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Inhibition of H3K27me3 Demethylases Promotes Plasmablast Formation

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ABSTRACT

B cell differentiation into Ab-secreting plasma cells requires transcriptional, metabolic, and epigenetic remodeling. Histone H3 lysine 27 trimethylation (H3K27me3), a histone modification associated with gene silencing, is dynamically regulated during B cell differentiation. Although several studies have focused on mechanisms involving the gain of this modification in plasmablasts (PB), the role of active demethylation of H3K27me3 by ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX) and Jumonji domain-containing protein 3 (JMDJ3) during B cell differentiation has not been examined. In this study, this process was assessed using a pharmacological inhibitor of UTX and JMDJ3, GSK-J4. Treatment of ex vivo stimulated mouse B cells with GSK-J4 led to an increase in PB frequency without affecting the ability of the newly formed PB to secrete Abs. Consistent with the role of UTX and JMDJ3 in promoting gene expression, the majority of differentially expressed were downregulated upon GSK-J4 treatment. Inhibitor treated cells upregulated genes associated with cell cycle and proliferation, which correlated with an increase in actively proliferating cells. Unexpectedly, a majority of the downregulated transcripts corresponded to genes that in the wild-type setting were genes that gain H3K27me3 and downregulated in PB. Together, our results show that UTX and JMDJ3 are required to restrain B cell differentiation and suggest that they function as a rheostat for H3K27me3 to control this process. ImmunoHorizons, 2021, 5: 918–930.

INTRODUCTION

Humoral immunity relies on the ability of naive B cells (nB) to differentiate into Ab secreting short-lived plasmablasts (PB) or long-lived postmitotic plasma cells (PC). To allow for robust Ab secretion and differentiation, B cells undergo substantial changes in their transcriptional profile as well as metabolism (1–4). nB and PC fates are regulated by distinct sets of transcription factors. Whereas PAX5 (5–8) and BACH2 (9, 10) promote the nB stage, BLIMP1 (11–13) and high levels of IRF4 (14, 15) promote PC formation. In addition, there is a growing appreciation for the epigenetic reprogramming that occurs during B cell differentiation (16, 17). This is well exemplified by the fact that differentiating B cells undergo cell division–coupled reprogramming of their accessible chromatin landscape and progressive DNA hypomethylation of their genome following stimulation with T cell–independent Ags (18, 19).
In addition to changes in DNA methylation to facilitate cell fate decisions, the distribution of histone modification also changes during B cell differentiation (18, 20, 21). Of particular note is the status of histone H3 lysine 27 trimethylation (H3K27me3) modifications at nB- and PB-specific genes. This histone modification is associated with a repressed chromatin state and gene silencing (22). H3K27me3 is deposited by EZH2 (23–25), a component of the PRC2 complex, and is enzymatically removed by two demethylases, ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX) and Jumonji domain-containing protein 3 (JMJD3) (26, 27). UTX and JMJD3 are also termed KDM6A and KDM6B, respectively. In a recent study, H3K27me3 was shown to be dynamically regulated during B cell differentiation with a roughly equal number of promoter regions that gain and lose this histone modification as B cells differentiate to PB (21). Deposition of H3K27me3 by EZH2 has been shown to play an important role during B cell development (28, 29), germinal center formation and maintenance (30–32), as well as PB formation in response to T-independent Ags (21). However, a significant gap in knowledge persists concerning the role of UTX and JMJD3 in the epigenetic regulation of B cell differentiation and PC formation.

UTX and JMJD3 facilitate H3K27me3 demethylation via their Jumonji C domain in an iron- and α-ketoglutarate–dependent manner (33, 34). This process occurs via direct hydroxylation of the methyl group, resulting in a formation of a hydroxymethyl intermediate, which is then released as a formaldehyde (35). UTX is X linked with a homolog, UTY, encoded on the Y chromosome. The demethylation activity of UTY is extremely low compared with UTX (26, 27, 36). In addition to their catalytic activity, UTX and JMJD3 influence gene expression through interactions with a host of chromatin regulators, including BRG1 (37, 38) and CHD4 (38, 39), p300 (40), and most notably the MLL complex, which promotes H3K4 methylation (41, 42). The tumor suppressor p53 also interacts with UTX and JMJD3 (43). UTX and JMJD3 have been shown to function in various biological processes, including early embryonic development (44–46). H3K27me3 demethylases were shown to facilitate resolution of bivalent promoters at retinoic acid–inducible genes (47) and derepression of inactive enhancers (40). Other roles have included cardiac development (48), hematopoiesis (49), M2 macrophage differentiation (50), and regulation of various T cell subsets (37, 51–56).

In a clinical setting, mutations in UTX lead to a rare, congenital disorder characterized by distinct facial features, developmental delay, intellectual disability, and multiorgan malfunctions (57, 58). Furthermore, mutations in UTX have been identified in diffuse large B cell lymphoma (DLBCL) (59) and multiple myeloma (60–62). Mutations in JMJD3 have been described in Hodgkin’s lymphomas (63) and DLBCL (64, 65). Together, this suggests that these enzymes are important regulators of B cell fate. Furthermore, changes in the expression of genes associated with PC and memory B cells have been reported following overexpression of JMJD3 in human germinatal center B cells (63). However, the role of UTX and JMJD3 in B cell differentiation has not been fully examined, thus leaving a significant gap in our knowledge of epigenetic regulation of PC formation.

In this study, we used a pharmacological inhibitor for UTX and JMJD3 to examine their role in regulating B cell differentiation using an ex vivo model system. The results showed that the demethylation enzymes are involved in controlling cell cycle, proliferation, and, ultimately, the frequency of B cells that differentiate into PB and are therefore critical for PB reprogramming and function.

MATERIALS AND METHODS

Mice

C57BL/6J mice (stock no. 000664) were purchased from The Jackson Laboratory and bred on site. All animals were housed by the Emory Division of Animal Resources following protocols approved by the Emory Institutional Animal Care and Use Committee.

Ex vivo differentiation

Naïve splenic B cells were magnetically enriched by negative selection using CD43 (Ly-48) MicroBeads (Miltenyi Biotec, 130-097-148) with >95% purity. Unless otherwise stated, purified B cells were cultured at 0.5 × 10⁶ cells/ml in B cell media (RPMI 1640, 10% heat-inactivated FBS, 0.05 mM 2-ME, 1X nonessential amino acids, 1X penicillin/streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate) supplemented with 20 μg/ml LPS (Sigma, L2630), 20 ng/ml IL-2 (BioLegend, 575406), and 5 ng/ml IL-5 (BioLegend, 581504). LPS and cytokines were supplemented with half of the above dose at 24 and 48 h of ex vivo culture as previously described (66). In some experiments, naïve B cells were stained with 5μM Cell Trace Violet (Life Technologies, C34557) prior to culturing. GSK-J4 (Sigma, SML0701) and GSK-J5 (Cayman Chemical, 12074) were dissolved in DMSO and diluted in B cell media. Cells were treated daily with 250 nM GSK-J4 or DMSO. For CD40L stimulation, cells were seeded at 10⁵ cells/ml in the above B cell media–containing CD40L (100 ng/ml, R&D Systems), IL-5 (5 ng/ml), and IL-4 (20 ng/ml, R&D Systems). Cultures were supplemented with the cytokines at each subsequent day of culture. For bromodeoxyuridine (BrdU) cell cycle analysis, cells were washed, resuspended in fresh media containing 10 μM BrdU, and incubated at 37°C for two hours. Cell proliferation analysis was then performed using the Phase-Flow FITC BrdU Kit (BioLegend, 370704) following the manufacturer’s protocol.

Flow cytometry

Cells were resuspended at 10⁶ per 100 μl FACS buffer (1X PBS, 1% BSA, and 2 mM EDTA) and blocked with anti-Fc (anti-CD16/CD32) (Tonbo Biosciences, 2.4G2). The following Abs were used for staining: B220-PE-Cy7 (Tonbo Biosciences, RA3-6B2), CD138-BV711 (BD, 281-2), GL7 eFluor660 (eBioscience, GL-7), CD11b-FITC (Tonbo Biosciences, MI-70), and Ghost
Dye Red 780 (Tonbo Biosciences, 13-0865) to assess viability. The Annexin V FITC Apoptosis Detection Kit (eBioscience, BMS500FI-100) was used to assess cell death. Cells were stained for 40 min and fixed using 1% paraformaldehyde.

Enrichment of CD138+ PB was performed by staining with CD138-APC (BD, 281-2), followed by magnetic enrichment using anti-APC MicroBeads (Miltenyi, no. 130-090-855). For RNA sequencing (RNA-seq), GL7+ cells were further enriched from the CD138-depleted fractions using GL7-PE (BioLegend, no.144608) and anti-PE MicroBeads (Miltenyi, no. 130-105-639).

Flow cytometry was performed on a BD LSRII using FACSDiva (version 6.2) and analyzed using FlowJo software. The following gating strategy preceded all flow cytometry analyses presented. Cells were gated on 1) lymphocytes (forward light scatter [FSC–area by side scatter [SSC–area]), 2) singlets (FSC-height by FSC-width), 3) singlets (SSC-height by SSC-width), and 4) live cells (Viability Dye negative), with 5) the exclusion of contaminating macrophages bearing CD11b (Supplemental Fig. 2A).

**Western blot**

Ex vivo cultured B cells were lysed on ice in RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% IGEPAL, 20% glycerol, and 50 mM Tris pH 8.0) for 20 min. Protein concentration was determined by a Bradford assay (BioRad). Primary Ab incubation was conducted at 4°C overnight, followed by several washes and a one-hour incubation with the secondary Ab. The following Abs were used: anti-UTX (Cell Signaling, 33510S), anti-JMJD3 (LSBio, C96528), anti-KDM5B (Abcam, ab181089), anti-KDM5C (Proteintech 14426-1-AP), and anti-ACTIN (Santa Cruz, sc69877). Blots were developed using the Immunobilon Cresendo HRP Substrate (Sigma, WBLURO100) and visualized on a Biorad ChemiDoc MP Imaging System.

**ELISA**

Equal numbers of DMSO- or GSK-J4–treated PB were cultured in fresh B cell media. After 2.5 h, the supernatant was collected and used to perform ELISA. ELISA plates (Sigma, M9410) were coated with goat anti-mouse Ig (Southern Biotechnology, 5300-05B) overnight at 4°C and blocked with 3% nonfat dry milk. Standard IgM Ab (Southern Biotechnology, 5300-01B) and collected media supernatants were incubated for 2 h at room temperature, followed by washes and incubation with HRP-conjugated goat anti-mouse secondary Ab (Southern Biotechnology 1021-05) for 2 h at room temperature. The plates were developed using the TMB ELISA peroxidase substrate (Rockland, 800-666-7625), and the reaction was stopped using 0.2 M sulfuric acid. Plates were read using a Synergy HT Multi-Mode Microplate Reader (BioTek).

**RNA-seq**

RNA was isolated from magnetically enriched PB and activated B cells (ActB) using Zymo Quick-RNA MicroPrep Kit (11-328M). Sequencing libraries were generated using mRNA HyperPrep Kit (KAPA Biosystems, KR1352) with 500 ng input RNA per sample. Final libraries were quality checked on a bioanalyzer, quantitated by quantitative PCR, pooled at equimolar ratio, and sequenced on a HiSeq2500 system (Illumina) using paired-end, 50-bp sequencing chemistry. TopHat2\(^1\) was used to map the raw sequencing reads to the mm9 mouse genome. For each sample, reads that overlapped exons of unique ENTREZ genes were annotated using the GenomicRanges (version 1.22.4) package in R/Bioconductor. Differential expression analysis was performed using the Bioconductor package edgeR using a false discovery rate (FDR) \(\leq 0.05\) and 1.5-fold change (log2 = 0.58) (Supplemental Table 1). Principal component analysis (PCA) was performed using the vegan package and the indicated Z-score–normalized gene list. For gene set enrichment analysis (GSEA) (67), all detected genes were ranked by multiplying the sign of fold change by the \(-\log_{10}\) \(p\) value.

**Data and code availability**

All sequencing data have been deposited in the NCBI Gene Expression Omnibus ( GEO) under the accession numbers GSE158139 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=GSE158139). Code and data processing scripts are available from the corresponding author upon request and at https://github.com/cdschar/.

**RESULTS**

**H3K27me3 demethylases are upregulated during B cell differentiation**

Previous work (21) described significant gains and losses in H3K27me3 modifications as B cells differentiate to PB in response to LPS, pointing to a potential role for the removal of these marks by histone demethylases. The expression of the two H3K27me3 demethylases (UTX and JMJD3) was examined in a previously published dataset (68), which quantified gene expression during in vivo differentiation of nB following LPS stimulation. Analysis of this data revealed that, compared with nB, Utx was upregulated several-fold in newly formed PB, whereas Jmj3d expression showed a modest, albeit statistically significant, increase in expression (Fig. 1A). Expression of UTX and JMJD3 was also examined in a second LPS-induced in vivo B cell–differentiation model dataset that correlated gene expression as a function of cell division (19). In that system, PB form after division 8 and are phenotypically recognized by expression of the PC maker CD138 (termed division 8+). Again, Utx expression was significantly higher in the division 8+ cells, which represent the newly formed PB compared with control earlier divisions (Fig. 1B). Furthermore, consistent with the changes in gene expression, the protein levels of UTX and JMJD3 were higher in PB derived from ex vivo cultures compared with nB and were not altered in expression by GSK-J4 treatment (Supplemental Fig. 1A). In a similar manner, other histone modifiers that are known to be functionally affected by GSK-J4 were not altered in expression by the inhibitor.
To determine whether change in H3K27me3 during B cell differentiation correlated with transcriptional differences, the change in promoter H3K27me3 enrichment (21) was plotted against the change in gene expression between PB and nB (68). Consistent with the repressive role of H3K27me3, the analysis revealed two major sets of genes: 1) genes that had high expression and low H3K27me3 levels in PB (Fig. 1C; green quadrant); and 2) genes that had low expression and high H3K27me3 in PB compared with nB (Fig. 1C; blue quadrant). H3K27me3 enrichment at the abovementioned gene groups was quantified (Fig. 1D). This analysis also revealed a group of genes that were upregulated in PB but exhibited a higher level of promoter H3K27me3 (Fig. 1C; gray shade) and are therefore not likely to be regulated by this histone modification, but rather by other epigenetic or transcriptional mechanisms.

To study the role of UTX and JMDJ3 during B cell differentiation, a pharmacological inhibitor, GSK-J4, known to inhibit the activity of these enzymes, was used (69). In this system, naive B cells were differentiated ex vivo with LPS, IL-2, and IL-5 as previously described (66).

**Treatment with GSK-J4 promotes PB formation**

To examine the effect of the inhibitor-mediated loss of UTX and JMDJ3 catalytic activity on B cell differentiation, nB were isolated and stimulated ex vivo with LPS, IL-2, and IL-5 in the presence of 250 nM GSK-J4 or DMSO control. After 3 d of culture, flow cytometry analysis revealed a significant increase in the frequency of CD138⁺ PB in the GSK-J4–treated cultures (Fig. 2A) with a small but significant increase in the B220⁺GL7⁺ ActB (Fig. 2B). Importantly, an increase in PB following GSK-J4 treatment was also observed when compared with the inactive control compound, GSK-J5 (Fig. 2C). To determine whether the observed phenotype was specific to TLR signaling, the effect of inhibitor treatment on PB formation was examined following stimulation with CD40L, IL-4, and IL-5 that mimics T cell–dependent B cell activation (70). Enhanced B cell differentiation was also observed following this mode of stimulation (Fig 2D).

To determine whether the UTX/JMJD3 inhibition affected the ability of cells to secrete Abs, CD138⁺ PB from the LPS cultures were magnetically enriched after 3 d of ex vivo culture and an equal number of cells was plated in fresh media. Ab secretion was then analyzed by ELISA and revealed no difference in the IgM Ab titers between the GSK-J4– and DMSO-treated PB (Fig. 2E), suggesting that UTX/JMJD3 regulates the process of B cell differentiation but not the Ab secreting function of PB.

**Inhibitor treatment alters B cell transcriptome**

To define the mechanism by which treatment with GSK-J4 promotes B cell differentiation, RNA-seq was performed on magnetically enriched nB, as well as ActB and PB derived from GSK-J4 or DMSO cultures at day 3 after LPS, IL-2, and IL-5 stimulation (Supplemental Fig. 2B). PCA revealed that the activation status was the major source of variation as principal component 1 separated nB from ActB and PB. Principal component 2 separated ActB from PB, whereas principal component 3 stratified cells based on GSK-J4 treatment status (Fig. 3A). Consistent with the results of PCA, hierarchical clustering of

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samples and differentially expressed genes (DEG) between GSK-J4 and DMSO revealed that samples stratified based on cell type rather than treatment status (Fig. 3B). Differential expression analysis of GSK-J4– and DMSO-treated cells (1.5-fold change, FDR < 0.05) revealed a skewing toward genes downregulated following drug treatment. In the ActB comparison, 253 genes were downregulated (downDEG) and 106 were upregulated (upDEG). In the PB comparison, there were 352 downDEG and 84 upDEG (Fig. 3C; Supplemental Table I). Furthermore, a common set of 113 genes was downregulated in both comparisons (Fig. 3D). The observed enrichment for downDEG is consistent with inhibition of UTX and JMJD3, which in the wild-type setting promote gene expression. GSK-J4 has been shown to act, although with weaker activity, on KDM5B and KDM5C, the H3K4me2/3 demethylases (69, 71). However, inhibition of H3K4me3 demethylases would be predicted to result in gene upregulation. Thus, this observation suggests that the inhibitor primarily acts on the UTX and JMJD3 demethylase pathway rather than others.

To identify the pathways altered following GSK-J4 treatment, GSEA (67) was performed on a ranked gene list for the ActB GSK-J4 versus ActB DMSO comparison. This analysis revealed downregulation of genes associated with hypoxia, TNF signaling, P53 pathways, and apoptosis (Fig. 3E). Examples of genes downregulated following inhibitor treatment included Cdkn2a, encoding p16Ink4a and p19 Arf, which inhibit the G1/S cell cycle transition (72) and regulate p53 stability (73), respectively. Other examples of downregulated DEG include Bnip3l, which promotes apoptosis (74), and Pdk1, which inactivates pyruvate dehydrogenase, thereby inhibiting the conversion of pyruvate into acetyl-CoA (75, 76) (Fig. 3F). Despite dysregulation of several apoptotic factors, there was no difference in the frequency of apoptotic cells following inhibitor treatment. A small but significant increase in necrotic cells at 48 h poststimulation was observed (Supplemental Fig. 1B, 1C), but this difference does not explain the differences observed in the number of PB formed (Fig. 2A, 2C).

Additionally, cell cycle and proliferation genes were upregulated following inhibitor treatment (Fig. 3G). Some of the pathways dysregulated have been previously shown to be downregulated in the absence of EZH2 (21) and are upregulated as B cells progress through cell division and differentiate to PB (19). This suggested that the PB program may be initiated early or more strongly in GSK-J4–treated cells. To test this, the
FIGURE 3. Inhibition of UTX and JMJD3 leads to global changes in gene expression.
RNA-seq was performed on magnetically enriched ActB and PB from DMSO or 250 nM GSK-J4–treated cultures as well as nB controls. (A) PCA of 10,031 genes differentially expressed in at least one comparison RNA-seq data. The percentage in parentheses is the proportion (Continued).
expression of genes that were previously described to constitute a PC transcriptional signature was examined in inhibitor-treated cells (77). GSEA analysis revealed upregulation of PC-signature genes in inhibitor-treated ActB and PB (Fig. 3H, 3I, Supplemental Fig. 1D). Taken together, GSK-J4 led to global changes in the B cell transcriptome, indicating a role for H3K27 demethylation in regulating the PB transcriptome.

**Inhibitor-treated cells exhibit increased proliferation**

The downregulation of Cdkn2a and genes in the p53 pathway combined with the upregulation of genes associated with cell cycle and proliferation following GSK-J4 treatment led to the hypothesis that inhibitor treatment results in enhanced proliferation. To examine whether treatment with GSK-J4 altered cell division kinetics, nB were stained with CellTrace Violet (CTV) and stimulated ex vivo with LPS, IL-2, and IL-5 in the presence of GSK-J4 or DMSO. Irrespective of treatment, cultured B cells underwent six cell divisions after 3 d of culture. However, analysis of PB frequency at each division revealed a significant increase in PB per division following inhibitor treatment, with PB increasing as early as division four (Fig. 4A). To examine whether inhibitor treatment altered cell cycle distribution in treated cells, at day 3 post–LPS stimulation, B cell cultures were pulsed with BrdU for 2 h and analyzed by flow cytometry. GSK-J4 treatment led to a significant increase in the frequency of cells in the S phase of the cell cycle (Fig. 4B). Further, analysis of BrdU by cell division revealed a significant increase in the frequency of BrdU+ cells at the early cell divisions (Fig. 4C). Overall, although GSK-J4 treatment does not alter the total number of cell divisions, it does lead to an increase in actively proliferating cells (S phase cell cycle) with a proportional reduction of cells in the G1 phase.
DEG are enriched for genes regulated by H3K27me3

To determine whether inhibition of UTX and JMJD3 predominately affects genes regulated by H3K27me3 during B cell differentiation, genes downregulated in PB following GSK-J4 treatment were overlaid on the scatterplot comparing PB/nB gene expression with change in H3K27me3 enrichment in Fig. 1. Consistent with the hypothesis that UTX and JMJD3 promote demethylation of H3K27me3 at genes upregulated in PB, several of GSK-J4 downDEG fell in the green quadrant described above. This group includes genes such as Slc7a3, which encodes a sodium-independent transporter of cationic amino acids (78, 79) (Fig. 5A, SD). Other genes included Cth (80), Lars2 (81), Ddt (82), Fut1, Tmed6 (83), Galk1 (84), Gstt1 (85), and Abcb8 (86), which are involved in various aspects of protein synthesis or protein modification, secretion, vesicular trafficking, and metabolism. Unexpectedly, the majority of the genes downregulated following inhibitor treatment correspond to genes that, in the wild-type setting, gain promoter H3K27me3 and are downregulated in PB (blue quadrant) (Fig. 5A). H3K27me3 levels at GSK-J4 downDEG in the respective quadrants were quantified (Fig. 5B). Thus, genes downregulated by inhibition of UTX and JMJD3 are predominately enriched for regions that, in the wild-type setting, gain H3K27me3 during B cell differentiation. Due to the gain in H3K27me3, these genes are likely regulated in part by EZH2, which is the counterpart to UTX/JMJD3. In fact, GSK-J4 downDEG in the blue quadrant corresponded to genes that were significantly upregulated in EZH2-deficient PB, and 29% were defined as DEG (21) (Fig. 5C).

Examples of genes that are downregulated following GSK-J4 treatment and have high levels of H3K27me3 in wild-type PB include Id3 (inhibitor of DNA-binding/differentiation 3), which forms heterodimers with E box proteins to inhibit their DNA binding (87) and is normally repressed during PC formation with concomitant accumulation of H3K27me3. Following GSK-J4 treatment, Id3 expression is super repressed in the PB (Fig. 5E, top). Spib, which regulates the ability of B cells to respond to external stimulation and inhibits germinal center B cell and PB formation (88), followed a similar path, normally accumulating H3K27me3 in PB and super repressed in GSK-J4–treated PB (Fig. 5E, bottom). Together, these data suggest that inhibition of UTX and JMJD3 leads to enhanced repression of a subset of B cell fate genes that gain H3K27me3, thus promoting PB formation.

To evaluate whether genes upregulated following inhibitor treatment were enriched for genes regulated by H3K4me3, we overlaid genes upregulated in PB following inhibitor treatment on a scatterplot comparing PB/nB gene expression (68) versus change in H3K4me3 enrichment (18) in a manner similar to Fig. 5A. A majority of the GSK-J4 upDEG corresponded to genes that exhibited minimal, if any, change in H3K4me3 methylation during B cell differentiation (Fig. 5F, 5G). Thus, the data strongly suggest the genes upregulated following inhibitor treatment are the result of inhibiting H3K4me3 demethylases.

DISCUSSION

This study investigated the role of histone H3K27me3 demethylation by UTX and JMJD3 on B cell differentiation through the use of GSK-J4, a pharmacological inhibitor that functions as a competitor for their substrate α-ketoglutarate (69). This inhibitor was previously shown to display high specificity for UTX and JMJD3 and acted at a lower specificity toward H3K4me3 demethylases (69, 71). GSK-J4 treatment of nB stimulated with LPS, IL-2, and IL-5 led an increase in the frequency of PB and dysregulation in gene expression. Despite promoting PB formation, GSK-J4 treatment had no influence on the ability of treated cells to secrete Abs. At the molecular level, GSK-J4 treatment during the differentiation process led to more genes being downregulated than expected. This observation suggests that the changes are likely driven by inhibition of UTX and JMJD3, which by removal of the inhibitory histone mark H3K27me3 normally promote gene expression. Some of these dysregulated genes included those associated with hypoxia, signaling, apoptosis, and P53 pathways, including cell cycle inhibitor Cdken2a (72). The transcription factors SPIB and ID3, which are known PB repressors (70, 88), were also downregulated and may account for the upregulation of PC-signature genes. Other genes found to be upregulated by GSK-J4 treatment during the differentiation process were associated with cell cycle and proliferation. These changes in gene expression were correlated with an increase of actively proliferating BrdU+ cells following GSK-J4 treatment of ex vivo differentiated nB. Thus, targeting cell cycle inhibitors and PB repressors are in part responsible for the increases in PB appearing after inhibition of these demethylases.

Epigenetic remodeling is necessary during B cell differentiation (18, 20, 21), and the histone modification H3K27me3 shows dynamic changes at thousands of loci with sites both gaining and losing the mark. A majority of GSK-J4 downDEG were associated with changes in promoter-localized H3K27me3 (although other changes can be observed in the gene body) during the differentiation process. This observation is consistent with the active and direct demethylase activities of UTX and JMJD3, in which a subset of GSK-J4 downDEG corresponded to genes that normally lose promoter H3K27me3 and gain expression as B cells differentiate. Surprisingly, a number of GSK-J4 downDEG matched genes that normally gain promoter H3K27me3 and are downregulated as B cells differentiate to PB. This included transcription factors known to repress the PB fate. Thus, UTX and JMJD3 might function as the rheostat or counterbalance for H3K27me3 by counteracting the activity of EZH2, the H3K27 methyltransferase. Taken together, these data suggest that the level of promoter H3K27me3 and gene expression during B cell differentiation is modulated by three distinct mechanisms: 1) direct control of a gene by either UTX/JMJD3 or
EZH2; 2) a balanced control in gene expression, in which both UTX/JMJD3 and EZH2 compete for the same genes, resulting in fine tuning of gene expression; or 3) indirect control of gene expression through passive loss of H3K27me3 through cell division, in which EZH2 is no longer recruited to a locus.

There is growing evidence that balance in the levels of histone modifications are necessary for proper B cell differentiation. Methylation of H3K4 is associated with gene activation and has been shown to play a critical role in B cells (89). Deletion of Kmt2d, the H3K4 methyltransferase, led to an increase in germinal center B cells as a result of the enhanced

FIGURE 5. DEG are enriched for genes regulated by H3K27me3 levels. (A) The log₂FC change in gene expression between PB and nB (68) was plotted against the log₂FC change in H3K27me3 between PB and nB (21) as in Fig. 1. DownDEG between GSK-J4 and DMSO-treated PB are represented by red dots. (B) Quantification of H3K27me3 levels in wild type nB and PB at GSK-J4 downDEG. (C) Average expression of GSK-J4 downDEG in the blue quadrant in (A) in EZH2-sufficient and -deficient PB (68). (D and E) Examples of GSK-J4 downDEG regulated by H3K27me3 levels. *FDR < 0.05 and >1.5-fold change, #FDR < 0.05 with <1.5-fold change. (F) The log₂FC change in gene expression PB and nB (68) was plotted against the log₂FC change in H3K4me3 between PB and nB (18). GSK-J4 upDEG in PB comparison are represented by red dots. (G) Quantification of H3K4me3 levels in wild type nB and PB at GSK-J4 upDEG.

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proliferation capacity of follicular B cells lacking Kmt2d (90). Deletion of Lsd1, the H3K4me1/2 demethylase, resulted in the opposite phenotype. B cells lacking LSD1 showed reduced proliferation and differentiation following T cell–independent stimulation (68), as well as reduced germinal center B cells (91). In a similar scenario, deletion of Ezh2 resulted in a reduction in germinal center and PC formation and reduced proliferation (21, 30, 31), whereas, in this study, inhibition of UTX and JMJD3 led to increased PB formation and enhanced cell proliferation. Together, these results suggest that a balanced level of opposing histone modifications is necessary for maintaining B cell fate and controlling cell proliferation.

The importance of balanced levels of histone modification is well exemplified by the fact that mutations in various epigenetic modifiers are frequently found in cancer (92). In particular, gain-of-function mutations in EZH2 have been identified in various human malignancies, including DLBCL, which result in increased promoter H3K27me3 methylation at cell cycle checkpoint genes, including CDKN1A and genes associated with germinal center exit (30). However, mutations in UTX are typically loss-of-function or deletion, which lead to a failure to demethylate H3K27me3. This results in increased levels of H3K27me3, possibly mimicking EZH2 hyperactivation (61, 93). Furthermore, attempts to re-establish a homeostatic level of this histone modification have proved to be an effective therapeutic avenue for human cancers, as EZH2 inhibitors are in clinical use and ongoing trials (94). A recent study has also proposed the use of EZH2 inhibitors for malignancies with UTX loss-of-function mutations. Treatment with EZH2 inhibitors led to reduced viability and cell cycle arrest of multiple myeloma cell lines lacking UTX and resulted in reduced tumor burden in vivo (62). Taken together, the balanced level of H3K27me3 is necessary to maintain homeostasis and prevent the development of human malignancies.

The role of UTX and JMJD3 has been examined in various cell types, including the hemopoietic lineage. Interestingly, several studies have revealed demethylase-independent roles for these enzymes in addition to their known role in active demethylation. The demethylase activity of UTX is required for the formation of invariant NKT cells (55, 56); however, in mouse embryonic stem cells, UTX cooperates with the MLL4 complex and p300 to convert enhancers from an inactive to an active state (40). This action occurs in the absence of the demethylase activity of UTX. In this setting, UTX promotes and enhances the activity of its binding partners to promote H3K4me1 and H3K27ac at enhancers (40). The inhibitor used in this study targets the catalytic activity of UTX and JMJD3, thus allowing for a targeted analysis of active H3K27me3 demethylation by both enzymes during B cell differentiation.

Although the presented data strongly suggest that the observed increase in PB is the result of the inhibition H3K27me3 demethylases, the GSK-J4 has also been shown to act on other histone demethylases (69, 71). As such, it remains possible that the observed increase in PB following GSK-J4 treatment is a cumulative effect of inhibiting multiple demethylases, including UTX and JMJD3. The development of new compounds specifically targeting the H3K27me3 demethylases is necessary to more definitely address the role of inhibition of UTX and JMJD3 in B cell differentiation.

Mutations in UTX and JMJD3 have been reported in various cancers, and several studies identified GSK-J4 as a potential therapeutic treatment for a subset of malignancies driven by gain-of-function mutations in these genes (64, 95, 96). Despite the promising effects of GSK-J4 as a cancer treatment, this drug is not currently used in any clinical trials, likely due to its nonspecific activity toward other demethylases. The work presented in this study would suggest that targeted UTX/JMJD3 inhibition could result in increased PB and PC formation that may exacerbate normal immune responses in treated patients.

In summary, the work described in this study demonstrates an important balance in the control of gene expression potentially regulated by the placement and removal of repressive histone modifications at histone H3K27. The dynamic changes in this histone modification at the targeted loci are likely critical for maintaining the cell fates as a B cell or as PC following their complex differentiation process.

DISCLOSURES

The authors have no financial conflicts of interest.

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REFERENCES


Supplemental Figure 1.

(A) Representative western blot and quantitation of KDM5C, KDM5B, UTX, and JMJD3 protein levels in naive B cells and plasmablasts from DMSO or GSK-J4 treated ex vivo cultures (left). Normalized to Actin control (right).

(B) Representative plots of Annexin V versus Viability in DMSO and 250nM GSK-J4 treated cultures after three days of ex vivo stimulation.

(C) Frequency of apoptotic, necrotic, and live cells in DMSO and 250nM GSK-J4 treated cells at 24hr (left), 48hr (middle), and 72hr (left) of ex vivo stimulation.

(D) GSEA for top 200 gene upregulated in PB (Haines et al. 2018) in GSK-J4 treated ActB (left) and PB (right) versus control. (E) The log$_2$FC change in H3K4me3 between PB and nB (Scharer et al. 2018) was plotted against the log$_2$FC change in gene expression between PB and nB (Haines et al. 2018). GSK-J4 upDEG in PB comparison were represented by red dots. (F) Quantification of the levels of H3K4me3 at GSK-J4 upDEG in wild type nB and PB from E.
Supplemental Figure 2.

(A) General gating strategy use for FLOW cytometry analyses to assess PB formation. (B) Representative FLOW cytometry plots for ActB and PB enrichments performed at day 3 of ex vivo culture. Purity is indicated as frequency of single cells.