

RESEARCH PAPER

The suppression of bromodomain and extra-terminal domain inhibits vascular inflammation by blocking NF- κ B and MAPK activation

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BACKGROUND AND PURPOSE

There is increasing evidence indicating that bromodomain and extra-terminal domain (BET) proteins play a critical role in the regulation of immune and inflammatory responses; however, their contribution to vascular inflammation has not yet been elucidated. In this study, we investigated the effect of inhibiting BET bromodomain on vascular inflammation and the underlying mechanisms.

EXPERIMENTAL APPROACH

HUVECs were isolated from fresh umbilical cords. JQ1, a specific BET bromodomain inhibitor, and Brd shRNA were used to evaluate the regulation of the BET proteins in vascular inflammation. Leukocyte adhesion to HUVECs was measured by an adhesion assay. Western blot or immunohistochemical analysis was used to detect the protein expression. Real-time PCR was used to evaluate mRNA expression. Leukocyte accumulation *in vivo* was determined by an acute lung inflammation model.

KEY RESULTS

BET bromodomain inhibition suppressed the expression of adhesion molecules induced by TNF- α - or LPS, including ICAM-1, VCAM-1 and E-selectin, and inhibited leukocyte adhesion to activated HUVEC monolayers. Treatment with JQ1 also attenuated the LPS-induced accumulation of leukocytes and expression of endothelial adhesion molecules in the acute lung inflammation model *in vivo*. Furthermore, BET bromodomain inhibition reduced the activity of p38 and JNK MAPKs and NF- κ B in TNF- α -stimulated HUVECs. TNF- α -induced NF- κ B activation was also blocked by inhibitors of p38 (SB203580) or JNK (SP600125).

CONCLUSIONS AND IMPLICATIONS

BET bromodomain is important for regulating endothelial inflammation. Strategies targeting endothelial BET bromodomain may provide a new therapeutic approach for controlling inflammatory-related diseases.

Abbreviations

BET, bromodomain and extra-terminal domain; ICAM-1, intercellular adhesion molecule 1; MPO, myeloperoxidase; VCAM-1, vascular cell adhesion molecule 1

Tables of Links

TARGETS	
Brd2	JNK1
Brd4	MPO
ERK	p38 α

LIGANDS	
JQ1	LPS
ICAM-1	SB203580
IL-6	TNF- α
IL-8	VCAM-1

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

The vascular endothelium separates and connects the bloodstream and all extravascular tissues. Endothelial dysfunction is associated with the development of inflammatory disorders, including rheumatoid arthritis, tumours, atherosclerosis, inflammatory bowel disease and sepsis (Aird, 2007; Gareus *et al.*, 2008; Roifman *et al.*, 2009; Khan *et al.*, 2010). An array of adhesion molecules, particularly vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and E-selectin, are up-regulated in the vascular endothelium under inflammatory conditions, and play critical roles in regulating the early events of leukocyte adhesion to endothelial cells (Ley *et al.*, 2007; Pober and Sessa, 2007).

Several signalling pathways play important roles in regulating endothelial inflammation. NF- κ B activation in the endothelium results in an increased expression of a series of inflammation-related genes, such as those encoding adhesion molecules and pro-inflammatory cytokines (Molestina *et al.*, 2000; Kempe *et al.*, 2005; Zhou *et al.*, 2007). Targeting NF- κ B-mediated activation of endothelial cells could provide a novel strategy for treating inflammation. The MAPK pathways consist of a family of highly conserved serine/threonine protein kinases that are believed to act as the regulators of key cellular processes including gene induction, cellular stress and inflammatory responses. There are three major classes of MAPKs in mammals, ERK, p38 and JNK (Johnson and Lapadat, 2002). TNF α -induced expression of adhesion molecules in the endothelium has been shown to be mediated by MAPKs (Read *et al.*, 1997; Hoefen and Berk, 2002; Nizamutdinova *et al.*, 2007; Liang *et al.*, 2011).

Bromodomain and extra-terminal domain (BET) proteins (Brd2, Brd3, Brd4 and Brdt) are a family of epigenetic adaptors that bind to acetylated lysine residues, thereby connecting acetylated chromatin and gene transcription (Filippakopoulos *et al.*, 2012). Brd proteins promote inducible gene transcription by the recruitment of transcriptional co-activators including positive transcription elongation factor b (Hargreaves *et al.*, 2009). Hence, BET bromodomain inhibition suppresses LPS-induced expression of pro-inflammatory cytokines in murine bone marrow-derived macrophages (Nicodeme *et al.*, 2010; Belkina *et al.*, 2013). Brd4 promotes the expression of a series of NF- κ B-mediated inflammatory genes in an acetylated lysine-310-dependent

manner (Huang *et al.*, 2009). These data indicate that BET bromodomain has the potential to affect inflammatory responses. However, the contribution of BET bromodomain to vascular inflammation remains to be defined. Therefore, in this study, we investigated the role of BET proteins in modulating endothelial inflammation and the underlying mechanisms.

Methods

Cell culture

HUVECs were isolated from fresh umbilical cords after treatment with collagenase. The cells were cultured in EBM2 media supplemented with 0.1% hEGF, 0.1% GA-1000 (gentamicin, amphotericin-B), 0.1% ascorbic acid, 0.1% R3-IGF-1, 0.1% heparin, 0.4% hFGF-B, 0.1% VEGF, 0.04% hydrocortisone and 2% FBS (Lonza, CA, USA) and used at passages 4 to 6 when the cells reached 80–90% confluence. The cells were grown in 5% CO₂ at 37°C, and the medium was replaced every 2 days. The isolation procedure of the HUVECs was approved by the Medical Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University, and was conducted in accordance with the recommendations of the Declaration of Helsinki. Informed consent was obtained for all of this study.

Endothelial-leukocyte adhesion assay

Endothelial-leukocyte adhesion was determined by the Cell Biolabs' CytoSelect™ Leukocyte-endothelium Adhesion Assay. HUVECs grown in gelatin-coated 24-well plates at 80–90% confluence were treated with JQ1 for 8 h or were transfected with shRNA for 48 h. The cells were treated with 10 ng·mL⁻¹ TNF- α for 4 h and LeukoTracker™ solution-loaded leukocytes were added to each well. Non-adherent cells were removed after 1 h of incubation. The adherent leukocytes were counted under an inverted fluorescence microscope, and the results from five separate fields per well were averaged (cells counted by three blinded observers). The final wash was aspirated and Lysis Buffer was added to each well containing the cells, and fluorescence was read by using a fluorescence plate reader at 480/520 nm.

Model of acute lung inflammation

All animal care and experimental protocols complied with guidelines for protection of animals used for scientific

purposes of Sun Yat-sen University. Animal experiments were approved by the Animal Care and Ethics Committee, Sun Yat-sen University, according to the *Guide for the Care and Use of Laboratory Animals*, which was published by the US National Institutes of Health. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015).

Male C57BL/6J mice (8 weeks old; average weight 25–29 g, obtained from Beijing Laboratory Animal Research Centre) were fed normal mouse chow and water *ad libitum*. All mice were reared and housed under standard conditions with air filtration ($20 \pm 2^\circ\text{C}$; 12 h light/12 h dark). The mice were randomly divided into three groups: control (10% DMSO, 0.2 mL), LPS and LPS+JQ1. Before inducing acute lung inflammation, JQ1 ($50 \text{ mg}\cdot\text{kg}^{-1}$) was given by i.p. injection for 5 days, and then LPS ($40 \text{ mg}\cdot\text{kg}^{-1}$, *E. coli* serotype O26:B6 endotoxin; Sigma-Aldrich) was administered i.p. to induce lung injury. Five hours after LPS administration, all animals were anaesthetised with $110 \text{ mg}\cdot\text{kg}^{-1}$ ketamine and $4.8 \text{ mg}\cdot\text{kg}^{-1}$ xylazine, and then killed to collect lung tissues for further analysis. The tissues were fixed in 10% neutral buffered formalin, and the paraffin sections ($5 \mu\text{M}$) were used to perform immunohistochemical analysis and were stained with haematoxylin and eosin (H&E); H&E staining was used to determine the lung injury score. The score was calculated by two blinded pathologists according to the following standards (Sun *et al.*, 2012): no injury, score 0; mild-to-moderate injury, score 0.1 to 2.5; severe injury, score 2.6 to 4.0. An Amplex Red Peroxidase Assay Kit (Invitrogen) was used to detect myeloperoxidase (MPO) activity in homogenates of murine lung tissue.

Retrovirus generation and transduction

Lentivirus- based shRNA vectors were constructed as described previously (Wang *et al.*, 2012). The sequences for control, Brd2 or Brd4 shRNA oligonucleotides are presented in Table 1. These shRNAs were obtained from Shanghai GenePharma (Shanghai, China). To generate virus, 293T cells were transfected, using Lipofectamine 2000 (Invitrogen), with plasmids encoding shRNA targeting Brd2, Brd4 ($1 \mu\text{g}\cdot\text{mL}^{-1}$), or control together with CMV-dR 8.2 packaging and CMV-VSVG envelope plasmids. After 48 and 72 h transfection supernatants containing virus were collected and used to infect HUVECs in the presence of $8 \mu\text{g}\cdot\text{mL}^{-1}$ polybrene.

Table 1

The sequences of Brd shRNA oligonucleotides

Oligo Sequence		
Brd2 shRNA	Forward	AATTCCTGCCTACAGGTTATGATTCTCGAGAATCATAACCTGTAGGCAGGGTTTTTAT
	Reverse	AAAAACCCTGCCTACAGGTTATGATTCTCGAGAATCATAACCTGTAGGCAGGG
Brd4 shRNA	Forward	AATTCCTGGAGATGACATAGTCTTACTCGAGTAAGACTATGTCATCTCCAGGTTTTTAT
	Reverse	AAAAACCTGGAGATGACATAGTCTTACTCGAGTAAGACTATGTCATCTCCAGG
Scrambled shRNA	Forward	AATTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTAT
	Reverse	AAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG

Overexpression of Brd2 and Brd4 in HUVECs

Brd2 and Brd4 expression cassettes were cloned into FG12 between the XbaI and XhoI sites to construct the overexpression lentiviral vectors. The sequences of these primers are as follows: Brd2, forward 5'-CTAGTCTAGAATGCTGCAAAACGTGACTC-3', reverse 5'-TCCGCTCGAGTTAGCCTGAGTCTGAATCAC-3'; Brd4, forward 5'-CTAGTCTAGA ATGTCTGCGGAGAGCGGCC-3', reverse 5'-TCCGCTCGAGTCAGGAGCAGC CA ACACAGC-3'. The correct construct was confirmed by means of DNA sequencing. HEK-293T cells were cotransfected with appropriate amounts of vector plasmid, the lentiviral packaging constructs pRSVREV and pMDLg/pRRE, and the VSV-G expression plasmid pHCMVG. The viral supernatants were harvested on days 2 and 3 after transfection, filtered with 0.4 μm filters, concentrated by ultracentrifugation, and then resolved in MEM medium. For Brd2 and Brd4 overexpression, ECs (50% confluent) were incubated with Brd2, Brd4 or green fluorescent protein viral preparations in the presence of $8 \text{ mg}\cdot\text{mL}^{-1}$ polybrene (Santa Cruz Biotechnology, Dallas, Tex) overnight, and then replaced with fresh medium. The efficiency of the infection was determined by western blotting for protein expression.

RNA isolation and quantitative PCR

After the designated treatments with JQ1 or transfection with shRNA for 48 h and then stimulation with TNF- α for 8 h, total RNAs were extracted using TRIzol (Sigma) and were reverse transcribed to cDNA by using miScript Reverse Transcription Kit. Quantitative real time PCR analysis for the expression of VCAM-1, ICAM-1, E-selectin, IL-6 and IL-8 was performed on cDNA using QuantiTect SYBR Green RT-PCR Kit on StepOnePlus™ Real Time PCR System (Applied Biosystems). Relative mRNA expression was normalized to the expression of GAPDH. The sequences of VCAM-1, ICAM-1, E-selectin, IL-6, IL-8, Brd2 and Brd4 are listed in Table 2.

Detection of cell culture supernatant level of IL-6 and IL-8

After the designated treatments with JQ1 or transfection with shRNA for 48 h and then treatment with TNF- α for 12 h, cell culture supernatants were collected for the determination of IL-6 and IL-8 by using ELISA kit (R&D Systems, USA).

Western blot analysis

Cells were lysed with cell lysis buffer (CST) for 15 min on ice, and lysates were centrifuged at $10\,000 \times g$ for 15 min at

Table 2

The sequences of RT-PCR primers

Sequences		
IL-6	Forward	ACTCACCTCTTCAGAACGAATTG
	Reverse	CCATCTTTGGAAGGTTTCAGGTTG
IL-8	Forward	ACTGAGAGTGATTGAGAGTGAGAC
	Reverse	AACCTCTGCACCCAGTTTTC
Brd2	Forward	ACTCACCTCTTCAGAACGA ATTG
	Reverse	CATCT TTGGAAGGTTTCAGGTTG
Brd3	Forward	ACTGAGAGTGATTGAGAGTGAGAC
	Reverse	AACCTCT GCACCCAGTTTTC
Brd4	Forward	CTCTGGAGTAATGTCACACCTCT
	Reverse	TGTTGGTC CACCTTTCATCTTC
Brdt	Forward	TGTAAAGAAACCTTCCTGCAA
	Reverse	TTTAAACACAGTATGCCCAA
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	TGGTGAAGACGCCAGTGGAA

4°C. Supernatants were incubated with 2 × laemmli sample buffer (Sigma) at 100°C for 5 min. Equal amounts of the samples were then separated by SDS-PAGE gel and transferred onto NC membranes and immunoblotted with the indicated antibodies: anti-VCAM1 (Abcam), anti-ICAM1 (Abcam), anti-E-selectin (Abcam) anti-JNK (Cell signalling), anti-phospho-JNK (Cell signalling), anti-p38 (Cell signalling), anti-phospho-p38 (Cell signalling), anti-ERK (Cell signalling), anti-phospho-ERK (Cell signalling), anti-NF-κB p65 (Cell signalling), anti-phospho-IκBα (Cell signalling), anti-IκBα (Cell signalling) and anti-phospho-IκB kinase (IKK) (Cell signalling).

Immunohistochemical analysis

Lung tissues were fixed overnight in 10% formalin, paraffin-embedded, cut into sections of 5 μm thickness and placed on 3-aminopropyltriethoxysilane-coated slides. The sections were then deparaffinized with xylene and rehydrated with ethanol, followed by antigen retrieval using microwave heating. Endogenous peroxidase activity was suppressed by incubating the samples with 1% hydrogen peroxide for 30 min. The sections were incubated with primary antibodies against VCAM1 (mouse monoclonal to VCAM1, 1:100) or ICAM1 (mouse monoclonal to ICAM1, 1:100) or MRP14 (mouse monoclonal to MRP14, 1:100) (all from Abcam, Cambridge, UK), respectively, at 4°C overnight. After being washed with PBS, the sections were incubated with the respective HRP-conjugated secondary antibodies, and substrates, and counterstained with haematoxylin.

Confocal laser scanning fluorescence microscopy

HUVECs on glass coverslips at 80–90% confluence were treated with JQ1 or DMSO for 8 h and then were stimulated with TNF-α for 30 min. Then, they were fixed with paraformaldehyde and permeated with 0.1% Triton X-100 in PBS. For detection of p65 NF-κB, the cells were

incubated with anti-p65 NF-κB antibody overnight and incubated with secondary antibodies for 1 h at room temperature. The cells were then incubated with DAPI and the coverslips were mounted on glass slides with antifade mounting media and examined using a confocal fluorescence microscopy (Zeiss LSM710).

MTT test for cell viability

HUVECs were pretreated for 24 h with JQ1 at different concentrations (50, 100 and 250 nM). The culture supernatants were removed, and the adherent cells were incubated for 30 min at 37°C with a solution of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) salt (1 mg·mL⁻¹ in PBS). The dark blue crystals of formazan produced were dissolved in acidified isopropanol, and the amount of formazan quantified by subjecting the samples to a test wavelength of 570 nm and a reference wavelength of 620 nm.

Flow cytometry

HUVECs were fixed in 2% paraformaldehyde and were analysed by flow cytometry using a FACSscan with CellQuest software (BD Bioscience, Oxford, United Kingdom). Cell apoptosis was assessed by labelling with annexin V (FITC) according to the manufacturer's instructions (BD Bioscience).

CCK-8 assay

Peripheral blood mononuclear cell (PBMCs) were seeded in 96-well plates, deprived of serum, and then treated with JQ1 as described in the text. At the end of the incubation, CCK-8 solution was added for 4 h and the absorbance at 450 nm (A 450 nm) was measured with a microplate reader.

Statistical analyses

The results are expressed as mean ± SEM. The present studies comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). The experimental procedures or treatment and data analyses were carried out with randomization and blinding. For *in vitro* studies, we performed a minimum of five independent experiments, where individual data points were based on at least technical duplicates each. For statistical analysis, we used normalized data to reduce the variations in the baseline between independent experiments, with the exception of data for pro-inflammatory cytokine secretion where the results were normalized to fold over control without TNF-α or LPS. Student's *t*-test was used to test compare the data from two groups. We compared ≥3 different groups with one way ANOVA, and if *F* reached significance two *post hoc* tests were used: the Dunnett *post hoc* test when comparing each group with control, or the Sidak *post hoc* test whenever a multiple group comparison was necessary. For *in vivo* studies, all groups were initially designed to contain 7 mice with a C57BL/6J background. We used nonparametric methods (Kruskal–Wallis test followed by Dunn's *post hoc* tests when *F* reached significance). *P* values less than 0.05 were considered statistically significant. Statistical analysis of data was performed by using GraphPad Prism 6.

Results

BET bromodomain inhibition suppresses TNF- α -induced expression of adhesion molecules

In our previous study (Huang *et al.*, 2016), we examined the expression patterns of BET proteins in endothelial cells and found that Brd2 and Brd4 are highly expressed. To rule out non-specific interference, we constructed 3 different sequences of shRNA oligonucleotides for Brd2 or Brd4. As shown in our previous study, transfection with all 3 shRNA oligonucleotides down-regulated endogenous Brd2 or Brd4 protein expression; however, the inhibitory effect of Brd2 shRNA-3 or Brd4 shRNA-3 was the most prominent. Accordingly, Brd2 shRNA-3 (Brd2 shRNA) or Brd4 shRNA-3 (Brd4 shRNA) was used for subsequent experiments. In the present study, we also determined the inhibitory efficiency of Brd2 shRNA and Brd4 shRNA on their endogenous expression in HUVECs (Figure S1).

To evaluate the ability of BET protein to affect the activation of endothelial cells, we determined the effect of BET inhibition on the expression of adhesion molecules in TNF- α -stimulated HUVECs by using JQ1 or Brd shRNA transfection. JQ1 treatment suppressed TNF- α -induced gene expression of VCAM-1, E-selectin and ICAM-1. Treatment with Brd2 or Brd4 shRNA also suppressed their expression respectively (Figure 1A and B). We further demonstrated that co-transfection with Brd2 and Brd4 shRNA decreased the expression of these adhesion molecules to a higher degree compared with the transfection of either of them alone (Figure 1B). The protein expression of VCAM-1, E-selectin and ICAM-1 was also decreased in cells treated with JQ1 compared with those treatment with DMSO alone (Figure 1C); moreover, the protein expression of these molecules was also inhibited by transfection with Brd2 shRNA or Brd4 shRNA (Figure 1D). Likewise, BET inhibition also reduced the mRNA expression of VCAM-1 and ICAM-1 in HUVECs in response to LPS treatment (Figure S2).

To determine the toxic effect of JQ1, the viability or apoptosis of HUVECs was evaluated by the MTT test or annexin V labelling assay. Treatment with JQ1 at concentrations of 50, 100 and 250 nM did not affect cell viability (Figure 1E) and apoptosis (Figure 1F), suggesting that the effects of JQ1 in our experiments were not associated with cellular cytotoxic effects.

BET bromodomain inhibition reduced leukocyte adhesion to activated HUVEC monolayers

Adhesion molecules are critical for the initiation, promotion and maintenance of leukocyte adhesion to the vascular endothelium; therefore, we performed an *in vitro* cell adhesion assay to evaluate the effect of BET inhibition on the interaction between leukocytes and HUVECs. As shown in Figure 2A and B, treatment with TNF- α increased the adhesion of human PBMC to HUVECs compared with the control group; however, this increase was suppressed by treatment with JQ1 or Brd2 shRNA or Brd4 shRNA. We also demonstrated that co-transfection with Brd2 shRNA and Brd4 shRNA decreased the adhesion of PBMC to HUVECs to a higher degree than treatment with Brd2 or Brd4 shRNA alone. Furthermore, the decreased adhesion was restored by the overexpression of Brd2 or Brd4 in their previously knocked-down cells (Figure 2C). In

addition, we determined whether JQ1 affected leukocyte viability so as to rule out the possibility that JQ1-induced reduction of PBMC adhesion to HUVECs is associated with reduced leukocyte viability. Our results showed that treatment with JQ1 for 8 h did not affect leukocyte viability (Figure 2D). Taken together, our findings suggest that BET protein is involved in the pro-inflammatory stimuli-induced expression of adhesion molecules and adhesion of leukocytes to activated HUVECs.

BET inhibition reduced the production of pro-inflammatory cytokines in HUVECs

Pro-inflammatory cytokines, including IL-6 and IL-8, play important roles in endothelial inflammation. We found that TNF- α -induced mRNA expression and secretion of IL-6 and IL-8 was reduced by JQ1 treatment in HUVECs (Figure 3A and B). Moreover, the mRNA expression and secretion of IL-6 and IL-8 were reduced in TNF- α -stimulated cells transfected with Brd2 shRNA or Brd4 shRNA. Co-transfection with Brd2 and Brd4 shRNA also reduced mRNA expression and secretion of IL-6 and IL-8 to a higher degree than transfection with Brd2 or Brd4 shRNA alone.

BET inhibition regulates NF- κ B signal pathways in activated HUVECs

In response to pro-inflammatory stimuli, NF- κ B pathway plays a critical role in regulating inflammatory responses in ECs (Molestina *et al.*, 2000). In inflammatory conditions, optimal classical activation of NF- κ B requires the activation of the IKK complex, which results in the phosphorylation, ubiquitination and degradation of the inhibitor of NF- κ B (I κ B), and phosphorylation of p65 (Perkins, 2007). To determine whether BET proteins are involved in the activation of NF- κ B in TNF- α -stimulated HUVECs, the cells were incubated with JQ1 for 8 h and then stimulated with TNF- α for 30 min. As shown in Figure 5A, JQ1 treatment blocked TNF- α -induced phosphorylation of IKK β . JQ1 treatment also suppressed the phosphorylation and degradation of I κ B α in TNF- α -stimulated cells (Figure 5B). We further found that TNF- α -induced nuclear accumulation of p65 protein was reduced in HUVECs treated with JQ1 (Figure 4C–F). Transfection with Brd2 or Brd4 shRNA also reduced the TNF- α -induced phosphorylation of IKK (Figure 4G). JQ1 also suppressed LPS-induced increase in IKK activity and nuclear translocation of p65 in HUVECs (Figure S3). These results suggest that BET bromodomain has an important role in regulating endothelial NF- κ B activation under inflammatory conditions.

BET inhibition affects the p38 and JNK MAPK signal in HUVECs

Since several MAPKs have been implicated in regulating endothelial inflammation, we determined whether BET inhibition affects the activation of MAPK signalling pathways. TNF- α treatment resulted in an increased phosphorylation of p38, JNK and ERK; however, JQ1 treatment decreased TNF- α -induced phosphorylation of p38 and JNK, but not ERK (Figure 5A). We also observed similar results from HUVECs transfected with Brd2 shRNA or Brd4 shRNA (Figure 5B). These data suggest that p38 and JNK are involved in the effects mediated by BET proteins on the inflammatory response in HUVECs.

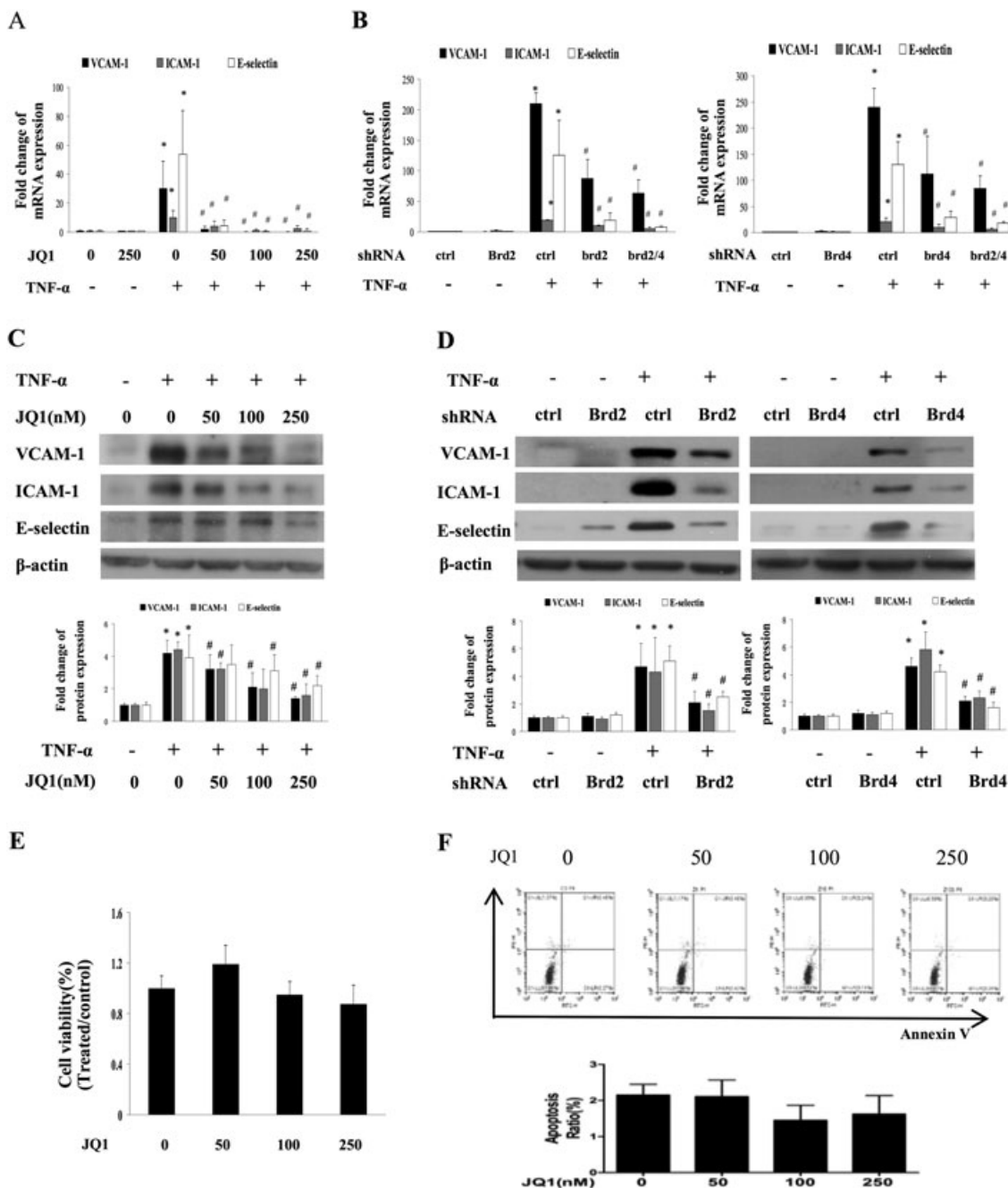


Figure 1

Effect of BET bromodomain inhibition on TNF- α -induced expression of adhesion molecules. (A and B) Real time qPCR analysis of VCAM-1, ICAM-1 and E-selectin mRNA expression from five independent experiments. HUVECs were treated with JQ1, or transfected with shRNA for Brd2, Brd4 or control, or co-transfected with Brd2 shRNA or Brd4 shRNA, and then stimulated with TNF- α (10 ng·mL⁻¹) for 8 h. The results were normalized to GAPDH and expressed as fold change over mean control. Data are representative of five independent experiments (mean \pm SEM; * P < 0.05 vs DMSO treatment (control) or control shRNA, # P < 0.05 vs TNF- α treatment; P values were obtained by one-way ANOVA). (C and D) Western blot analysis of VCAM-1, ICAM-1 and E-selectin in HUVECs treated with different concentrations of JQ1 (C) or transfected with shRNA for Brd2, Brd4 or control (D) after treatment with 10 ng·mL⁻¹ TNF- α for 12 h. Data are a combination of densitometric analyses from five independent experiments (mean \pm SEM; * P < 0.05 vs control or control shRNA, # P < 0.05 vs TNF- α treatment; P values were obtained by one-way ANOVA.) (E) Effect of JQ1 on viability of HUVECs. HUVECs were treated with JQ1 for 48 h. HUVECs' viability was detected by MTT test. Data are a combination from five independent experiments, (mean \pm SEM, one-way ANOVA test). (F) Effect of JQ1 on apoptosis of HUVECs. HUVECs were treated with JQ1 for 48 h. Annexin V labelling was used to determine cell apoptosis by flow cytometry. Data are shown are (upper panel) representative or (lower panel) combined results from five independent experiments (mean \pm SEM, one-way ANOVA test).

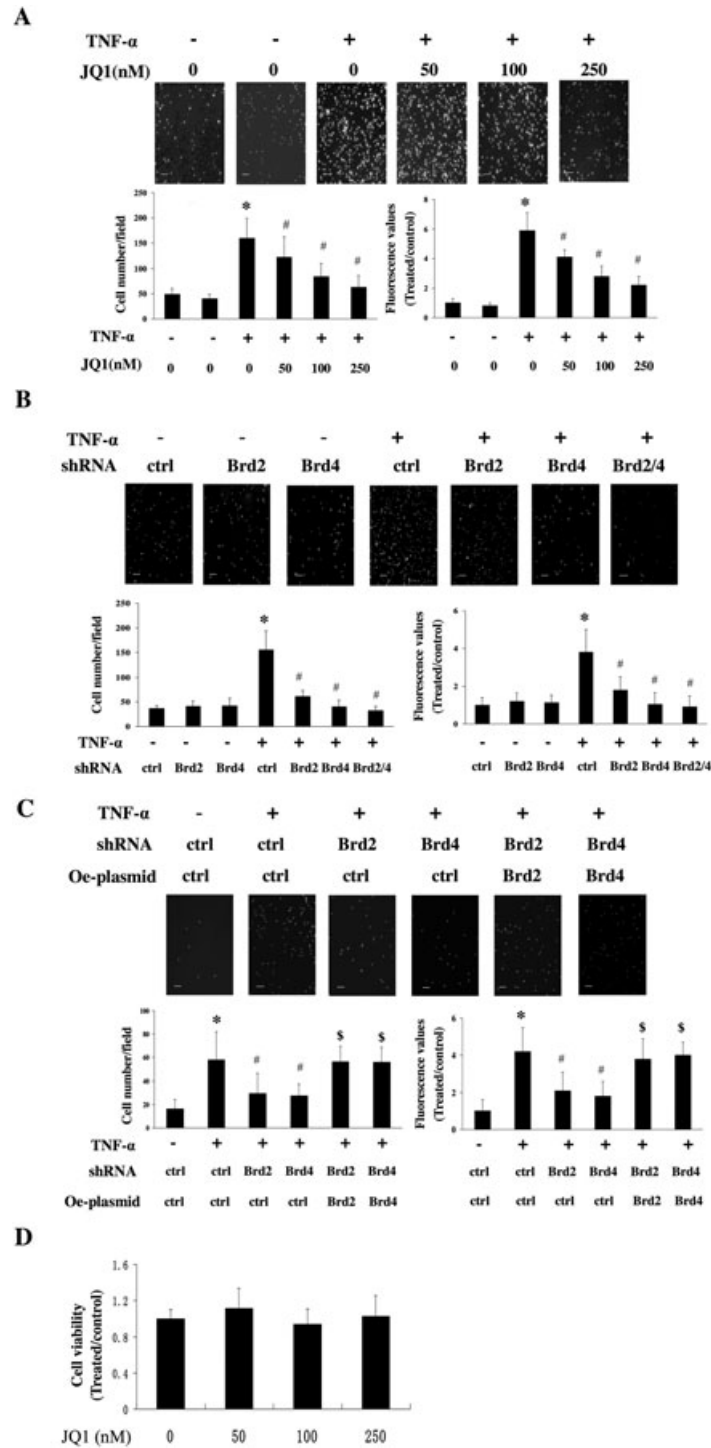
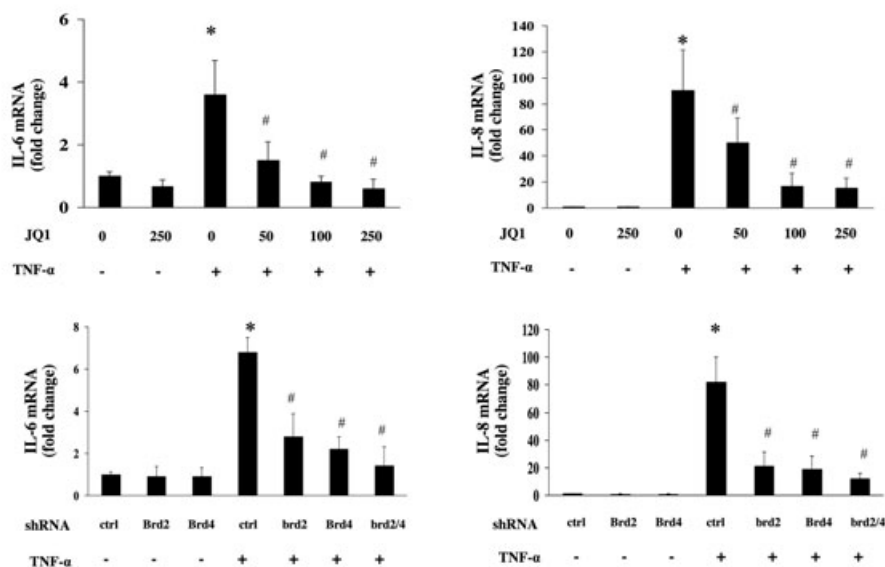


Figure 2

Effect of BET bromodomain inhibition on TNF- α -induced leukocyte adhesion to activated EC monolayers. (A and B) Effect of BET inhibition on the adhesion of PBMCs to TNF- α -activated HUVECs. Upper panels: representative photo images of PBMCs adhering to HUVECs treated with JQ1 (A) or transfected with shRNA for Brd2, Brd4 or control, or co-transfected with shRNA for Brd2 and Brd4 (B) in the presence of TNF- α (10 ng·mL⁻¹). Scale bars: 100 μ m. The lower panels show data for the number and fluorescence values of adhered cells from five independent experiments (mean \pm SEM; * P < 0.05 vs control or control shRNA, # P < 0.05 vs TNF- α treatment; P values were obtained by one way ANOVA.). (C) Effect of overexpression of Brd2 or Brd4 on adhesion of PBMCs to TNF- α -activated HUVECs. Plasmids containing Brd2 or Brd4 (Oe plasmid) were transfected in their previously knocked-down HUVECs, and then the cells were treated with TNF- α (10 ng·mL⁻¹) for 4 h. The data shown are from five independent experiments (mean \pm SEM; * P < 0.05 vs control or control plasmid, # P < 0.05 vs TNF- α treatment; $\S P$ < 0.05 vs Brd2 or Brd4 shRNA, one-way ANOVA test). (D) Effect of JQ1 on viability of PBMCs. PBMCs were treated with JQ1 for 8 h. PBMCs' viability was detected by CCK8 test from five independent experiments (mean \pm SEM, one-way ANOVA test).

A



B

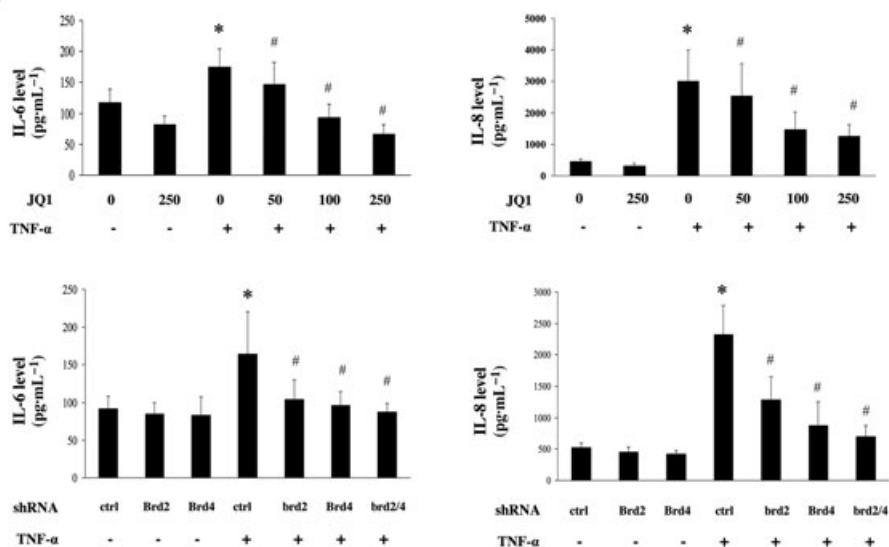


Figure 3

Effects of BET inhibition on the production of pro-inflammatory cytokines in HUVECs. HUVECs were treated with DMSO (as the control), or different concentrations of JQ1 for 8 h or transfected with shRNA for Brd2, Brd4 or control, and then incubated with or without 10 ng·mL⁻¹ TNF-α for 12 h (for IL-6 and IL-8 mRNA) or 24 h (for IL-6 and IL-8 secretion). (A–B) Effect of BET inhibition on the expression of IL-6 and IL-8. Gene expression of IL-6 and IL-8 was determined by quantitative real-time PCR (A). Data were normalized to GAPDH. The levels of the IL-6 and IL-8 in cultured cell supernatants were measured by ELISA (B). Data were obtained from five independent experiments (mean ± SEM; **P* < 0.05 vs control or control shRNA, #*P* < 0.05 vs TNF-α treatment; one-way ANOVA test).

Previous studies have shown that the MAPK pathway is involved in regulating NF-κB activation (Liang *et al.*, 2011; Liu *et al.*, 2012). Therefore, we investigated whether a MAPK inhibitor affected the activity of IKK, a critical upstream protein of NF-κB pathway. As shown in Figure 5C, inhibitors of p38 and JNK reduced the activity of IKK. We also found that both of the inhibitors decreased the TNF-α-induced expression of VCAM-1 ICAM-1 and E-selectin in HUVECs (Figure 5D). Our findings suggest that BET inhibition

prevents the activation of NF-κB in endothelial cells by, at least in part, decreasing upstream MAPK activity.

BET bromodomain inhibition suppresses leukocyte infiltration in vivo

The preceding results showing the effects of BET inhibition on vascular leakiness (Huang *et al.*, 2016), and adhesion prompted us to investigate whether BET proteins play a

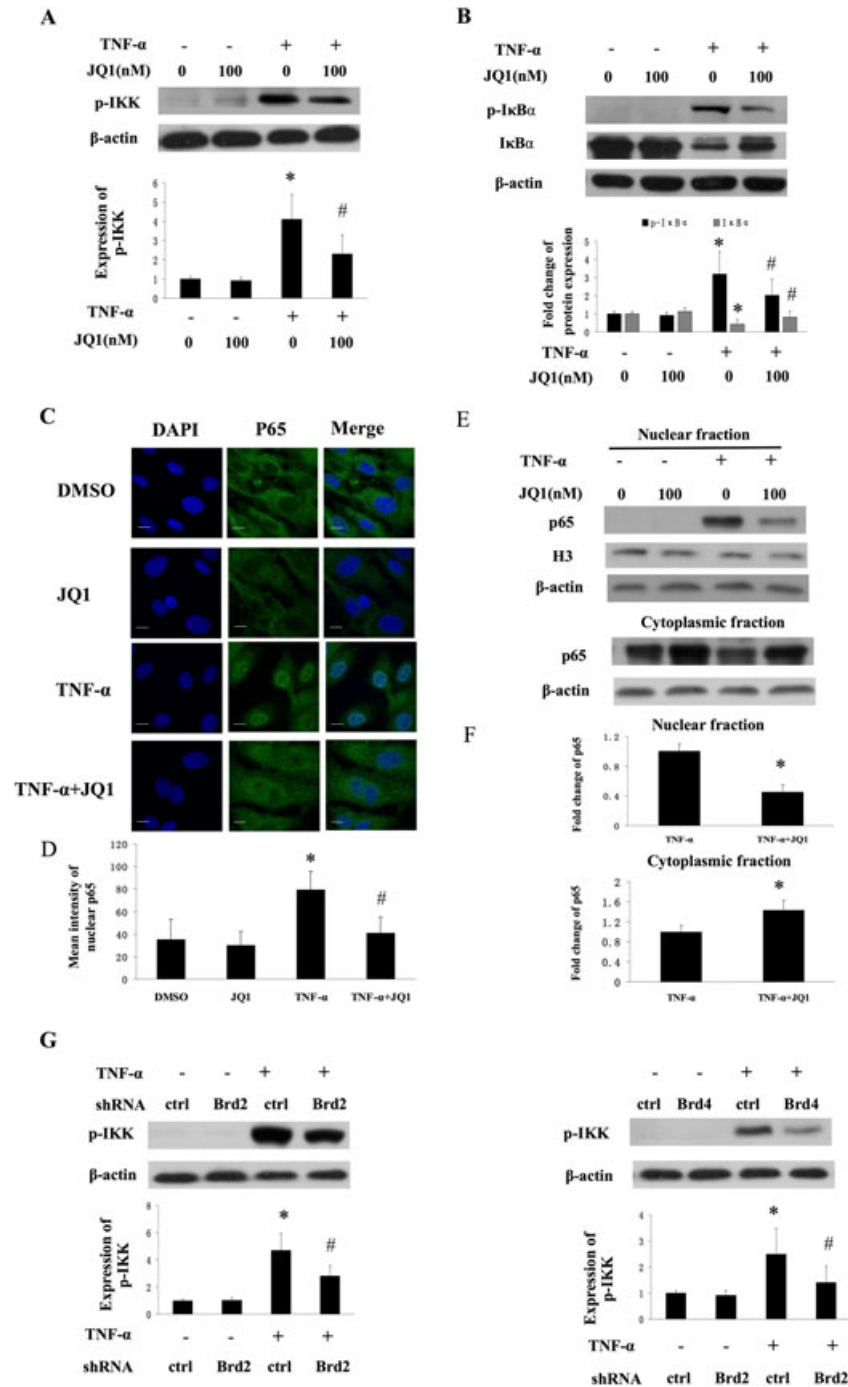


Figure 4

Effect of BET inhibition on TNF- α -induced NF- κ B activation in HUVECs. (A and B) Western blot analysis of phosphorylated IKK or phosphorylated and degraded I κ B α in HUVECs treated with 10 ng·mL⁻¹ TNF- α for 15 min in the presence of DMSO or JQ1. Data represent relative quantities obtained by densitometry (lower panel) from five independent experiments (mean \pm SEM; * P < 0.05 vs DMSO, # P < 0.05 vs TNF- α treatment; one-way ANOVA test). (C and D) Effect of JQ1 on nuclear translocation of p65. HUVECs were treated with TNF- α (10 ng·mL⁻¹) for 30 min in the presence or absence of JQ1. The representative images show immunofluorescence staining analysis of p65 localization (green). Nuclei were stained with DAPI. (D) Mean intensity of nuclear p65 protein from 5 independent experiments (mean \pm SEM; * P < 0.05 vs DMSO, # P < 0.05 vs TNF- α alone; one-way ANOVA test). (E and F) The expression of p65 protein, detected by western blot analysis, in nuclear and cytoplasmic fractions isolated from HUVECs. Results of semiquantitative densitometry of nuclear p65 protein expression are shown in (D), from five independent experiments (mean \pm SEM; * P < 0.05 vs TNF- α alone, Student's *t*-test). (G) Western blot analysis of TNF- α -induced phosphorylation of IKK β in HUVECs transfected with Brd2 shRNA or Brd4 shRNA or control shRNA in the presence or absence of TNF- α stimulation. Results of semiquantitative densitometry of protein expression are shown in the right panel, from five independent experiments (mean \pm SEM; * P < 0.05 vs control shRNA, # P < 0.05 vs TNF- α treatment; one-way ANOVA test).

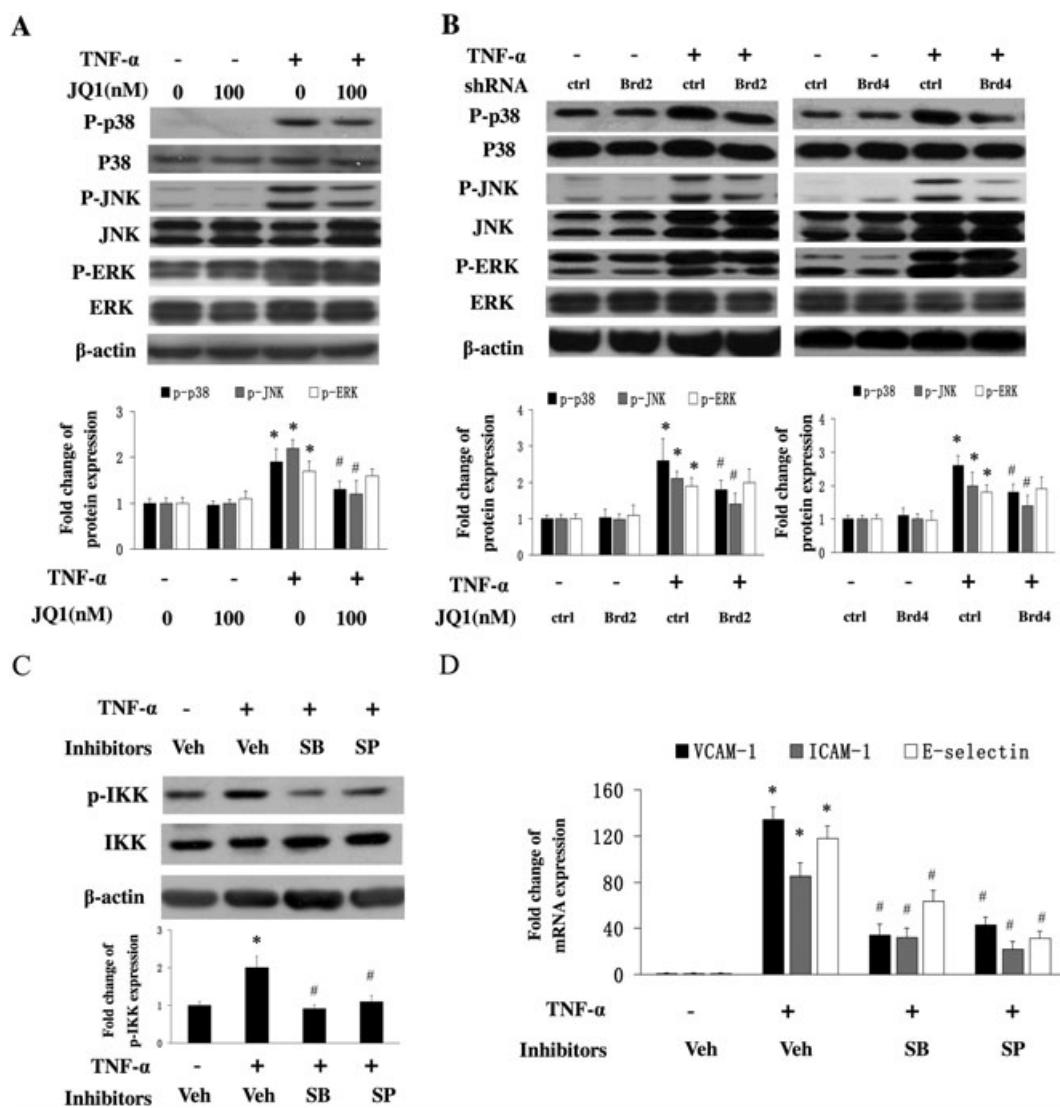


Figure 5

Effect of BET inhibition on the activation of the MAPK signalling pathway in HUVECs. (A and B) HUVECs were pretreated with DMSO or JQ1 for 8 h followed by TNF- α (10 ng·mL⁻¹) for 10 min. The expression of phosphorylated p38, JNK and ERK was measured by western blot analysis. The results of semiquantitative densitometry of protein expression are shown (lower panel) from five independent experiments (mean \pm SEM; **P* < 0.05 vs DMSO or control shRNA, #*P* < 0.05 vs TNF- α alone; one-way ANOVA test). (C) Effects of p38 and JNK inhibitors on the activity of IKK. HUVECs were treated with DMSO (as vehicle, Veh) or p38 inhibitor SB203580 (SB) or JNK inhibitor SP600125 (SP) for 3 h followed by TNF- α (10 ng·mL⁻¹) for 15 min. The results of semiquantitative densitometry of phosphorylated IKK expression are shown in the lower panel from five independent experiments (mean \pm SEM; **P* < 0.05 vs DMSO, #*P* < 0.05 vs TNF- α alone; one-way ANOVA test). (D) Effect of p38 or JNK inhibitor on the expression of adhesion molecules. HUVECs were treated with DMSO or SB203580 or SP600125 for 3 h followed by TNF- α (10 ng·mL⁻¹) for 8 h. Real time qPCR analysis of the expression of adhesion molecules mRNA from 5 independent experiments (mean \pm SD; **P* < 0.05 vs DMSO, #*P* < 0.05 vs TNF- α alone; one-way ANOVA test).

role in leukocyte infiltration through permeable endothelium *in vivo*.

Acute inflammation is an essential host response mediated by the infiltration of circulating leukocytes from the peripheral blood to remove pathogens. We determined the effect of JQ1 administration on the acute inflammatory response in murine lungs challenged with LPS. It has been demonstrated that JQ1 powerfully inhibits BET bromodomain function and displays excellent effects when administered once-daily (Filippakopoulos *et al.*, 2011). Also

no obvious toxicity was observed in adult mice injected with JQ1 50–100 mg⁻¹·kg⁻¹·day⁻¹. Therefore, in the present study, the mice were administered 50 mg⁻¹·kg⁻¹ of JQ1 once a day. LPS treatment resulted in a robust increase in leukocyte accumulation in lungs 5 h after its administration; however, JQ1 administration attenuated this LPS-induced leukocyte accumulation (Figure 6A). The expression of migration inhibitory factor-related protein 14 (MRP14), a marker neutrophils and monocytes, was decreased in mice treated with JQ1 (Figure 6 F and G). The activity of MPO, a peroxidase enzyme

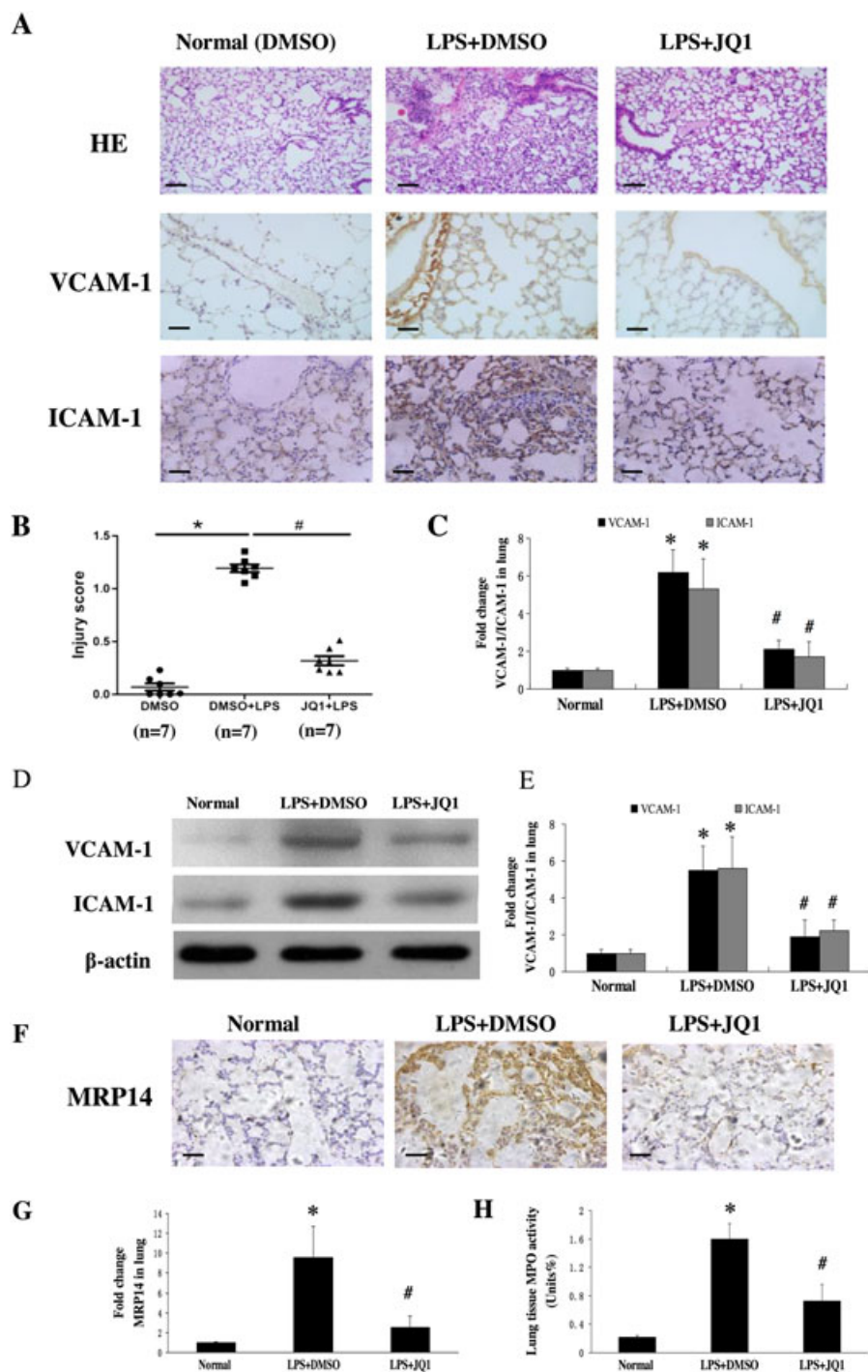


Figure 6

Effect of BET bromodomain inhibitor JQ1 on leukocyte infiltration *in vivo*. (A) Effect of JQ1 on the acute inflammatory response in murine lungs challenged with LPS. Mice were administered DMSO (as control) or JQ1, *i.p.*, for 5 days and then treated with or without LPS ($40 \text{ mg} \cdot \text{kg}^{-1}$, *i.p.*) for 5 h. The lung tissues were collected for staining of H&E (upper panel), VCAM-1 (middle panel) and ICAM1 (lower panel). Scale bars: $50 \mu\text{m}$. (B) LPS-induced lung injury was determined by lung injury scoring ($n = 7$ mice per group). Kruskal–Wallis test was used for the statistical analysis of injury scores ($*P < 0.05$ vs DMSO, $^{\#}P < 0.05$ vs LPS alone). (C) Quantification of VCAM-1 staining in lung tissue. Data represent mean \pm SEM from seven independent experiments ($*P < 0.05$ vs normal, $^{\#}P < 0.05$ vs LPS alone; Kruskal–Wallis test). (D and E) Lung tissues were collected for detection of VCAM-1 and ICAM-1 protein expression by western blot analysis. Results of semiquantitative densitometry of protein expression are shown from seven independent experiments ($*P < 0.05$ vs normal (DMSO), $^{\#}P < 0.05$ vs LPS alone; Kruskal–Wallis test). (F) The lung tissues were collected and stained for MRP14. (G) Quantification of MRP14 staining in lung tissues. Data represent mean \pm SEM, $*P < 0.05$ versus normal, $^{\#}P < 0.05$ versus LPS alone (Kruskal–Wallis test). (H) Lung tissues were collected and evaluated for MPO activity ($n = 7$ mice per group). Values represent mean \pm SEM ($*P < 0.05$ versus normal; $^{\#}P < 0.05$ versus LPS; Kruskal–Wallis test).

abundantly present in neutrophils, was decreased in JQ1-treated animals (Figure 6E). Furthermore, *in vivo* administration of JQ1 decreased the elevated expression of VCAM-1 and ICAM-1 in lung tissue of mice treated with LPS (Figure 6 A and C). In addition, we observed that JQ1 treatment did not affect weight and survival of mice, and no animals died after LPS administration (data not shown). Our findings indicate that BET proteins might play an important role in regulating endotoxin-induced vascular inflammation and leukocyte accumulation.

Discussion

In the present study, we demonstrate that BET bromodomain is involved in endothelial inflammatory responses by regulating the expression of VCAM-1, ICAM-1, E-selectin, IL-6, IL-8 and the adhesion of leukocytes to HUVECs. Furthermore, BET inhibition attenuated IKK-mediated activation of the NF- κ B pathway in TNF α -treated endothelial cells. It was also shown that BET inhibition suppresses the activation of p38 and JNK MAPKs. TNF- α -induced NF- κ B activation was also blocked by inhibitors of p38 or JNK inhibitor. Our findings identify BET bromodomain as a new epigenetic modulator of vascular inflammation.

ECs play a critical role in maintaining the homeostasis of the vascular endothelium, particularly in the modulations of leukocyte trafficking and vascular permeability. The adhesion molecules mediate the attachment of leukocytes to the vascular endothelium and transendothelial migration and are critical early stages in the responses to inflammatory stimuli and associated diseases (Berlin *et al.*, 1995; Kansas, 1996; Ley *et al.*, 2007). Our findings that BET inhibition powerfully suppresses the expression of endothelial adhesion molecules and pro-inflammatory cytokines and the attachment of leukocytes to endothelial cells suggest that BET bromodomain might be involved in regulating the early events of vascular inflammation. Since the induction of VCAM-1, ICAM-1, E-selectin, IL-6 and IL-8 in ECs is mainly modulated by the NF- κ B signalling pathway (Hou *et al.*, 1994; Manning *et al.*, 1995), our results showing that suppression of BET inhibited NF- κ B activation further support the notion that BET bromodomain is essential for controlling vascular inflammation. In line with these findings, recent studies indicate that BET bromodomain suppression modulates the transcription of NF- κ B target genes in macrophages (Nicodeme *et al.*, 2010), renal tubular epithelial cells (Zhang *et al.*, 2012), tumour cells (Wu *et al.*, 2013; Zou *et al.*, 2014) and rheumatoid fibroblast-like synoviocytes (Xiao *et al.*, 2016).

In addition to the NF- κ B pathway, the MAPK pathway is important in regulating the expression of endothelial adhesion molecules (Read *et al.*, 1997; Hoefen and Berk, 2002; Liang *et al.*, 2011). In this study, we found that treatment with the BET inhibitor JQ1 or specific shRNA for Brd2 or Brd4 reduced the TNF α -induced activation of p38 and JNK, but not ERK in HUVECs, suggesting the effects of BET bromodomain on endothelial inflammation are mediated by the activation of p38 and JNK. However, in contrast to our findings, a recent study showed that Brd2 is involved in regulating ERK1/2 phosphorylation but does not affect the activities of JNK and p38MAPK in 3T3-L1 cells (Zang *et al.*, 2013). This

suggests the effects of BET bromodomain on the MAPK pathway are cell type-specific.

We hypothesized initially that BET bromodomain contributes to vascular inflammation owing to its direct regulation of the transcriptional activity of NF- κ B, as found in recent studies in HIV-associated kidney disease (Zhang *et al.*, 2012) and macrophages (Nicodeme *et al.*, 2010). However, in contrast to findings from a recent report (Brown *et al.*, 2014), we demonstrated, unexpectedly, that BET inhibition reduces the phosphorylation of IKK, phosphorylation and degradation of I κ B α and nuclear translocation of p65 in TNF- α -stimulated HUVECs. These data indicate that the nuclear Brd proteins modulate NF- κ B activity by, at least partly, interfering in the early event of cytoplasmic IKK activation. Indeed, recent studies have also shown that BET inhibition reduces IKK activity in rheumatoid fibroblast-like synoviocytes (Xiao *et al.*, 2016) and diffuse large beta cell lymphoma (Ceribelli *et al.*, 2014).

Previous studies have indicated that MAPK pathways are involved in the modulation of NF- κ B activation in TNF- α -stimulated endothelial cells (Liang *et al.*, 2011; Liu *et al.*, 2012; Olejarsz *et al.*, 2014), therefore, in the present study, we determined whether the effects of BET inhibition on activation of the IKK/NF- κ B signal is mediated by MAPK pathways. We found that p38 and JNK inhibitors decreased the activity of IKK and reduced the TNF- α -induced expression of VCAM-1, ICAM-1 and E-selectin in HUVECs. These observations suggest that BET bromodomain regulates IKK-mediated NF- κ B activation through, at least in part, the p38 and JNK MAPK pathways. However, the detailed mechanism(s) of action for the inhibitory effect induced by the suppression of BET on MAPK signalling remains unclear. BET bromodomains localize genome-wide to promoter and enhancer regions (Hargreaves *et al.*, 2009; Chapuy *et al.*, 2013). It is well documented that several extracellular stimuli including inflammatory cytokines can induce the activation of MAPK pathways, which occurs in the cytoplasm of endothelial cells. However, BET proteins are highly enriched in nuclear super enhancers, and JQ1 disrupts the formation of the chromatin complexes, essential for the expression of many genes, by interfering with the binding of BET bromodomain to acetylated histones. This implies that nuclear located BET proteins regulate cytoplasmic activation of the MAPK pathway, most likely, in an indirect manner in TNF- α -stimulated ECs. That is to say, the effect of inhibiting BET on the MAPK pathway may be through a decrease in the transcription of genes encoding signalling proteins that function in the phosphorylation of p38 and JNK or their upstream proteins such as MAPKK and MKK. Another possibility is that BET proteins may mediate 'inside-out' signalling from the nucleus to the cytoplasm to modulate activation of the MAPK pathways. In addition, we cannot eliminate the possibility that BET proteins might shuttle out of the nucleus and interact directly with acetylated signal proteins that control the activation of MAPKs in the cytoplasm of ECs. However, this possibility seems to be unlikely because we did not find that TNF- α -induced the translocation of Brd4 from the nucleus to cytoplasm (data not shown). In the future, it would be interesting to explore further the mechanism by which the nuclear BET bromodomain activates the cytoplasmic MAPK signalling pathway.

In addition, we observed that JQ1 treatment resulted in a slight but not significant reduction in the expression of adhesion molecules and cytokines, and leukocyte adhesion in the absence of TNF- α or LPS; these data indicate that JQ1 does not affect the function of unstimulated ECs. However, the possibility that these observations might be due to the low baseline expression of adhesion molecules and cytokines cannot be ruled out.

Conclusions

We have identified BET bromodomain as a target that regulates endothelial inflammation by modulating the MAPK/NF- κ B signalling pathway. Strategies targeting BET bromodomain might provide a new therapeutic approach for controlling inflammatory-related diseases.

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Author contributions

H.S.X., X.Y.Y., G.Q.C and L.Q.L. conceived and designed the experiments. M.C.H., S.Z., Y.Y.Z and M.H.S. performed most of the biological experiments. M.C.H., Q.Q. and Y.J.X. analysed the data. H.S.X. wrote the main manuscript text. All the authors reviewed the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.13657>

Figure S1 Targeted depletion of Brd2 and Brd4 in HUVECs. HUVECs were transfected with shRNA for Brd2 (shBrd2) or Brd4 (shBrd4) oligonucleotide or control (shC). After 72 h of transfection, cells were lysed, and mRNA (A) and protein (B) expression of Brd2 or Brd4 was assessed by real time qPCR and Western blot analysis respectively. Values for mRNA expression represent mean SEM from 5 independent experiments. * $P < 0.05$ vs control shRNA, Student t-test).

Figure S2 Effect of BET bromodomain inhibition on LPS-induced expression of adhesion molecules and proinflammatory cytokines. A and B, HUVECs were pretreated with JQ1, and then incubated with 10 ng/mL LPS for 8 h. The mRNA expression of adhesion molecules (A) and proinflammatory cytokines (B) were determined by Real time qPCR. Data were obtained from 5 independent experiments (mean SEM; * $P < 0.05$ vs control, # $P < 0.05$ vs TNF- α treatment; one-way ANOVA test).

Figure S3 Effect of BET bromodomain suppression on LPS-induced activation of NF- κ B signal by HUVECs. A, HUVECs were treated with DMSO or JQ1 (100 nM) for 8 h followed by LPS (10 ng/mL) for 15 min. The phosphorylated I κ B kinase (IKK) expression was measured by Western blot analysis.

Semiquantitative densitometry of p-IKK expression is shown in right panel from 5 independent experiments (mean SEM; * $P < 0.05$ vs control, # $P < 0.05$ vs TNF- α treatment; one-way ANOVA test). B and C, Effect of JQ1 on nuclear translocation of p65. HUVECs were treated with 10 ng/mL LPS for 30 min in the presence or absence of JQ1 (100 nM).

The images show immunofluorescence staining analysis of p65 localization (green). Nuclei were stained with 40,6-diamidino-2-phenylindole (DAPI). Panel C shows mean intensity of nuclear p65 protein (mean SEM) from 5 independent experiments, * $P < 0.05$ vs DMSO, # $P < 0.05$ vs LPS (one-way ANOVA test).