



# The JMJD3 histone demethylase inhibitor GSK-J1 ameliorates lipopolysaccharide-induced inflammation in a mastitis model

Received for publication, January 9, 2022, and in revised form, April 29, 2022. Published, Papers in Press, May 6, 2022.  
<https://doi.org/10.1016/j.jbc.2022.102017>

Jing-Jing Wang<sup>1,2,‡</sup>, Xia Wang<sup>1,2,‡</sup>, Yin-Er Xian<sup>1,2</sup>, Zi-Qing Chen<sup>1,2</sup>, You-Peng Sun<sup>1,2</sup>, Yi-Wu Fu<sup>1,2</sup>, Zhi-Kai Wu<sup>1,2</sup>,  
Pei-Xuan Li<sup>1,2</sup>, Er-Shun Zhou<sup>1,2</sup>, and Zheng-Tao Yang<sup>1,2,\*</sup>

From the <sup>1</sup>College of Life Science and Engineering and <sup>2</sup>Guangdong Provincial Key Laboratory of Animal Molecular Design and Precise Breeding, Foshan University, Foshan, Guangdong, China

Edited by Dennis Voelker

Jumonji domain-containing 3 (JMJD3/KDM6B) is a histone demethylase that plays an important role in regulating development, differentiation, immunity, and tumorigenesis. However, the mechanisms responsible for the epigenetic regulation of inflammation during mastitis remain incompletely understood. Here, we aimed to investigate the role of JMJD3 in the lipopolysaccharide (LPS)-induced mastitis model. GSK-J1, a small molecule inhibitor of JMJD3, was applied to treat LPS-induced mastitis in mice and in mouse mammary epithelial cells *in vivo* and *in vitro*. Breast tissues were then collected for histopathology and protein/gene expression examination, and mouse mammary epithelial cells were used to investigate the mechanism of regulation of the inflammatory response. We found that the JMJD3 gene and protein expression were upregulated in injured mammary glands during mastitis. Unexpectedly, we also found JMJD3 inhibition by GSK-J1 significantly alleviated the severity of inflammation in LPS-induced mastitis. These results are in agreement with the finding that GSK-J1 treatment led to the recruitment of histone 3 lysine 27 trimethylation (H3K27me<sub>3</sub>), an inhibitory chromatin mark, *in vitro*. Furthermore, mechanistic investigation suggested that GSK-J1 treatment directly interfered with the transcription of inflammatory-related genes by H3K27me<sub>3</sub> modification of their promoters. Meanwhile, we also demonstrated that JMJD3 depletion or inhibition by GSK-J1 decreased the expression of toll-like receptor 4 and negated downstream NF- $\kappa$ B proinflammatory signaling and subsequently reduced LPS-stimulated upregulation of *Tnfa*, *Il1b*, and *Il6*. Together, we propose that targeting JMJD3 has therapeutic potential for the treatment of inflammatory diseases.

Mastitis, characterized by the inflammation of parenchyma in mammary glands, is one of the most common inflammatory diseases affecting dairy cattle worldwide (1). Mastitis is caused by several factors including viruses (2), mycoplasmas (3), or protozoa (4), among which gram-positive and gram-negative bacteria are the most common causes. As the central outer surface membrane component of gram-negative bacteria,

bacterial lipopolysaccharide (LPS) acts as extremely strong stimulators of innate or natural immunity during mastitis. Upon LPS stimulation, immune cells are activated and recruit to the mammary gland to produce inflammatory mediators, thus triggering excessive inflammation and mammary tissue injury. In the immune defenses of the mammary gland, the innate immune response plays a prominent role, which detects and defends against the invading pathogens to provide the initial defense. If the first defense is broken, the inflammatory response is activated to create a protective effect, but uncontrolled or excessive inflammation leads to mammary gland injury (5). Epigenetics could be one such mechanism that dynamically regulates gene expression or chromosome structure without altering the DNA sequence. Epigenetic mechanisms, including histone modification, DNA methylation, noncoding RNA regulation, and chromatin remodeling, occur in the pathogenesis of many diseases. Histone modification is one of the main epigenetic regulators, which is a potent modulator of innate immunity and inflammation (6). There have been many reports of crosstalk between inflammation and histone modification during mastitis. In a mice model for *Staphylococcus aureus*-induced mastitis, *S. aureus* infection upregulated the acetylation level at histone H3K9 and H3K14 residues in the mammary gland (7). In *Escherichia coli*-induced mastitis mice, *E. coli* infection leads to histone hyperacetylation (H3K14 and H4K8) at the promoter of immune genes (8). Moreover, we previously showed that lysine-specific demethylase 1 (LSD1) is a potential epigenetic determinant to regulate inflammatory responses in mastitis (9). Here, we focused on the epigenetic regulation of another histone demethylase, Jumonji domain-containing 3 (JMJD3), in LPS-induced mastitis.

Previous studies have demonstrated that epigenetic regulation affects inflammatory gene expression (10, 11). Several epigenetic mechanisms, including histone modification, DNA methylation, and nuclear positioning, control chromatin structure and regulate gene expression. Among these mechanisms, histone acetylation and methylation are the most common histone modifications in the control of inflammatory gene expression (12). JMJD3, also known as LSD 6B (KDM6B), is identified as histone 3 lysine 27 (H3K27) demethylase (13). Furthermore, H3K27 trimethylation (H3K27me<sub>3</sub>) is associated

<sup>‡</sup> These authors contributed equally to this work.

\* For correspondence: Zheng-Tao Yang, [yangzhengtao01@sina.com](mailto:yangzhengtao01@sina.com).

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with a condensed chromatin conformation and exerts a repressive epigenetic mark (14). The upregulation of JMJD3 demethylates H3K27me3 to H3K27me2 or H3K27me1, thus leading to the removal of the methylation mark from H3K27 and activates gene transcription (15). Until now, studies have primarily investigated the role of JMJD3 in inflammation. In LPS-activated vascular endothelial cells, the expression of JMJD3 upregulated significantly in the nucleus, which synergizes with NF- $\kappa$ B to activate gene expressions (16). Another study suggests that the upregulation of JMJD3 enhances the proinflammatory response and immunopathology during respiratory syncytial virus infection (17). Moreover, the inhibition of JMJD3 attenuates interleukin (IL)-1 $\beta$ -induced chondrocytes damage and protects osteoarthritis cartilage (18). Here, we use the JMJD3-selective pharmacological inhibitor, GSK-J1, to clarify the role of JMJD3 in response to LPS-induced inflammation, exploring its molecular mechanisms of regulating inflammatory gene expression.

In the present study, it was found that JMJD3 was upregulated in the injured mammary gland, but the administration of JMJD3 inhibitor GSK-J1 significantly alleviated the severity of mammary gland damage. Therefore, the authors hypothesized that JMJD3 was involved in the epigenetic regulation of the inflammatory response in mastitis. To characterize the epigenetic response, the expression of JMJD3 and H3K27me3 in response to LPS exposure was analyzed. Through *in vitro* experiments, GSK-J1 treatment led to the recruitment of H3K27me3 at the promoters of *Tnfa*, *Il1b* and *Il6*, and toll-like receptor 4 (*Tlr4*), while also inhibiting the activation of Tlr4–NF- $\kappa$ B signaling pathways and the expression of inflammatory cytokines in LPS-stimulated mouse mammary epithelial cells (MECs). These data established a positive correlation between JMJD3 and the inflammatory response and identified the epigenetic mechanism of JMJD3 in LPS-induced mastitis, which not only provides a new avenue for understanding the

relevant mechanisms but would also help to identify novel targets for mastitis.

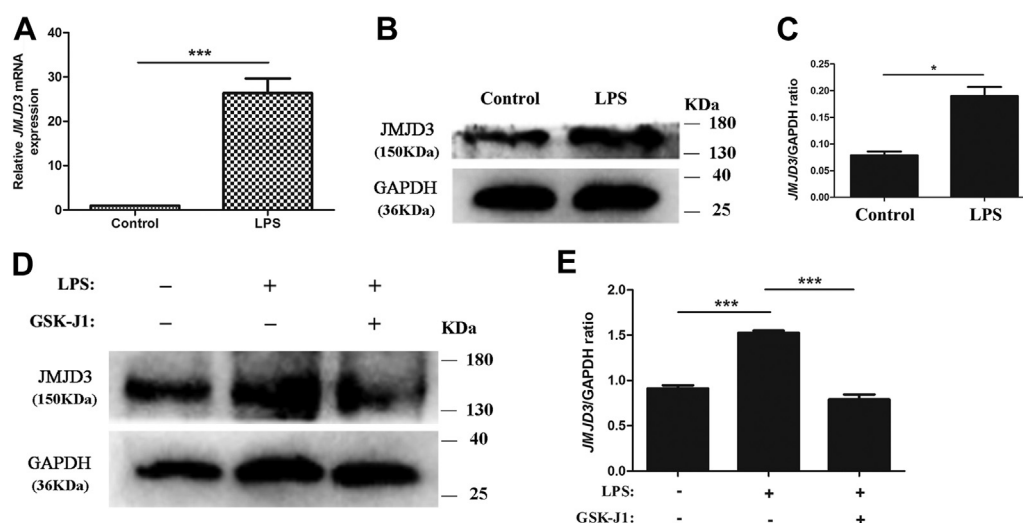
### Results

#### Expression of JMJD3 is positively associated with the inflammatory response of LPS-induced mastitis

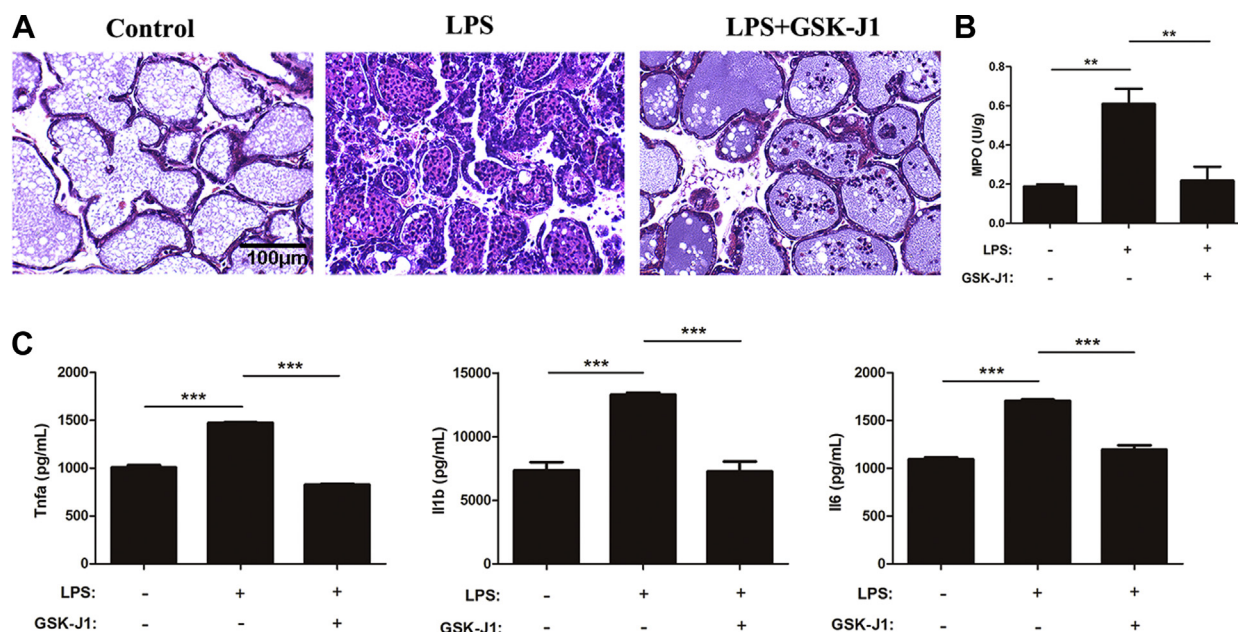
It is well known that JMJD3 has been implicated in regulating gene expression during an inflammatory response. Previous studies have shown that multiple stimuli could induce the expression of JMJD3. In the current study, we establish an LPS-induced mastitis model, where the gene and protein levels of JMJD3 are measured by quantitative RT-PCR and Western blot analysis. As shown in Figure 1, A–C, JMJD3 is strongly upregulated following LPS treatment. The results suggest that JMJD3 may be involved in the inflammation in response to LPS. To further clarify the effect of JMJD3 on regulating inflammatory response, JMJD3 is downregulated specifically in mammary glands *via* the administration of a selective histone demethylase JMJD3 inhibitor, GSK-J1. The results show that GSK-J1 significantly inhibits the level of JMJD3 expression after LPS exposure (Fig. 1, D and E).

#### JMJD3 inhibition alleviates LPS-induced inflammatory response *in vivo*

Next, we further confirm the correlation between JMJD3 and LPS-driven inflammation. The mammary gland histology shows that GSK-J1 treatment results in the decreased infiltration of inflammatory cells and alleviates the severity of the mammary gland (Fig. 2A), as well as results in a reduction of myeloperoxidase (MPO) activity (Fig. 2B). Moreover, a decrease in proinflammatory cytokines (*Tnfa*, *Il1b*, and *Il6*) is observed in the mammary gland of GSK-J1-treated mice (Fig. 2C). Therefore, these data show decreased inflammatory



**Figure 1. Expression of JMJD3 is positively associated with the inflammatory response of LPS-induced mastitis.** A–C, gene and protein expression of JMJD3 was detected in untreated and LPS-induced mice using qRT-PCR and Western blotting. GAPDH was used as a loading control. The Western blotting data were quantified by densitometry and were normalized to GAPDH. D and E, total protein extracts from mammary glands treated with GSK-J1 were used to detect JMJD3 by Western blotting. GAPDH was used as a loading control. The Western blotting data were quantified by densitometry and normalized to GAPDH. Values represent mean  $\pm$  SEM ( $n = 6$ ). \* $p < 0.05$ ; \*\*\* $p < 0.001$  as determined by one-way ANOVA followed by Tukey's test. JMJD3, Jumonji domain-containing 3; LPS, lipopolysaccharide; qRT-PCR, quantitative RT-PCR.



**Figure 2. Inhibition of JMJD3 alleviates LPS-induced inflammatory response *in vivo*.** A, representative images of histological analysis of mammary gland stained with H&E. The mammary glands were harvested, buffered formalin fixed, and paraffin embedded. Then, 5  $\mu$ m sections were stained with H&E for histologic examination. B, MPO activity in the mammary gland was measured by MPO kits. C, the release of a set of genes in the mammary gland was analyzed by ELISA. Values represent mean  $\pm$  SEM ( $n = 6$ ).  $**p < 0.01$ ,  $***p < 0.001$  as determined by one-way ANOVA followed by Tukey's test. JMJD3, Jumonji domain-containing 3; LPS, lipopolysaccharide; MPO, myeloperoxidase.

responses by JMJD3 downregulation, clarifying a direct link between JMJD3 and LPS-driven inflammation.

#### Inhibition or knockdown of JMJD3 reduces inflammatory cytokine expression

The MEC model system was used to study the immune response during mastitis. Here, we establish the model system *in vitro* to explore the underlying mechanisms of the epigenetic regulation of JMJD3 in LPS-stimulated MECs. The potential cytotoxicity of GSK-J1 on mouse MECs is analyzed by Cell Counting Kit-8 (CCK-8) assay. As shown in Figure 3A, at the concentration of 100  $\mu$ M, GSK-J1 has significant cytotoxicity, and 0.1  $\mu$ M, 1  $\mu$ M, or 10  $\mu$ M was chosen as a subsequent use of the concentration. Furthermore, the mRNA expressions of *Tnfa*, *Il1b*, and *Il6* were analyzed, where the key proinflammatory cytokines that are known to exacerbate inflammatory response were elevated in mastitis. Our study shows that the JMJD3 inhibition by GSK-J1 during LPS exposure leads to a significant reduction in *Tnfa*, *Il1b*, and *Il6*. (Fig. 3, B–D). Meanwhile, we transfect silencer predesigned siRNA sequences into MECs by RNAiMAX transfection reagent. As observed by fluorescent microscopy, the green fluorescent signal is observed 6 h after transfection, suggesting that the uptake of the siRNA by MECs is highly efficient (Fig. 4A). At 24 h post-transfection, real time PCR analysis and Western blotting reveals that JMJD3 siRNA results in a significant decrease in JMJD3 mRNA and protein (JMJD3 siRNA) compared to the negative control (NC) group (scrambled siRNA) (Fig. 4, B and C). The three cytokine expressions were also quantified; the findings were consistent with the results in Figure 4, D–F.

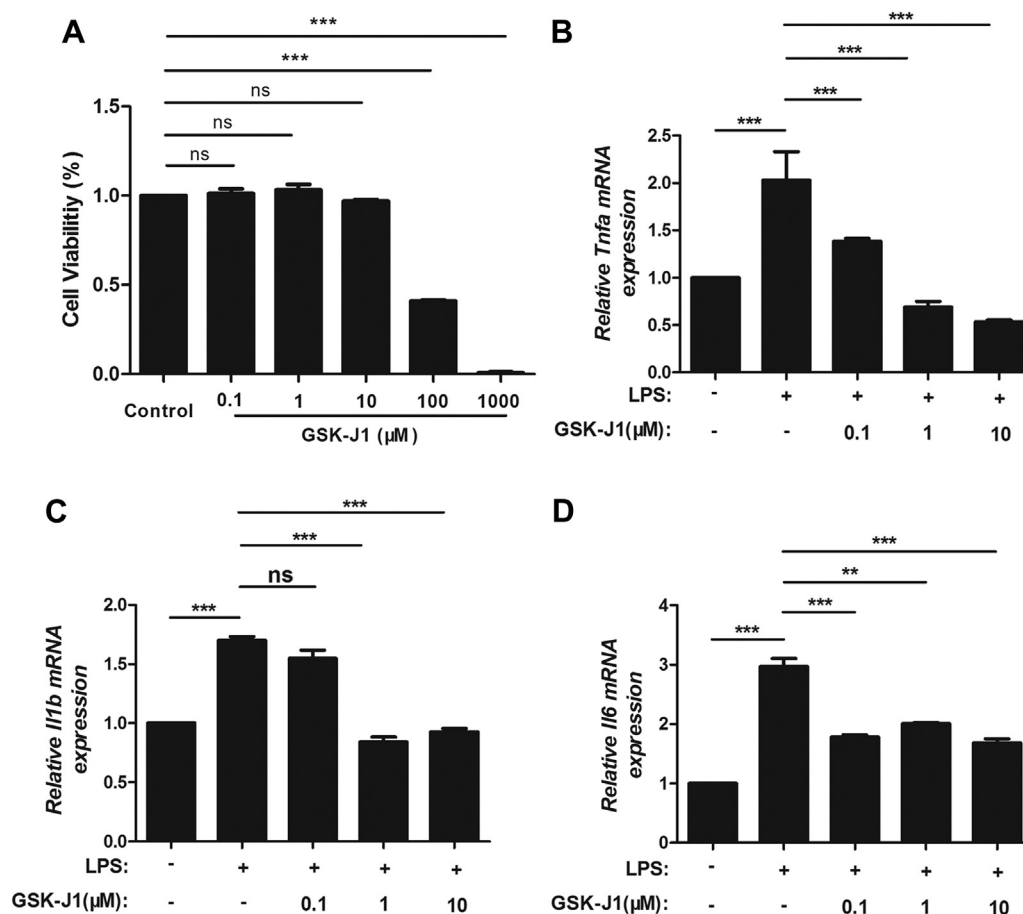
#### JMJD3 epigenetically regulates inflammatory cytokine expression

It has been established that LSD JMJD3 specifically demethylates H3K27me3 to activate genes (19). To explore the function of JMJD3 during mastitis, we examined the level of H3K27me3 in LPS-stimulated MECs. Treatment with GSK-J1 decreases JMJD3 expression and increases H3K27me3 status, suggesting LPS induced JMJD3 in a histone demethylase-dependent manner (Fig. 5A). Furthermore, to determine if an increased JMJD3 epigenetically regulates inflammatory gene expression, H3K27me3 (an inhibitory marker) on the gene promoters of *Tnfa*, *Il1b*, and *Il6* was examined, following LPS stimulation. The chromatin immunoprecipitation (ChIP)–PCR analysis reveals that LPS abolishes the recruitment of H3K27me3, while the GSK-J1 treatment results in an increase in the level of H3K27me3 on the promoter of the three genes (Fig. 5B). These data may indicate that inflammatory gene transcription is controlled, at least partially, by JMJD3.

#### Tlr4 engages JMJD3-mediated inflammation in MECs

Prior research has demonstrated that external stimuli, including LPS, can drive JMJD3 expression. Given that Tlr4 is an important receptor for activation of transcription factors and chromatin-modifying enzymes (20), the effects of H3K27me3 of JMJD3 inhibition on the Tlr4 promoter were also examined. It was determined that JMJD3 inhibition led to the recruitment of H3K27me3 after LPS administration (Fig. 6A). The further inhibition or knockdown of JMJD3 displayed a reduced level of Tlr4 following LPS treatment (Fig. 6, B and C). In addition, Tlr4 and the downstream pathways are closely associated with the inflammatory

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**Figure 3. Inhibition of JMJD3 reduces inflammatory cytokines expression.** A, the cell viability was determined by the CCK8 assay. B, expressions of a set of genes in MECs were analyzed by qRT-PCR. Values represent mean  $\pm$  SEM ( $n = 6$ ). \*\* $p < 0.01$ , \*\*\* $p < 0.001$  as determined by one-way ANOVA followed by Tukey's test. JMJD3, Jumonji domain-containing 3; MEC, mammary epithelial cell; qRT-PCR, quantitative RT-PCR.

response during mastitis (21, 22). Then the relevance of the relationship between JMJD3 and inflammation-associated signaling pathways was investigated. The results showed that JMJD3 inhibition significantly reduced the expression of Tlr4 and phosphorylation level of p65 and I $\kappa$ B *in vitro* (Fig. 6D). Taken together, the detailed results of the present study indicate JMJD3 regulates inflammatory genes in mastitis *via* an H3K27me3-mediated mechanism and that JMJD3 may be controlled by the Tlr4–NF- $\kappa$ B–mediated pathway.

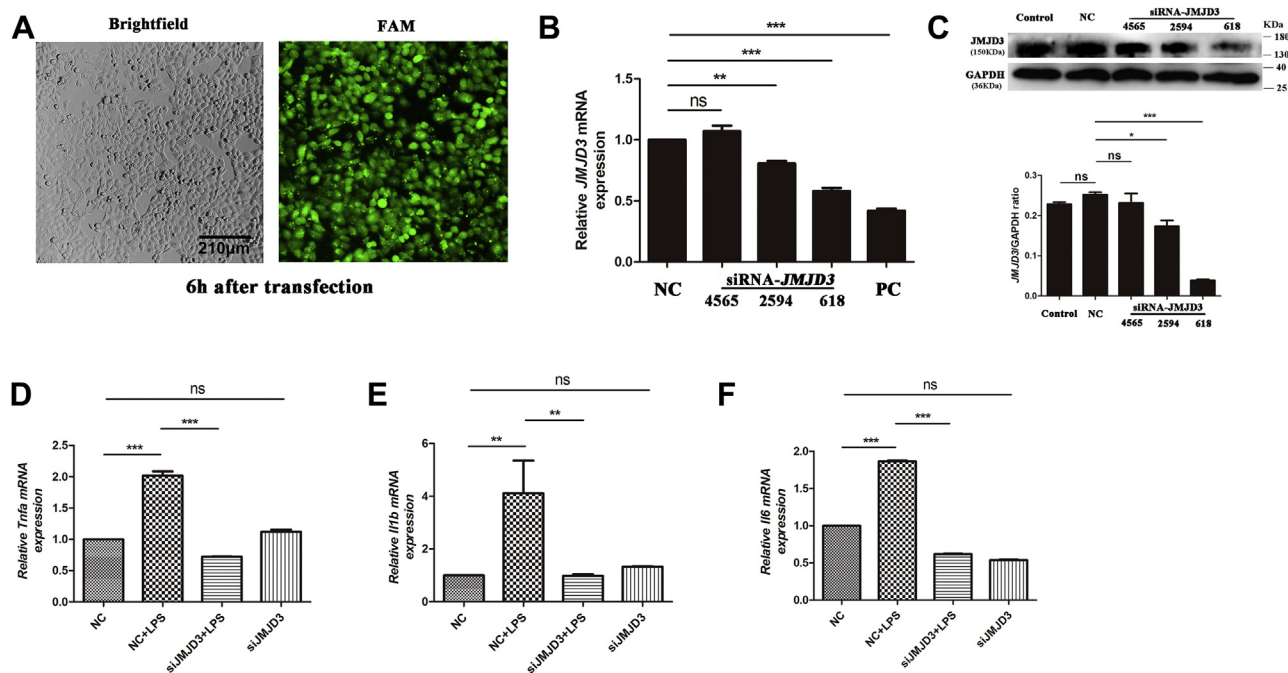
### Discussion

Mastitis is a complex disease that mainly involves the interaction of the host response and pathogen factors, which is the result of a combination of various factors (23). Previous studies conducted by the authors focused on the innate immunity mechanisms during mastitis (24, 25). The innate inflammatory response participates in the initial stages of defense of the mammary gland to infectious pathogens. Once the pathogens invade, polymorphonuclear neutrophilic leukocytes migrate from blood vessels to the infected site. Then, pattern recognition receptors (including Tlr, NOD-like receptors, and RIG-1-like receptors) are activated to mediate the recognition of pathogens, which finally initiate the innate

immune response (26, 27). Recently, an increasing amount of attention has been given to the emerging field focusing on the epigenetic regulation of innate immune response. However, the role of epigenetic regulation in innate immunity and inflammatory response during mastitis remains unknown. As a histone demethylase, JMJD3 was found to be upregulated in the mammary gland during mastitis. The pharmacological inhibition of JMJD3 by GSK-J1 mediated the inflammatory response and alleviated mammary gland injury. Here, our findings provide evidence supporting JMJD3 as an epigenetic regulator contributing to the regulation of the inflammatory factor in mastitis.

Many studies have indicated that epigenetic modulation (including histone methylation, acetylation, and phosphorylation, among others) is involved in the epigenetic control of immune responses (28, 29). Moreover, it is well established that epigenetic regulation is a crucial mechanism in the control of the inflammatory response *via* site-specific histone acetylation and methylation (30, 31). JMJD3, also referred to as KDM6B, is a crucial histone demethylase that has known functions in cancer (32), development (33), physiology (13), immune disease (34–36), infectious diseases (37, 38), and aging-related diseases (39, 40). H3K27 trimethylation is a key epigenetic gene modifier, which represses gene transcription

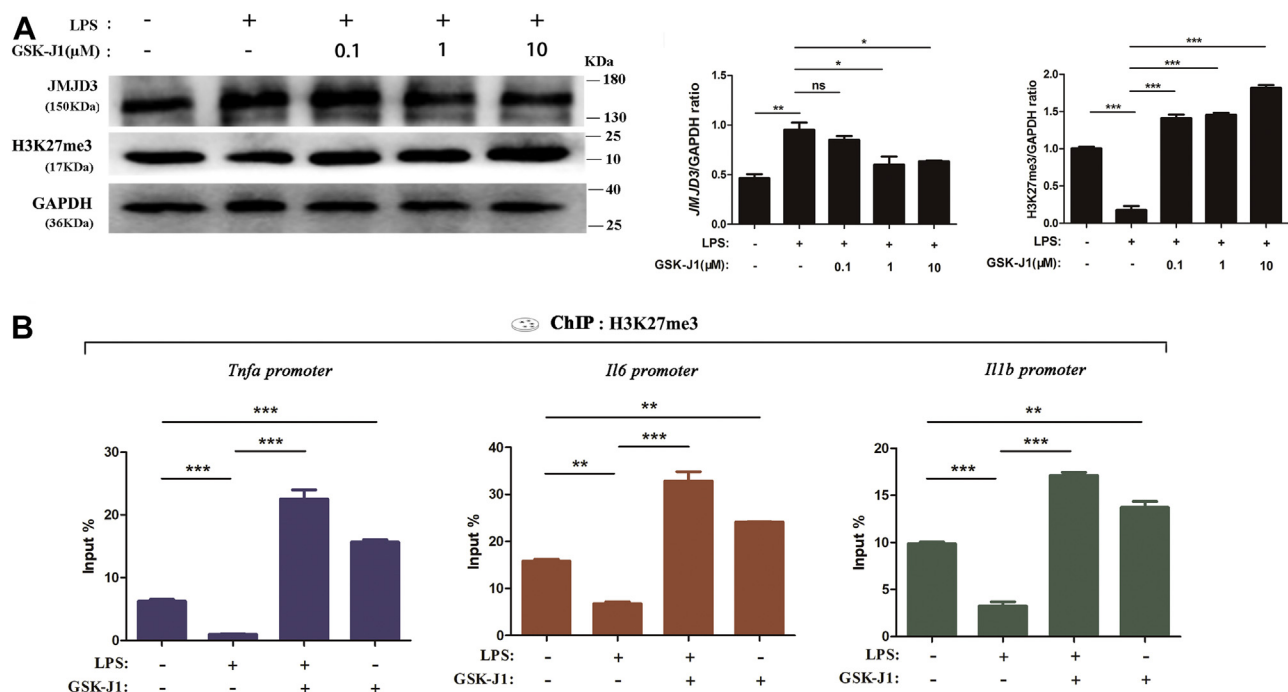
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**Figure 4. Knockdown of JMJD3 reduces inflammatory cytokines expression.** Effects of transfection with JMJD3 siRNA in MECs. *A*, transfection efficiency of FAM-labeled scrambled siRNA delivery, the green fluorescent signal is observed 6 h after transfection by fluorescent microscopy. *B* and *C*, relative mRNA and protein expression of JMJD3 were analyzed by qRT-PCR and Western blotting (NC, negative control; PC, positive control). *D–F*, expressions of a set of genes in MECs were analyzed by qRT-PCR. Values represent mean  $\pm$  SEM ( $n = 6$ ). \* $p < 0.5$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  as determined by one-way ANOVA followed by Tukey's test. JMJD3, Jumonji domain-containing 3; MEC, mammary epithelial cell; qRT-PCR, quantitative RT-PCR.

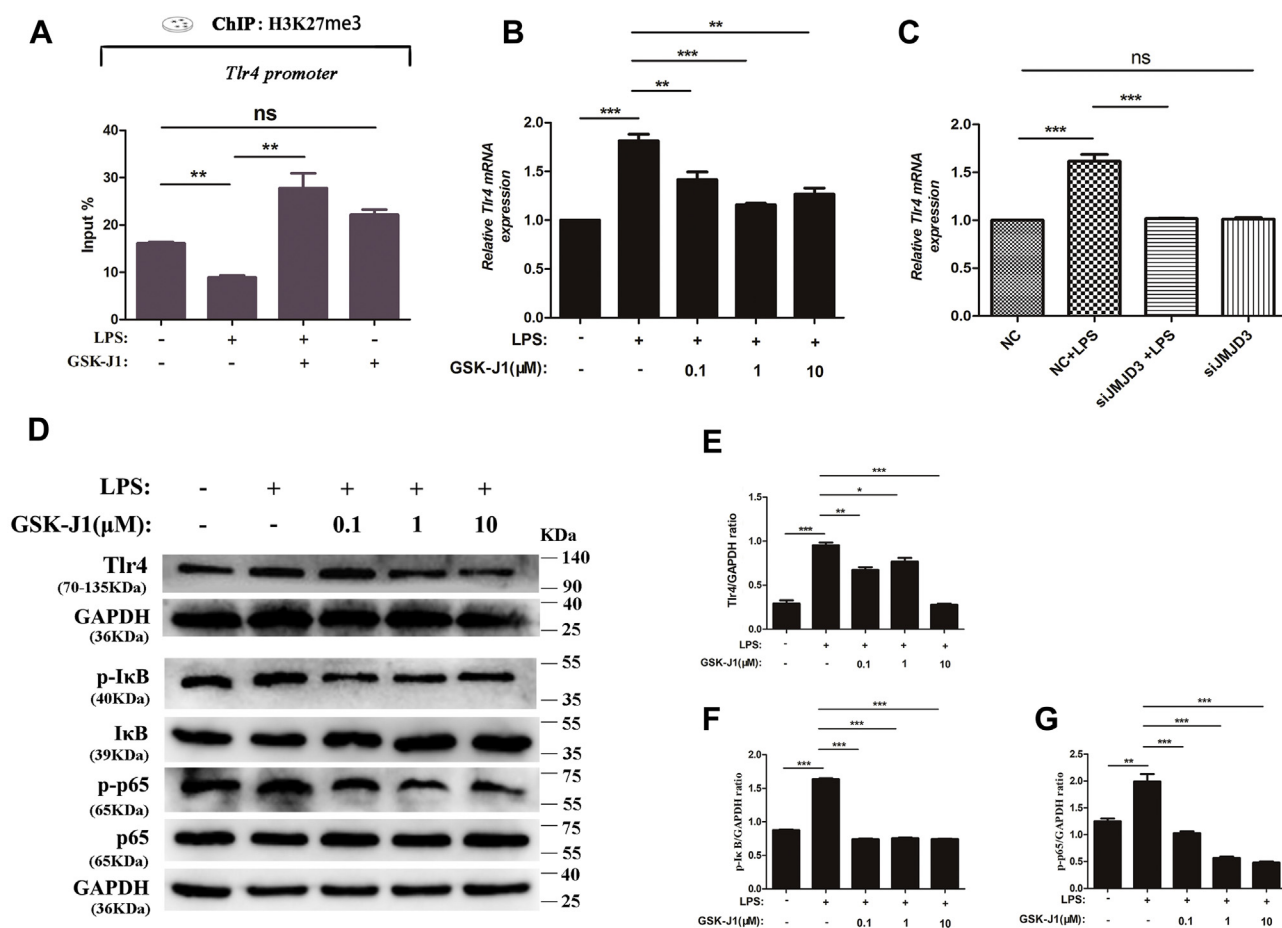
and is implicated in inflammation and disease pathogenesis (39, 41). JMJD3 is a known H3K27 demethylase that results in the removal of the repressive methylation mark from

H3K27me3 to active gene transcription (17). Herein, we report that JMJD3 is positively correlated with the inflammatory response during mastitis, and the administration of a JMJD3



**Figure 5. JMJD3 epigenetically regulates inflammatory cytokines expression.** *A*, LPS-stimulated MECs were analyzed by Western blotting using antibodies specific to JMJD3 and H3K27me3. GAPDH was used as a loading control. The Western blotting data were quantified by densitometry and were normalized to GAPDH. *B*, MECs were collected and DNA was extracted for ChIP analysis. The level of H3K27me3 on the inflammatory cytokine promoters was measured after GSK-J1 administration in MECs with LPS treatment using the indicated antibodies. Values represent mean  $\pm$  SEM ( $n = 6$ ). \* $p < 0.5$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  as determined by one-way ANOVA followed by Tukey's test. ChIP, chromatin immunoprecipitation; JMJD3, Jumonji domain-containing 3; LPS, lipopolysaccharide; MEC, mammary epithelial cell.

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**Figure 6. Tlr4 engages JMJD3-mediated inflammation in MECs.** *A*, the level of H3K27me3 on Tlr4 promoters after GSK-J1 administration in MECs with LPS treatment was measured using ChIP assay. *B* and *C*, expressions of Tlr4 after JMJD3 inhibition or knockdown in MECs were analyzed by qRT-PCR. *D*, LPS-stimulated MECs were analyzed by Western blotting using antibodies specific to Tlr4, phosphor-NF-κB p65, phosphor-IκBα, p65, and IκBα. GAPDH was used as a loading control. *E–G*, the Western blotting data were quantified by densitometry and were normalized to GAPDH. Values represent mean ± SEM ( $n = 6$ ). \* $p < 0.05$ ; \*\*\* $p < 0.001$  as determined by one-way ANOVA followed by Tukey's test. ChIP, chromatin immunoprecipitation; JMJD3, Jumonji domain-containing 3; LPS, lipopolysaccharide; MEC, mammary epithelial cell; qRT-PCR, quantitative RT-PCR; Tlr4, toll-like receptor 4.

specific inhibitor, GSK-J1, alleviates mammary gland injury. As shown by pathological observation, the infiltration of inflammatory cells in the mammary gland was significantly reduced and inflammatory cytokine expression induced by the LPS challenge was compromised after the administration of GSK-J1. The inhibitory effects on inflammatory cytokines were also observed when JMJD3 was inhibited or knocked down in MECs, indicating JMJD3 serves as a potential target for inflammation. Furthermore, *Tnfa*, *Il1b*, and *Il6* are recognized as representative inflammatory mediators during mastitis, and the mechanism underlying the epigenetic regulation of JMJD3 on inflammatory gene expression was further explored *in vitro*. The ChIP-PCR analysis revealed that the GSK-J1 treatment improved H3K27me3 recruitment at the promoter of inflammatory genes after the LPS challenge, including *Tnfa*, *Il1b*, and *Il6*.

TLRs are a family of 13 receptors known as pattern recognition receptors and play a key role in the innate immune response. The TLRs are activated by pathogen-associated molecular patterns that are structurally conserved molecules present on the surfaces of bacteria and viruses. The activation of these TLRs by pathogens results in the downstream

activation of genes involved in the production of proinflammatory factors. Epigenetics is concerned with changes in gene expression due to modifications to the structure of chromatin, which affect gene regulation by repressing or enhancing the transcription factor binding through different mechanisms. The activation of the Tlr4 signaling pathway is critical for controlling local mammary gland inflammation and a cascade of immune responses in mouse models of mastitis (42). Given the importance of Tlr4 on the inflammatory response during mastitis, we investigated the effect of JMJD3 in regulating Tlr4/NF-κB in mastitis. Our previous study has demonstrated that Tlr4 and signaling are activated in mastitis mice, where curcumin and geniposide attenuate the inflammatory response by suppressing Tlr4 and downstream signaling pathways (22, 43). The role of epigenetic modifications on TLRs has been examined in the regulation of Tlr4 within wound healing, demonstrating that mix-lineage leukemia-1 (MLL1) regulated Tlr4 expression in wound myeloid cells (44). Moreover, in lung adenocarcinoma cells, KDM3A promotes inhibitory cytokine secretion by participating in the Tlr4 regulation of Foxp3 transcription (45). Within the current study, we showed that JMJD3 inhibition decreased the

expression of JMJD3 and increased methylation in H3K27me3 driven by LPS at the Tlr4 promoter. The dependence of Tlr4 on JMJD3 further supported that JMJD3 inhibition and knockdown significantly decreased Tlr4 expression. The aforementioned findings indicate that JMJD3 regulated Tlr4 expression in LPS-driven MECs. Previous studies have demonstrated that JMJD3 directly interacts with NF- $\kappa$ B to mediate the transcription of inflammatory genes and regulate keratinocyte wound healing (39, 46). We next investigated the relevance of the relationship between JMJD3 and NF- $\kappa$ B signaling pathways, where JMJD3 inhibition significantly reduced the phosphorylation level of p65 and I $\kappa$ B. Taken together, our results indicate JMJD3 regulates Tlr4 expression, leading to the secretion of inflammatory genes. In the present study, our results identified specific methylation signatures associated with mammary gland inflammation in mastitis, demonstrating a regulatory role for JMJD3 in mastitis. The administration of the JMJD3 inhibitor GSK-J1 significantly decreased the inflammatory response in LPS-induced mastitis, mainly by controlling the methylation status of H3K27me3 in inflammatory genes and regulating the Tlr4/NF- $\kappa$ B-mediated pathway. The present study suggested that targeting JMJD3 with GSK-J1 has a therapeutic potential for the treatment of inflammatory diseases.

### Experimental procedures

#### Materials

LPS from *E. coli* O55:B5 was purchased from Sigma-Aldrich (catalog no.: L2880). Histone demethylase inhibitor GSK-J1 (catalog no.: S7581) was from Selleck.cn. ELISA MAX Deluxe Set Mouse Tnfa (catalog no.: 430904), Il6 (catalog no.: 431304), and Il1b (catalog no.: 432604) were purchased from Biologend Enabling Legendary Discovery. MPO assay kits were obtained from Nanjing Jiancheng Bioengineering Institute. The JMJD3/KDM6B antibody (#61388, 1:1000 dilution for Western blot analysis) was obtained from Active Motif Enabling Epigenetics Research. Tri-Methyl-Histone H3 (Lys27) rabbit mAb (#9733, 1:1000 dilution for Western blot analysis), phosphor-NF- $\kappa$ B p65 rabbit mAb (#3033, 1:1000 dilution for Western blot analysis), NF- $\kappa$ B p65 XP rabbit mAb (#8242, 1:1000 dilution for Western blot analysis), phosphor-I $\kappa$ B $\alpha$  rabbit mAb (#2859, 1:1000 dilution for Western blot analysis), I $\kappa$ B $\alpha$  mouse mAb (#4814, 1:1000 dilution for Western blot analysis), antimouse immunoglobulin G (IgG) horseradish peroxidase-linked antibody (#7076, 1:2000 dilution for Western blot analysis), and anti-rabbit IgG horseradish peroxidase-linked antibody (#7074, 1:2000 dilution for Western blot analysis) were purchased from Cell Signaling Technology. The GAPDH mouse monoclonal antibody (BM3876, 1:1000 dilution for Western blot analysis) was obtained from Boster Biological Technology Co, Ltd. SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) (#9003s) was obtained from Cell Signaling Technology. Lipofectamine RNAiMAX transfection reagent was purchased from Thermo Fisher Scientific. All other chemicals are reagent grade.

#### Mouse MEC culture

Mouse MEC line HC11 used in the experiment was obtained from Stem Cell Bank, Chinese Academy of Science. Cells were cultured in a 25 ml cell culture flask with a Dulbecco's modified Eagle's medium/F12 medium (Hyclone) containing 10% bovine serum (Clark Bioscience) and 1% penicillin-streptomycin (Hyclone). When cells reached 85% density at the bottom of the culture flask, the cells were digested with trypsin, and the digestion was terminated with the culture medium immediately after the cells fell off. The cells were then divided into a new cell culture flask. Cells that passed to the third generation were used for experimentations. Throughout the process, the cells were always cultured in a cell incubator containing 5% carbon dioxide at 37 °C.

#### Animals

Pregnant BALB/c female mice were obtained from Guangdong Medical Laboratory Animal Center. All animal feeding and operation procedures in this study were carried out in accordance with the animal ethics committee of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Foshan. Animals were housed in a controlled environment with a temperature of 22 to 23 °C and 12:12 h light-dark cycle.

#### Experimental design

The lactating mice were used in the experiments and GSK-J1 (Selleck, S7581) was used as the inhibitor of JMJD3. The mice were randomly divided into three groups ( $n = 6$  per group): control, LPS treatment, and 1 mg/kg GSK-J1 pretreatment, followed by LPS treatment (GSK-J1+LPS). The methodology of the LPS-induced mastitis in mice has been described previously (25). In brief, on day 10 of mice lactation, the pups were separated from their mother. Mice of the GSK-J1 group were administered with the defined concentrations of GSK-J1 (1 mg/kg) by i.p. injection. One hour after treatment with GSK-J1, LPS (0.2 mg/ml) was injected into the fourth inguinal mammary gland. Twenty-four hours after LPS injection, the mice were sacrificed with ketamine (30 mg/kg) and xylazine (6 mg/kg), and the mammary glands were collected for further analysis.

#### Histopathology

Twenty-four hours after LPS administration, the lactating mice were sacrificed, fixed with 10% formalin solution and embedded in paraffin. A series of organic solvents were used for dehydration, hardening, transparency, and infiltration, subsequently stained by H&E and observed under a microscope.

#### ELISA and MPO assay

We detected the level of *Tnfa*, *Il1b*, and *Il6* in the breast tissue following the instructions of the enzyme linked immunosorbent assay kits. All diluted antibodies, standards, and

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samples were added to the ELISA plate in turn. The plate was sealed and incubated at room temperature (RT) for 2 h with shaking, and the plate was washed four times. Freshly mixed 3,3',5,5''-tetramethylbenzidine substrate solution was added to each well for 15 min in the dark; 100  $\mu$ l stop solution was added to each well, and the absorbance at 450 nm and 570 nm was read within 15 min. Then, the changes in MPO in the mouse mammary tissues were evaluated by using the MPO detection kit in accordance with the instructions of commercial reagents. Briefly, the mammary glands were ground and centrifuged at 15,000 rpm for 10 min to obtain the supernatant. The required samples were configured according to the operations schedule. An ultraviolet spectrophotometer was used to analyze the absorbance at 460 nm.

### CCK8 assay

The effect of GSK-J1 on the viability of mouse MECs was tested with the CCK-8 kit. In short, after the cells were seeded on a 96-well culture plate until the cells adhered to the wall, GSK-J1 of different concentrations was added to incubate the cells for 18 h and then CCK-8 solution was added to incubate for 2 h. Finally, the absorbance of the culture plate was detected by a microplate reader at a wavelength of 450 nm, and the viability of the mouse MECs was assayed.

### RNAi-PCR

The cells were plated on 6-well plates at 70% to 90% confluence before transfection and were then transfected with siRNA targeting JMJD3, NC, or positive control using Lipofectamine RNAiMAX transfection reagent. The efficiency of the knockdown of the specific genes was confirmed with real-time PCR after 24 h. The siRNA oligonucleotides were designed and synthesized by Shanghai GenePharma Co, Ltd. The sequence of JMJD3-specific siRNA, mouse GAPDH, Ambion A fluorescently labeled negative control siRNA, and JMJD3 are shown in Table 1.

### ChIP-PCR

The chromatin immunoprecipitation assay was performed using the ChIP Kit as described previously (47). Briefly, to crosslink the proteins to DNA, cells were incubated in 1%

formaldehyde for 10 min at RT and then 10 $\times$  glycine was added. Cells were collected and continued with nuclei preparation and chromatin digestion. Micrococcal nuclease was added to digest DNA to a length of approximately 150 to 900 bp, and the cells were then sonicated with several pulses to break the nuclear membranes. Cell lysates were clarified by centrifugation at 9400g for 10 min at 4  $^{\circ}$ C. The supernatant containing chromatin was diluted, and a 10  $\mu$ l sample of the diluted chromatin was removed, which is the 2% input sample. The remaining chromatin fractions were added to the immunoprecipitating antibody: positive control histone H3 (D2B12) XP rabbit mAb, NC normal rabbit IgG, JMJD3 antibody, Tri-Methyl-Histone H3 (Lys27) rabbit mAb, and Tlr4 rabbit mAb overnight at 4  $^{\circ}$ C with rotation. Following this, immune complexes were precipitated with ChIP-Grade Protein G magnetic beads. The pellet protein G magnetic beads complex were washed with a series of buffers (low salt wash and high salt wash) in a magnetic separation rack. The chromatin was eluted from the antibody/protein G magnetic beads and crosslinking was reversed by adding proteinase K and undergoing incubation at 65  $^{\circ}$ C. The DNA was purified using spin columns and was subjected to real-time PCR. Primers were designed using the Ensembl genome browser to search for the promoters of *Tnfa*, *Il1b*, and *Il6*. The primer sequences are available in Table 2.

### Real-time PCR

The total RNA from the mouse MECs was extracted by using Trizol reagent and then the reverse transcription of RNA was performed according to the complementary DNA (cDNA) synthesis kit in accordance with the manufacturer's instructions. The total RNA was reverse transcribed to obtain synthetic cDNA. Finally, real-time quantitative PCR was carried out based on the synthesized cDNA using SYBR Green PCR Master Mix (Roche), and the program settings were set follows: 95  $^{\circ}$ C for 2 min, 95  $^{\circ}$ C for 5 s, and then 55  $^{\circ}$ C for 30 s for 40 cycles. The gene expression levels were analyzed by the  $2^{-\Delta\Delta CT}$  method. The sequences of the mouse primers are shown in Table 3.

### Western blotting

Mammary gland tissue and mouse MECs were ground and lysed by the protein extraction reagent containing protease and phosphatase inhibitor cocktail, centrifuged at 12,000 rpm at 4  $^{\circ}$ C for 10 min in a centrifuge, and then the protein concentration was quantified according to the bicinchoninic

**Table 1**  
Primers used in the study (RNAi-PCR)

Gene	Primer	Sequence 5'>3'
Kdm6b-4565	Sense	CAGGCCACCAAGAGAAUAATT
	Antisense	UUAUUCUCUUGGUGGCCUGTT
Kdm6b-618	Sense	CCCUAACAAACCCUAUUAUTT
	Antisense	AUAAUAGGGUUUGUUAGGGTT
Kdm6b-2495	Sense	GGCUGGCAAACAUCAUGAATT
	Antisense	UUCAUGAUGUUUGCCAGCCTT
Mouse GAPDH	Sense	CACUCAAGAUUGUCAGCAATT
	Antisense	UUGCUGACAAUCUUGAGUGAG
Negative control	Sense	UUCUCCGAACGUGUCACGUTT
	Antisense	ACGUGACACGUUCGGAGAATT
Negative control FAM	Sense	UUCUCCGAACGUGUCACGUTT
	Antisense	ACGUGACACGUUCGGAGAATT
JMJD3	Sense	AAGGAGGAGGAGCAGCAGCAG
	Antisense	GTGGTGGTGGTGGTGGTTGTG

**Table 2**  
Primers used in the study (ChIP-PCR)

Gene	Primer	Sequence 5'>3'
Tnfa	Sense	CCTCAGTTGGTCCATGGGATCC
	Antisense	GGTCTTCCAAGGATTCCCCTCC
Il6	Sense	GGTGGACAGAAAACAGGAAGTAG
	Antisense	TTGTTGAAGTTTCTTTGATTCATGGTA TATGGAAG
Il1b	Sense	TACACTGATACCTGCATACTGTGTGTG
Tlr4	Antisense	GGGTTTTCTGGGTTAGCTGTAC
	Sense	AACGGTGTCTACTTGGGTGT
	Antisense	TGGCATCTGGGAAGAGACTG



**Table 3**  
Primers used in the study

Gene	Primer	Sequence 5'>3'
Tnfa	Sense	GCGACGTGGAAGTGGCAGAAG
	Antisense	GCCACAAGCAGGAATGAGAAGAGG
Il6	Sense	TGGGACTGATGCTGGTGACA
	Antisense	ACAGGTCTGTTGGGAGTGGT
Il1b	Sense	TCGCAGCAGCACATCAACAAGAG
	Antisense	TGTCATGTCCTCATCCTGGAAGG
Tlr4	Sense	TACAAAATCCCGACAACCTCC
	Antisense	GCTGCCTAAATGCCTCAGGG

acid method by the manufacturer's instructions. The quantified protein was separated by SDS-agarose gel electrophoresis and then the protein was transferred to the polyvinylidene difluoride (PVDF) membrane under the action of voltage. After the protein transfer, the PVDF membrane was treated with skim milk and the primary antibody was added to incubate it overnight at 4 °C. Then, it was washed three times with Tris-buffered saline with Tween 20 and incubated with a secondary antibody corresponding to the primary antibody for 2 h. Finally, all the images were captured by the developer using a chemiluminescent substrate on the PVDF membrane.

### Statistical analysis

The data obtained were analyzed by GraphPad Prism 5 (version 5.0, GraphPad InStat Software). All experimental results were expressed by mean ± SEM. The statistical significance of differences between the two groups was determined using Student's *t* test for data that passed a normality test. Differences between more than two groups were evaluated by one-way ANOVA and Tukey's test. A *p* value <0.05 was considered significant.

### Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

**Acknowledgments**—This study was supported by grants from the National Natural Science Foundation of China (No. 31772721).

**Author contributions**—Z.-T. Y. and J.-J. W. conceptualization; Z.-T. Y. and J.-J. W. methodology; Z.-T. Y. and J.-J. W. validation; J.-J. W., X. W., Y.-E. X., Y.-P. S., Y.-W. F., Z.-Q. C., Z.-K. W., P.-X. L., and E.-S. Z. investigation; J.-J. W., X. W., Y.-E. X., Y.-P. S., Y.-W. F., Z.-Q. C., Z.-K. W., P.-X. L., and E.-S. Z. resources; Z.-T. Y. and J.-J. W. writing—original draft; Z.-T. Y. and J.-J. W. writing—review & editing.

**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: CCK-8, Cell Counting Kit-8; cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; IgG, immunoglobulin G; Il, interleukin; JMJD3, Jumonji domain-containing 3; LPS, lipopolysaccharide; LSD1, lysine-specific demethylase 1; MEC, mammary epithelial cell; MPO,

myeloperoxidase; NC, negative control; PVDF, polyvinylidene difluoride; Tlr, toll-like receptor.

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