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ABSTRACT

BET proteins are a group of epigenetic regulators controlling transcription through reading acetylated histone tails and recruiting transcription complexes. They are considered as potential therapeutic targets in many distinct diseases. A novel synthetic bromodomain and extraterminal domain (BET) inhibitor, JQ1, was proved to suppress oncogene transcription and inflammatory responses. The present study was aimed to investigate the effects of JQ1 on inflammatory response and bone destruction in experimental periodontitis. We found that JQ1 significantly suppressed lipopolysaccharide (LPS)-stimulated inflammatory cytokine transcription, including interleukin (IL)-1 β , IL-6, and tumor necrosis factor α (TNF- α), as well as receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclast markers, such as c-Fos, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), tartrate-resistant acid phosphatase (TRAP) and cathepsin K *in vitro*. JQ1 also inhibited toll-like receptors 2/4 (TLR2/4) expression and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) phosphorylation and nuclear translocation. Chromatin immunoprecipitation and quantitative polymerase chain reaction (ChIP-qPCR) revealed that JQ1 neutralized BRD4 enrichment at several gene promoter regions, including NF- κ B, TNF- α , c-Fos, and NFATc1. In a murine periodontitis model, systemic administration of JQ1 significantly inhibited inflammatory cytokine expression in diseased gingival tissues. Alveolar bone loss was alleviated in JQ1-treated mice because of reduced osteoclasts in periodontal tissues. These unprecedented results suggest the BET inhibitor JQ1 as a prospective new approach for treating periodontitis.

KEY WORDS: periodontitis, BRD4 protein, epigenetics, anti-inflammatory agents, osteoclasts, experimental animal models.

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BET Inhibitor JQ1 Blocks Inflammation and Bone Destruction

INTRODUCTION

Periodontitis is a bacteria-initiated chronic inflammatory disease that causes alveolar bone destruction, tooth mobility, and even tooth loss. Tissue degradation and bone destruction are known as the consequence of unshackled inflammatory responses against bacteria challenge (Van Dyke, 2007; Darveau, 2010). Inflammatory gene expression is delicately controlled through epigenetic pathways, including a distinct group of chromatin readers from the bromodomain and extraterminal domain (BET) family, among which are the ubiquitously expressed proteins, BRD2, BRD3, and BRD4. The tandem bromodomains of these proteins enable them to “read” and bind to the acetylated histones. The binding of BET proteins, especially BRD4, to the promoter of target genes recruits DNA-binding transcription complexes, including the mediator complex and the positive transcription elongation factor b (pTEFb) (Dey *et al.*, 2003; Wu and Chiang, 2007). By controlling transcription of many disease-related genes, BET proteins are considered as potential therapeutic targets in many distinct diseases (LeRoy *et al.*, 2008; Hargreaves *et al.*, 2009; Fowler *et al.*, 2011).

Several BET inhibitors act as therapeutic agents against target genes through blocking the interaction between BET proteins and acetylated histones. A cell-permeable small molecule named JQ1 can competitively occupy the recognition pocket of bromodomains and displace BET proteins, prominently BRD4, from chromatin (Filippakopoulos *et al.*, 2010). JQ1 was proved efficient in suppressing leukemic cells and tumor growth (Delmore *et al.*, 2011; Mertz *et al.*, 2011; Zuber *et al.*, 2011; Ott *et al.*, 2012). JQ1 also reduces inflammatory cytokine production by disrupting the interaction between BET proteins and target genes in lipopolysaccharide (LPS)-stimulated macrophages and protects mice from LPS-induced endotoxemic shock (Belkina *et al.*, 2013).

Considering the significant impact of BET inhibition on inflammation, we hypothesized that JQ1 may prevent inflammation and the related bone destruction caused by periodontitis. In the present study, we investigated the effects of JQ1 on the inflammation response and bone destruction in an experimental periodontitis model. Analysis of our data showed that JQ1 arrests both inflammatory and osteoclastogenic gene expression *in vitro*. The JQ1 treatment suppresses the inflammation and bone destruction in mice with periodontitis. Taken together, our results suggest that JQ1 may be exploited as a novel therapeutic agent against inflammation-related bone disorders such as periodontitis.

MATERIALS & METHODS

Cells, Reagents, and Bacteria

RAW264.7 cells from a mouse macrophage cell line (ATCC® TIB-71™, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco®, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA). LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich) was dissolved in medium to a concentration of 100 µg/mL. Recombinant murine receptor activator of nuclear factor kappa-B ligand (RANKL) was diluted to 100 µg/mL (PeproTech Inc., Rocky Hill, NJ, USA). JQ1 (generously provided by the Bradner Laboratory of Harvard Medical School) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). *Porphyromonas gingivalis* (wild-type strain 33277, ATCC®) was grown and maintained in Schaedler Broth (Thermo Fisher Scientific) in an anaerobic chamber with 85% N₂, 10% H₂, and 5% CO₂ at 37°C. For induction of experimental periodontitis, sterile 6-0 silk sutures were placed in Schaedler Broth containing *P. gingivalis* and incubated in an anaerobic chamber with 85% N₂, 10% H₂, and 5% CO₂ at 37°C for 2 days prior to use.

Induction of LPS-stimulated Inflammation and RANKL-induced Osteoclast Differentiation

RAW264.7 cells were cultured to 80% confluence in 6-well plates (5×10^5 per well) and challenged with LPS (100 ng/mL) to stimulate inflammatory responses. The cells were pre-treated with JQ1 (250 nM) or DMSO of the same volume. Cells were collected for RNA extraction after 1, 4, 24, and 48 hr of incubation, and real-time polymerase chain reaction (PCR) was performed for the measurement of inflammatory cytokines, interleukin-1β (IL-1β), IL-6, tumor necrosis factor alpha (TNF-α), IL-10, transforming growth factor beta (TGF-β), inducible nitric oxide synthase (iNOS), IL-18, leukemia inhibitory factor (LIF), and macrophage chemokine (C-C motif) ligands 2 (CCL2), CCL3, and CCL4 expression. The dynamic expression patterns of toll-like receptor 2 (TLR2) and TLR4 in 48 hr were examined by real-time PCR and Western blot.

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) phosphorylation and nuclear translocation were measured by Western blot.

RAW264.7 cells in 6-well plates (6×10^4 per well) were supplemented with RANKL (PeproTech, 50 ng/mL) and cultured for 6 days to induce osteoclast differentiation. JQ1 (250 nM) was added on day 1, 2, or 3. The specific osteoclast markers (c-Fos; nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 [NFATc1], TNF receptor associated factor 6 [TRAF6]; tartrate-resistant acid phosphatase [TRAP]; and cathepsin K) were detected by real-time PCR.

Real-time PCR and Western Blot

Real-time PCR and Western blot were performed as described in the Appendix.

Immunofluorescence

To detect the change of NF-κB distribution in the cytoplasmic and nuclear areas, we performed immunofluorescence staining as described in the Appendix.

Chromatin Immunoprecipitation (ChIP) and Quantitative PCR

Approximately 5×10^7 RAW264.7 cells were stimulated by LPS (100 ng/mL) with or without JQ1 (250 nM) for 4 hr. Chromatin was immunoprecipitated by BRD4 antibody (Bethyl Laboratories, Montgomery, TX, USA) and analyzed by quantitative PCR.

Mice and Experimental Periodontitis Model

This study was performed in accordance with the animal protocol approved by the Institutional Animal Care and Use Committee at Tufts University, as well as the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines for animal research. In total, 31 twelve-week-old male C57BL/6J mice (Stock Number 000664, The Jackson Laboratory, Bar Harbor, ME, USA) were randomly assigned to 3 groups: periodontitis group (infected with *P. gingivalis*, $n = 11$), JQ1 group (infected with *P. gingivalis* and receiving JQ1 therapy, $n = 8$), and the control group ($n = 12$). Mice were anesthetized by an intraperitoneal injection of ketamine (80 mg/Kg) and xylazine (10 mg/Kg). A 6-0 silk suture pre-soaked in *P. gingivalis* broth was placed around the necks of both maxillary second molars and knotted mesio-buccally. Ligatures were changed every other day on days 3, 5, and 7 to sustain a sufficient microbial load (Amar *et al.*, 2007). Mice in the JQ1 group were treated with daily intraperitoneal injections of JQ1 (50 mg/Kg), while mice in the periodontitis group received a similar volume of carrier containing 5% DMSO. Both JQ1 and DMSO were diluted by 10% 2-hydroxypropyl-β-cyclodextrin carrier (Sigma-Aldrich). Mice in the control group received neither the periodontal ligature nor any intraperitoneal injections. All mice were humanely euthanized 10 days after the initial ligature application.

Histological Analysis, Quantitative PCR, and Alveolar Bone Loss

Hemi-maxillary bone was fixed in 10% formalin at 4°C overnight and demineralized in a 10% ethylenediaminetetraacetic acid (EDTA)-buffered solution (pH 7.0) for 14 days at room temperature. Samples were embedded in paraffin and serial sections of 5-µm thickness were obtained in a mesial-distal direction and stained with hematoxylin and eosin (HE) or TRAP (Sigma-Aldrich). TRAP-positive cells with multiple nuclei along the alveolar bone surface were identified as active osteoclasts and normalized by the bone surface length. Using the SPOT Advanced Image Analysis System (SPOT Imaging Solutions, Sterling Heights, MI, USA), we determined the number of osteoclasts along the alveolar bone surface. The results are presented by the alveolar bone surface number of osteoclasts per millimeter.

Gingival tissues separated from the other half of the maxillary bone were immediately frozen in liquid nitrogen and stored

at -80°C for RNA isolation. The gingival sample from each mouse was homogenized separately by being ground in liquid nitrogen with a pestle and mortar. RNA was isolated by means of the QiaShredder and RNeasy Kit (Qiagen) and reverse-transcribed to cDNA for real-time PCR.

The maxillary bone was de-fleshed *via* boiling for 15 min and bleached in 3% H_2O_2 overnight. The air-dried maxillary bone was stained by Methylene Blue (0.003%, Sigma-Aldrich), and both buccal and lingual sides were photographed to test for alveolar bone loss. The distance from the cement-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 6 sites (mesio-buccal, mid-buccal, disto-buccal, mesio-palatal, mid-palatal, and disto-palatal) around the second molar and normalized by Image-Pro Plus 6.0 Software (Media Cybernetics, Warrendale, PA, USA). The alveolar bone loss data represent the mean in millimeters of the 6 measured sites.

Statistical Analysis

Data are presented as mean $\pm$ SEM. Statistical analysis was administered with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Differences between groups were assessed by one-way analysis of variance (ANOVA) with a Student's *t* test and were considered significant when $p < .05$.

RESULTS

JQ1 Inhibited Inflammation and Osteoclast Differentiation

The inflammatory cytokines, including IL-1 β , IL-6, TNF- α , and IL-10, were significantly enhanced immediately after LPS challenge, and the JQ1 treatment significantly inhibited IL-1 β , IL-6, TNF- α , and IL-10 production at 1, 4, 24, and 48 hr. The increased IL-18, iNOS, and LIF were also reduced by JQ1 at different time points. The LPS-enhanced expression of macrophage chemokines such as CCL2, CCL3, and CCL4 was significantly decreased by JQ1 at 4, 24, and 48 hr. However, TGF- β was not significantly changed under any conditions (Fig. 1A). ChIP assay with the BRD4 antibody revealed BRD4 binding to chromatin at the TNF- α promoter and NF- κ B promoter under LPS stimulation. JQ1 treatment decreased BRD4 binding to the transcription start site (TSS) around the promoter region of both TNF- α and NF- κ B (Fig. 1B).

When RAW264.7 cells were induced to osteoclasts by RANKL, the expression of NFATc1, TRAP, and cathepsin K

Figure 1. The expression of LPS-induced cytokines and chemokines was suppressed by JQ1 treatment. **(A)** Gene expression relative to GAPDH in RAW264.7 cells treated with LPS+Vehicle (DMSO) (LPS, white) or LPS+JQ1 (JQ1, black) at indicated time points was compared with that in the control (DMSO only) group (Con, grey). **(B)** ChIP-qPCR was performed with BRD4 antibody and TSS specific primers. * $p < .05$ between Con and LPS; * $p < .05$ between LPS and JQ1. JQ1 inhibited osteoclast-specific gene expression in response to RANKL. **(C)** RAW264.7 cells were induced to osteoclasts by RANKL for 6 days, and JQ1 was added on the 1st, 2nd, or 3rd day. The osteoclast-specific gene levels were determined by qPCR. **(D)** ChIP-qPCR was performed with BRD4 antibody and TSS specific primers. * $p < .05$ between Con and RANKL; * $p < .05$ between RANKL and JQ1. (The experiments were repeated 3 times.)

was significantly higher than in control. JQ1 treatment significantly reduced the RANKL-induced osteoclast markers, including c-Fos, NFATc1, TRAP, and cathepsin K (Fig. 1C). ChIP analysis found BRD4 evacuation from the c-Fos and NFATc1 promoters in JQ1-treated cells in accordance with the reduced expression of osteoclast markers (Fig. 1D).

The TLR2 mRNA level was noticeably elevated by LPS within 4 hr, while the TLR4 mRNA level was not obviously changed. However, JQ1 treatment significantly attenuated TLR2/4 mRNA expression (Fig. 2A). Western blot analysis showed that JQ1 treatment quickly and consistently reduced the LPS-induced TLR2/4 protein levels over a 12-hour time period (Fig. 2B). Additionally, Western blot analysis demonstrated that NF- κ B (p65) phosphorylation promoted by LPS was inhibited by JQ1, while the NF- κ B (p65) protein level remained unchanged by LPS or JQ1 (Fig. 2C). The LPS stimulation rendered NF- κ B translocation into the nucleus within 5 min, and JQ1 treatment

Figure 2. JQ1 inhibited the TLR2/4-NF-κB pathway in LPS-treated macrophages. **(A)** Relative expression of TLR2/4 mRNA induced by LPS (black) was compared with LPS+JQ1 (white) at 0, 0.5, 1, 2, 4, 12, 24, and 48 hr. * $p < .05$ between baseline and the indicated time; * $p < .05$ between LPS only and LPS+JQ1. **(B)** TLR2/4 protein levels were determined at 1/4, 1/2, 1, 2, 4, and 12 hr and balanced by β-actin. **(C)** Relative NF-κB phosphorylation was analyzed by Western blot at 1/4, 1/2, 1, 2, 4, and 12 hr. **(D)** Nuclear NF-κB level was compared between the LPS and LPS+JQ1 groups at 5, 15, 30, and 60 min. **(E)** Immunofluorescence analysis of NF-κB nuclear translocation. NF-κB protein was labeled with FITC (green), and nuclei were labeled with DAPI (blue).

attenuated nuclear NF-κB (Fig. 2D). In accordance with the Western blot, immunofluorescence assay also found that nuclear signals of NF-κB in JQ1-treated cells were weaker than in LPS-treated cells (Fig. 2E).

JQ1 Reduces Bone Destruction in Experimental Periodontitis

Compared with the increased cytokine expression levels of IL-1β, IL-6, TNF-α, IL-17, IL-10, and TGF-β in gingival tissue from the periodontitis group, the gingival tissue from the JQ1-treated group had significantly reduced cytokine expression levels (Fig. 3A). Moreover, the enhanced matrix metalloproteinase 9 (MMP-9) and RANKL expression in the periodontitis group was also neutralized by JQ1 treatment ($0.1 > p > .05$) (Fig. 3B). Morphometric analysis showed advanced alveolar

bone resorption in the periodontitis group, and histological results showed interproximal bone destruction and inflammatory infiltration in the periodontitis and JQ1 groups (Fig. 3C). As a specific feature of periodontitis, the alveolar bone loss in the control, periodontitis, and JQ1 groups was 0.11 ± 0.04 mm, 0.31 ± 0.06 mm, and 0.22 ± 0.02 mm, respectively. JQ1 treatment significantly reduced the bone destruction caused by periodontitis (Fig. 3D). More active osteoclasts were found in the periodontal tissue from the periodontitis group than from the control group, and JQ1 treatment significantly decreased osteoclast formation in the periodontal tissue (Fig. 3E).

DISCUSSION

BET proteins are a group of epigenetic regulators controlling transcription through reading acetylated histone tails and recruiting

transcription complexes. Numerous small-molecule BET inhibitors were developed to target diverse BET members in cancer and inflammatory diseases (Muller *et al.*, 2011; Prinjha *et al.*, 2012). For instance, a pan-BET inhibitor I-BET has been proven to protect against endotoxic shock and infectious sepsis (Nicodeme *et al.*, 2010). Another BET inhibitor can interrupt T-cell-mediated inflammatory response (Bandukwala *et al.*, 2012). Among these BET inhibitors, JQ1 attracts the most attention for its significant efficacy in hematological malignancies (Dawson *et al.*, 2012). Recent discoveries demonstrate even wider prospective applications for BET inhibitors, in that JQ1 attenuates lung fibrosis (Tang *et al.*, 2013), endotoxemic shock (Belkina *et al.*, 2013), NO synthesis, and innate immunity in mice (Wienerroither *et al.*, 2014).

Analysis of our data shows that JQ1 reduces inflammatory responses both *in vitro* and *in vivo*. While suppressing most of the pro-inflammatory cytokines, JQ1 also inhibited IL-10 production, a known anti-inflammatory cytokine, in JQ1-treated cells and mice. The down-regulated MMP-9 and RANKL in gingival tissue suggest that JQ1 protects mice from connective tissue degradation and bone destruction. The alleviated bone destruction and decrease of osteoclasts in JQ1-treated mice agree with the reduced production of osteoclast markers *in vitro*. The anti-inflammatory and anti-osteoclastogenic effects of JQ1 are dependent on the interaction between BRD4 and target genes. NF- κ B, known as a master transcription factor, regulates a number of pro-inflammatory gene productions and contributes to the etiology and progression of inflammatory disorders (Pasparakis, 2009). Its pro-inflammatory activity is dependent on the association between its acetylated lysine 310 and BRD4 in complex with pTEFb and RNA polymerase II, which form an active transcription complex (Huang *et al.*, 2009). Analysis of our data proves that BRD4 uncoupling from NF- κ B promoter is associated with down-regulation of pro-inflammatory cytokine expression, which agrees with previous reports (Nicodeme *et al.*, 2010; G Zhang *et al.*, 2012). Moreover, the NF- κ B downstream transcription factors, c-Fos and NFATc1, are involved in RANKL-induced osteoclastogenesis. Although there are no previous reports showing the role of BRD4 in osteoclastogenesis, the BRD4/c-Fos association has been determined in mitogen stimulation (Byun *et al.*, 2009). We discovered that the interactions between BRD4 with c-Fos and NFATc1 may account for the inhibition of osteoclast-specific markers.

Figure 3. JQ1 administration inhibited gingival cytokine expression, alveolar bone loss, and osteoclast formation in periodontal tissues due to periodontitis. Cytokines (A), MMP-9, and RANKL (B) levels in gingival tissue were analyzed by qPCR. Morphological, HE staining, and TRAP staining results from different groups are shown (C). The alveolar bone loss was 0.11 ± 0.04 mm (Con), 0.31 ± 0.06 mm (PD), and 0.22 ± 0.02 mm (JQ1) (D). Osteoclast number in periodontal tissue was 1.49 ± 1.21 (Con), 9.69 ± 1.35 (PD), and 6.72 ± 1.43 (JQ1) (E). The osteoclasts are indicated by blank arrows (F). * $p < .05$ between Con and PD; * $p < .05$ between PD and JQ1. N = 8-12 mice/group in A, B, D. N = 6 mice/group in E.

Our results support that JQ1 evacuating BRD4 from specific gene promoters renders anti-inflammatory and anti-osteoclastogenic effects, without ruling out that some effects of JQ1 are derived from inhibiting other members of the BET family. BRD2 is involved in IL-6 transcription, and JQ1 can inhibit BRD2-mediated IL-6 expression (Belkina *et al.*, 2013). Nevertheless, BRD4 protein attracts more attention for its crucial role in transcription control of many disease-related genes, and it is the most complementary target of JQ1 (Filippakopoulos *et al.*, 2010; Jung *et al.*, 2014). For these advantages, many researchers have applied JQ1 as a BRD4 inhibitor in cancer and inflammatory disease studies (G Zhang *et al.*, 2012; W Zhang *et al.*, 2012; Tang *et al.*, 2013).

Though JQ1 inhibits inflammatory responses under many challenged conditions, the molecule showed no significant suppression in the same inflammatory cytokines without LPS stimulation (Belkina *et al.*, 2013). Analysis of the data suggests that the anti-inflammatory effects of JQ1 are restricted to the inflammatory responses with

abnormally high expression of inflammatory cytokines. No negative side-effect other than weight loss was verified in the systemic application of JQ1 (Matzuk *et al.*, 2012). These features make JQ1 a potential drug candidate against inflammatory and bone-destructive diseases through epigenetic regulation. Further studies will be needed to explore the mechanisms of JQ1 in inflammation and bone destruction.

In conclusion, this study focused on the effects of JQ1 on periodontitis. Furthermore, to our knowledge, this is the first study to apply the BET inhibitor JQ1 to an inflammatory disease model associated with bone destruction. JQ1 inhibited inflammatory responses and alveolar bone loss, suggesting JQ1 as a potential therapeutic approach against periodontitis.

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