of tazemetostat is affected by the gene mutations in lymphoma cells, we evaluated mutations in 29 representative genes in FL34 in the five FL cell lines based on the RNA-seq analysis. These FL cell lines carried mutations in 13 of the 29 genes examined, including those coding for different epigenetic modifiers, but only one (FL218) carried an EZH2-activating mutation (Figure 1E). According to these results, it is demonstrated that tazemetostat has a function of upregulating HL-related genes in FL cell lines, regardless of EZH2 mutations.

Figure 1 CCL17 gene expression is enhanced in B-cell lymphoma lines by tazemetostat. A, Fold changes in the expression levels of seven candidate genes in B-cell lymphoma lines treated with tazemetostat (5 µM, 4 d). Gene expression levels were examined by qPCR and normalized to the expression level of ACTB. Fold changes in gene expression in cells treated with tazemetostat compared with DMSO-treated controls were calculated. B, Results of the changes in CCL17 gene expression levels of B-cell lymphoma lines treated with tazemetostat (black bars) compared with DMSO-treated controls (gray bars). CCL17 gene expression levels were examined by qPCR and normalized to the expression level of ACTB. The vertical bars indicate standard deviation of the duplicates. C, Gene expression alterations induced by tazemetostat treatment in 5 FL cell lines. Genes upregulated by tazemetostat were enriched in the genes that were expressed higher than 2-fold in Hodgkin lymphoma (HL) than in follicular lymphoma (FL) cell lines (upper panel), and genes downregulated by tazemetostat were enriched in the genes that were expressed lower than 0.5-fold in HL than in FL cell lines (lower panel). D, Gene set enrichment analysis (GSEA) enrichment plot for five FL cell lines with versus without tazemetostat treatment for genes reported to be upregulated in Hodgkin/Reed-Sternberg (H/RS) cells microdissected from HL tissues. E, Representative gene mutations in five FL cell lines. All of them are positive for t(14;18) translocation.
(A) | FL | DLBCL | BL | MCL |
|---|---|---|---|---|
| CCL17 | FL18 | FL218 | FL318 | FL518 | FL618 | DLBCL2 | SUDHL6 | HBL-1 | Raji | Daudi | Grandpa51 |
| TNFRSF25 | FL18 | FL218 | FL318 | FL518 | FL618 | DLBCL2 | SUDHL6 | HBL-1 | Raji | Daudi | Grandpa51 |
| IL22RA1 | FL18 | FL218 | FL318 | FL518 | FL618 | DLBCL2 | SUDHL6 | HBL-1 | Raji | Daudi | Grandpa51 |
| ID2 | FL18 | FL218 | FL318 | FL518 | FL618 | DLBCL2 | SUDHL6 | HBL-1 | Raji | Daudi | Grandpa51 |
| BACH2 | FL18 | FL218 | FL318 | FL518 | FL618 | DLBCL2 | SUDHL6 | HBL-1 | Raji | Daudi | Grandpa51 |
| ITGAV | FL18 | FL218 | FL318 | FL518 | FL618 | DLBCL2 | SUDHL6 | HBL-1 | Raji | Daudi | Grandpa51 |
| ID3 | FL18 | FL218 | FL318 | FL518 | FL618 | DLBCL2 | SUDHL6 | HBL-1 | Raji | Daudi | Grandpa51 |

Genes expressed higher in HL than in FL:
- CCL17 gene expression

Genes expressed lower in HL than in FL:
- NES 1.38
- FDR q-value 0.17
- FWER p-value 0.09

(B) CCL17 gene expression

(D) Enrichment plot: HLRELATED_GENES

(E) Genes expressed higher in HL than in FL

| FL18 | FL218 | FL318 | FL518 | FL618 |
|---|---|---|---|---|
| KMT2D | CREBBP | EZH2 | ARID1A | EP300 |
| SMARCA4 | TNFRSF14 | CXCR4 | STAT6 | IRF8 |
| ETS1 | CCND3 | TP53 | t(14;18) |

Missense | Nonsense | Translocation
3.2 | Tazemetostat enhances CCL17 promoter activity by EZH2 inhibition leading to demethylation of H3K27me3

EZH2 functions as a transcriptional repressor by catalyzing histone H3K27 trimethylation; therefore, we hypothesized that the CCL17 promoter region is enriched in repressive histone modification H3K27me3, and tazemetostat induces CCL17 gene expression by inhibiting EZH2 and leading to H3K27 demethylation of the promoter. We performed ChIP of FL318 cells with an anti-H3K27me3 antibody and quantified the CCL17 promoter region in the precipitated samples by qPCR. We found that the CCL17 promoter region was more highly marked with H3K27me3 in FL318, and treatment of the cells with tazemetostat led to its reduction (Figure 2A). These results indicated that CCL17 promoter activity is repressed by H3K27me3, and tazemetostat is able to reverse this epigenetic repression by inhibiting the function of EZH2.

As tazemetostat upregulated the gene expression of FL cells more similar to H/RS cells, we hypothesized that different epigenetic regulation partly determines the difference in FL and HL gene expression. Chromosome band 9p24 is reported to be frequently amplified in HL cells, and histone demethylase KDM4C/JMJD2C is identified as an oncogene in this amplicon. We found that knockdown of KDM4C in two HL cell lines, L428 and HDLM2, downregulated the expression of CCL17 (Figure 2B). According to these findings, it is suggested that the gene expressions of FL and HL cells are partly reciprocally regulated by the histone modifications, and alterations in epigenetic regulation can reverse these gene expressions.

3.3 | Tazemetostat enhances CCL17 secretion in B-cell lymphoma lines

CCL17 is a chemokine that is physiologically produced by antigen-presenting cells and leads to the activation and migration of T cells expressing its receptor CCR4. To evaluate whether tazemetostat treatment enhances CCL17 production in B-cell lymphomas, we examined CCL17 levels in the supernatant of the representative four B-cell lymphoma lines cultured with or without tazemetostat. We found that CCL17 levels were increased in FL18, FL318, and Raji cells in parallel with the concentration of tazemetostat, and costimulation with CpG further enhanced its production (Figure 3). In SUDHL6, CCL17 was not produced solely by tazemetostat, but concurrent treatment with CpG induced CCL17 secretion.

3.4 | CCL17 produced by B-cell lymphoma cells enhances migration of T cells with potential cytotoxic activity

We next performed an in vitro transwell T-cell migration assay to examine whether enhancement of CCL17 production in B-cell lymphoma with tazemetostat treatment can promote chemotaxis of T cells. T cells of healthy donors were placed in the upper chambers of the transwell culture plate, and the lower chambers were filled with a supernatant of FL318 cells cultured in conditions with various tazemetostat concentrations. After culturing T cells for 12 hours, the number of T cells that migrated to the lower chambers was calculated. We found that the number of T cells that...
migrated increased when the lower chamber was filled with supernatant from FL318 cells treated with higher tazemetostat concentrations (Figure 4A). In contrast, cell migration was suppressed when T cells were pretreated with CCR4-blocking antibody, indicating that T-cell migration was mainly driven by CCL17 produced by FL318 cells. Moreover, we were able to detect CCL17 secretion from in vitro–cultured primary B-cell lymphoma cells in parallel with the concentration of tazemetostat, and CCL17 secreted by primary lymphoma cells similarly had the function of inducing T cell migration (Figure 4B).

Evaluation of migrated CD4⁺, CD8⁺, and Treg-cell subsets using an MTT assay and flow cytometry demonstrated that although CCR4 is typically expressed on Treg cells, migrated cells contained an MTT assay and flow cytometry demonstrated that although the amounts of precipitated DNA relative to the input are demonstrated. The vertical bars indicate standard deviation of the duplicates (**P < .01; ***P < .001; Student’s t-test). B, KDM4C was knocked down in two Hodgkin lymphoma (HL) cell lines, L428 and HDLM2, by two different KDM4C shRNA constructs. KDM4C and CCL17 expression levels were evaluated by qPCR and normalized to the expression level of ACTB. The vertical bars indicate standard deviation of the duplicates (**P < .01; Student’s t-test)

3.5 | CCL17 expression parallels the T-cell–rich microenvironment in human B-cell lymphomas

Our observation that an EZH2 inhibitor has a function of inducing CCL17 secretion by B-cell lymphoma cell lines led us to question whether CCL17 can be expressed and involved in the regulation of immune microenvironment in human B-cell lymphomas. We analyzed publicly available FL and DLBCL databases to elucidate the relationship of EZH2 activity, CCL17 expression, and T-cell signatures in human B-cell lymphoma samples. In the FL and GCB-DLBCL datasets, EZH2-activating mutations (Y641F/N/S/H/C, A677G, and A687V) were found in six out of 40 FL samples and 35 out of 278 GCB-DLBCL samples, and these samples presented with a higher EZH2-activated signature and lower CCL17 gene expression (Figure 5A). In ABC-DLBCL, on the other hand, the number of samples with EZH2-activating mutations was too small to analyze (three out of 252 samples). We further found that CCL17 expression levels were significantly inversely correlated with a high EZH2-activated signature in FL and GCB-DLBCL but not in ABC-DLBCL (Figure 5B). Notably, CCL17 expression levels were significantly associated with not only the Treg cell signature but also CD4⁺ and CD8⁺ T-cell signatures in FL and GCB-DLBCL (Figure 5B). On the other hand, CCL17 expression has no or weaker association with other cell signatures such as follicular dendritic cells, myeloid cells, or stromal cells (Figure S1). These results were considered to correspond well with our findings that CCL17 produced by B-cell lymphoma induces T-cell chemotaxis and promotes a T-cell–rich tumor microenvironment.

4 | DISCUSSION

Epigenetic modification regulates the transcription of many genes, plays critical roles in cell development and differentiation, and is also
**FIGURE 3** CCL17 secretion is induced by tazemetostat in B-cell lymphoma lines. B-cell lymphoma lines (FL18, FL318, SUDHL6, Raji) were cultured with tazemetostat at the indicated concentration (0.2, 1, and 5 μM) or DMSO for 4 d, with or without 1 μM CpG stimulation for the last 1 d. Cell supernatants were then collected and examined for CCL17 protein levels by ELISA. The vertical bars indicate standard deviation of the duplicates (*P < .05, **P < .01; ***P < .001; NS, not significant; Student’s t-test).

**FIGURE 4** CCL17 secreted from B-cell lymphoma cells enhances T cell migration. A, Chemotaxis of T cells was evaluated using a chamber plate separated with 5-μm-pore inserts. Peripheral T cells from healthy donors were plated in the upper chambers, and after a 12-h incubation, T cells that migrated to the bottom chambers were quantified using an MTT assay. In the indicated samples, T cells were incubated for 1 h with an anti-CCR4 antibody before plating for migration. Results from two different T-cell donors are shown. The vertical bars indicate standard deviation of the duplicates (*P < .05; **P < .01; NS, not significant; Student’s t-test). B, Primary B-cell lymphoma cells were cultured with CD40L-transfected L cells and tazemetostat at the indicated concentration, and cell supernatants were examined for CCL17 by ELISA (left panel). Chemotaxis of T cells toward CCL17 secreted from primary B-cell lymphoma cells was evaluated with the same method as in Figure 4A (right panel). In the indicated samples, T cells were preincubated for 1 h with an anti-CCR4 antibody. The vertical bars indicate standard deviation of the duplicates (**P < .01; ***P < .001; NS, not significant; Student’s t-test). C, Cell numbers of the T-cell subsets migrated toward the supernatant of FL318 treated with DMSO or tazemetostat (5 μM). Mean of the data from two different T-cell donors are shown (*P < .05; **P < .01; ***P < .001; Student’s t-test). D, Interferon (IFN)-γ production of T cells that migrated toward the CCL17-expressing B-cell lymphoma line. T cells that migrated toward the supernatant of FL318 treated with DMSO or tazemetostat (5 μM) were cultured with FL318. In the indicated wells, anti-PD-1 antibody was added. After culturing for 3 d, supernatants were examined for IFN-γ production by T cells by ELISA. Mean of the data from two different T-cell donors are shown (*P < .05; **P < .01; NS, not significant; Student’s t-test).
(A) FL

EZH2 activating mutation

Log2(FPKM+1) of CCL17

EZH2 activating mutation

EZH2 activating mutation

EZH2 activating mutation

EZH2 activating mutation

(B) FL

GCB-DLBCL

ABC-DLBCL

EZH2-activated signature

EZH2-activated signature

EZH2-activated signature

EZH2-activated signature

Treg cell signature

CD4+ T cell signature

CD8+ T cell signature

Log2(FPKM+1) of CCL17

Log2(FPKM+1) of CCL17

Log2(FPKM+1) of CCL17
involved in the pathogenesis of a variety of malignancies. In this report, we have demonstrated that the EZH2 inhibitor tazemetostat leads to CCL17 upregulation in B-cell lymphomas.

CCL17 is typically overexpressed in H/RS cells and is responsible for the T-cell–rich microenvironment of HL. In addition to CCL17, we have found a positive correlation between gene upregulation by tazemetostat in five FL cell lines and those reported to be overexpressed in H/RS cells microdissected from HL tissues. The CCL17 promoter region is found to be enriched in H3K27me3, and EZH2 inhibition induces its demethylation and activates CCL17 gene transcription. On the other hand, knockdown of KDM4C in HL cell lines is shown to downregulate CCL17 expression. According to these findings, it is suggested that the gene expressions of FL and HL cells are partly reciprocally regulated by histone modifications, and alterations in epigenetic regulation can reverse these gene expressions. It is clinically recognized that, in some rare instances, lymphoma patients develop two distinct types of lymphoma called composite lymphomas, and many of the composite cases with HL and B-cell lymphoma are clonally related. Based on our findings, it can be speculated that HL and B-cell lymphoma may evolve concurrently from the same lymphoma clone when two different epigenetic modifications occur during the developmental process.

We have also shown that enhanced secretion of CCL17 from B-cell lymphoma cells by tazemetostat promoted the recruitment and IFN-γ secretion of T cells against lymphoma cells, and the addition of anti-PD-1 antibody further enhanced the IFN-γ secretion of T cells that migrated toward CCL17. Finally, we analyzed human lymphoma databases and found that the CCL17 expression level inversely paralleled the EZH2 activation signature and significantly paralleled the T-cell–rich microenvironment in FL and GCB-DLBCL.

The high efficacy of PD-1 blockade therapy in HL has been demonstrated in several clinical studies and in clinical practices. In contrast, B-cell malignancies are less sensitive to PD-1 blockade therapy, which could be partly explained by the scarcity of tumor-infiltrating T cells that can readily exert antitumor immune responses. Our findings suggest that an EZH2 inhibitor can induce T-cell inflamed lymphoma microenvironment and is expected to be utilized for amplifying the effect of immunotherapy.

We observed CCL17 upregulation by tazemetostat in all 11 B-cell lymphoma lines tested, irrespective of their histologic origin. In the analysis of FL and GCB-DLBCL databases, CCL17 expression levels were shown to significantly inversely parallel the EZH2-activated signature and positively correlate with both CD4+ and CD8+ T-cell signatures. On the other hand, the association among CCL17 expression levels, EZH2-activated signature, and T-cell signatures was unclear in ABC-DLBCL samples. Although we do not have any clear explanation, there may be additional factors that affect the extent of tumor-infiltrating T cells in ABC-DLBCL. As our findings are mainly based on the experiment of cell lines, further analysis is necessary to find out whether the EZH2 inhibitor actually has a function in upregulating CCL17 and increasing tumor-infiltrating T cells in B-cell lymphoma patients.

In summary, we found that the EZH2 inhibitor tazemetostat upregulates CCL17 expression in B-cell lymphoma cells and facilitates T-cell recruitment, which have the potential to exert antilymphoma responses. We propose that EZH2 inhibition in B-cell lymphomas promotes T-cell infiltration into the tumor microenvironment. Although further studies are warranted, our findings provide a rationale for the combination of an EZH2 inhibitor with immunotherapy.

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CONFLICT OF INTEREST
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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