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BET Protein Function Is Required for Inflammation: Brd2 Genetic Disruption and BET Inhibitor JQ1 Impair Mouse Macrophage Inflammatory Responses

Anna C. Belkina,*,† Barbara S. Nikolajczyk,† and Gerald V. Denis*

Histone acetylation regulates activation and repression of multiple inflammatory genes known to play critical roles in chronic inflammatory diseases. However, proteins responsible for translating the histone acetylation code into an orchestrated proinflammatory cytokine response remain poorly characterized. Bromodomain and extraterminal (BET) proteins are "readers" of histone acetylation marks, with demonstrated roles in gene transcription, but the ability of BET proteins to coordinate the response of inflammatory cytokine genes through translation of histone marks is unknown. We hypothesize that members of the BET family of dual bromodomain-containing transcriptional regulators directly control inflammatory genes. We examined the genetic model of *brd2 lo* mice, a BET protein hypomorph, to show that Brd2 is essential for proinflammatory cytokine production in macrophages. Studies that use small interfering RNA knockdown and a small-molecule inhibitor of BET protein binding, JQ1, independently demonstrate BET proteins are critical for macrophage inflammatory responses. Furthermore, we show that Brd2 and Brd4 physically associate with the promoters of inflammatory cytokine genes in macrophages. This association is absent in the presence of BET inhibition by JQ1. Finally, we demonstrate that JQ1 ablates cytokine production in vitro and blunts the "cytokine storm" in endotoxemic mice by reducing levels of IL-6 and TNF-α while rescuing mice from LPS-induced death. We propose that targeting BET proteins with small-molecule inhibitors will benefit hyperinflammatory conditions associated with high levels of cytokine production. ***The Journal of Immunology*****, 2013, 190: 3670–3678.**

Regulation of inflammatory gene expression is tightly controlled through chromatin "readers" that specifically bind histone posttranslational modifications and provide a scaffold, which, in addition to sequence-specific transcription factors, is an integral component of the transcriptional activation complex (1). The biological effects of chromatin-dependent, multiprotein complexes include both transcriptional coactivation and corepression of inflammatory genes in differentiated adult cells (2); thus chromatin readers play critical roles in exquisitely tuned inflammatory responses to a variety of immune system stimuli.

Proinflammatory stimuli such as bacterial endotoxin (LPS) arouse extensive transcriptional reprogramming through their ability to activate acetylation of ε-amino groups of nucleosomal histone lysines, a general mark of gene activation (3–5). The acetylated lysine residues are recognized by chromatin readers, many of which contain a conserved structure designated the bromodomain. Bromodomains are highly conserved, left-twisted bundles of four α-helices, with a hydrophobic cleft between two conserved loops that connect the helices (6). The motif uses hydrogen bonding, often at asparagine residues, to bind to acetylated histones (7). In humans, there are at least 40 bromodomain proteins (8, 9), which include histone acetyltransferases, helicases, scaffolding proteins, and other cofactors that control gene transcription. These findings raise the possibility that bromodomain proteins regulate acetylated, histone-packaged inflammatory genes through multiple downstream mechanisms to significantly contribute to outcomes from proinflammatory stimuli.

The bromodomain and extraterminal domain (BET) family is a distinct group of bromodomain proteins that in mammals includes Brd2, Brd3, and Brd4, all of which are ubiquitously expressed in mammalian tissues (10–13). Brd2 and Brd4 have been extensively studied in the context of cell-cycle control (14–18) and transcription elongation (19–21), but potential roles in inflammatory responses have not been explored well. Establishing links between BET proteins and inflammation has become clinically critical owing in part to recent drug development efforts, which have shown that drugs able to interrupt interactions between Brd4 and thienodiazepines (22) have efficacy in BET protein–related cancers (23, 24). JQ1 was the first drug developed that specifically interacts with the hydrophobic pocket of the BET bromodomain to block interaction between multiple BET proteins (Brd2/3/4) and acetylated histones (9). JQ1 effectively prompted squamous differentiation and reduced tumor volume of Brd4-dependent, human NUT midline carcinoma xenografts in mice (9), and was proved efficient to block growth of various leukemic cells (25–27).

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Abbreviations used in this article: BET, bromodomain and extraterminal domain; BM, bone marrow; BMDM, bone marrow–derived macrophage; ChIP, chromatin immunoprecipitation; eGFP, enhanced GFP; IR, insulin resistance; KC, keratinocyte chemoattractant; qRT-PCR, quantitative RT-PCR; SAA, serum amyloid A; shRNA, short hairpin RNA; siRNA, small interfering RNA; WT, wild-type.

However, other possible applications of JQ1, including those targeting BET protein functions in nonmalignant cells, are untested (reviewed in Ref. 28). Establishing the importance of BET proteins in inflammation is a first critical step toward evaluating the possibility that JQ1 may be exploited as a next-generation anti-inflammatory treatment.

Considering the involvement of BET proteins in control of the most fundamental cell growth and proliferation processes, it is not surprising that multiple attempts to create mouse strains of BET gene knockouts have not succeeded. Both Brd2 and Brd4 knockouts have early lethality phenotypes (13, 29–31). We have generated a mouse model with gene disruption of *Brd2* that exhibits a hypomorph phenotype with expression of about half the wild-type (WT) level of Brd2 in all tissues tested. These "*brd2 lo*" hypomorphs develop a complex whole-body phenotype, the key feature of which is severe obesity without insulin resistance (IR) (13). Given that inflammation from the macrophage compartment is critical for obesity-associated IR (32, 33), these data predicted that appropriate levels of BET protein expression in macrophages may be a required component of inflammation in obesity, among other inflammatory diseases (reviewed in Ref. 33).

To establish rigorously the link between BET protein function and inflammation, we investigated the inflammatory response of macrophages derived from *brd2 lo* mice in detail and showed that low Brd2 levels severely blunt proinflammatory cytokine production. Complementary studies that test BET protein knockdown and the BET inhibitor JQ1 in vivo demonstrate that BET proteins play important roles in acute inflammatory responses. Finally, we show that the functions of BET proteins in inflammation are regulated by direct contact with the promoter chromatin of a select subset of cytokine genes. Taken together, these studies establish a role for BET proteins in mouse macrophage stimulation and justify further testing of BET protein–targeting drugs in chronic inflammatory diseases.

Materials and Methods

Mice

C57BL/6J (The Jackson Laboratory) and BALB/cJ (Charles River) males, 6–8 wk of age, were maintained in our specific pathogen–free animal facility according to institutional guidelines, with protocols approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine. The *brd2 lo* mice were generated as previously reported (13). NF-κB^{EGFP} mice (34) were obtained from Christian Jobin (University of North Carolina, Chapel Hill, NC). In these mice, a single copy of enhanced GFP (eGFP) reporter was placed under control of *cis*-NF-κB^{EGFP} and was inserted 5′ of the X-linked hypoxanthine phosphoribosyltransferase locus. Male hemizygous mice were used for the experiments. Sacrifice was by isoflurane narcosis, followed by cervical dislocation in accordance with recommendations of the American Veterinary Association.

Reagents

DMEM plus glutamine and 25 mM glucose was from Mediatech. Penicillin, streptomycin, and Fungizone were purchased from Life Technologies. LPS from *E. coli* clone 0111:B4 was purchased from Sigma-Aldrich. JQ1(+) and JQ1(-) were a generous gift from James Bradner (Dana-Farber Cancer Institute, Boston, MA). For in vitro assays, we prepared 10 mM stock solutions of JQ1 in DMSO that were diluted in PBS before addition to cell culture medium. For in vivo studies, 50 mg/ml JQ1 stock in DMSO was diluted 1:10 with 10% (w/v) solution of 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) immediately prior to administration.

Cell isolation from spleen and bone marrow for flow cytometry

Splenocytes were isolated from freshly harvested, minced spleens of *brd2 lo* mice or littermate controls by gently pressing the spleens through a 70-μm cell strainer (BD Biosciences), followed by treatment with 1× RBC lysis buffer (eBioscience). Bone marrow (BM) cells were isolated as described below. Cells were counted and stained with directly conjugated monoclonal Abs against CD3, CD4, CD8, B220, CD11b, and Gr-1 in the presence of anti-mouse CD16/CD32 from eBioscience. All stains were performed along with isotype controls. Flow cytometry was performed on an LSRII cytometer (BD), and data were analyzed from 50,000 to 100,000 gated events using FlowJo 8.7 (TreeStar).

Isolation, culture, and stimulation of BM-derived macrophages

Femora, tibiae, and sterna were isolated under sterile conditions. Bones were crushed in a sterile glass Dounce homogenizer to liberate BM cells into a suspension of RPMI 1640 medium buffered with 20 mM HEPES, pH 7.4, and supplemented with 10% FBS, penicillin, streptomycin, Fungizone, and 50 μM 2-ME. Bone fragments were removed by sterile filtration with 70-μm cell strainers (BD), and erythrocytes were lysed as above. Nucleated BM cells were recovered and plated overnight in 75-ml flasks to allow resident macrophages to attach, and then suspension cells were plated in DMEM supplemented as above. To initiate differentiation, the medium was supplemented with 50 ng/ml recombinant M-CSF (eBioscience) for 5–7 d. Equal numbers of macrophages were seeded into 6-, 12-, or 24-well plates prior to stimulation with *E. coli* LPS 0111:B4 (50 ng/ml; Sigma-Aldrich). IFN-γ priming was performed with 100 U/ml IFN-γ (eBioscience). At indicated time points, cell-free supernatants were removed for multiplex protein/cytokine analysis, and/or RNA was isolated from adherent cells.

NF-κB luciferase reporter assay

RAW264.7 cells were transfected using Lipofectamine 2000 (Invitrogen) and components of the Cignal NFκB Reporter (luc) Kit (SA Biosciences) plus either pSiBrd2 (13) or control vectors. Cells were harvested 48 h after transfection, and luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega) on a TD-20/20 Luminometer (Turner Biosystems).

Protein assays

The supernatants from bone marrow–derived macrophage (BMDM) or RAW264.7 cultures, or serum, were collected and frozen in aliquots. Cytokines were determined using Singleplex reagent kits (Invitrogen/Life Sciences) according to the manufacturer's instructions and a Bio-Plex 200 (Bio-Rad) reader. Serum was diluted with Singleplex Assay Diluent buffer prior to analysis or was analyzed for serum amyloid A (SAA) by ELISA (Immunology Consultants Laboratory). For TNF-α intracellular staining, we used a Fixation and Permeabilization Buffer Set (eBioscience). RAW264.7 cells were collected from the plates in trypsin-free ice-cold PBS supplemented with 2 mM EDTA. Cells were washed, fixed, and permeabilized according to the manufacturer's protocol, then stained with allophycocyanin–anti–TNF-α (eBioscience). Labeled cells were analyzed by flow cytometry, as described above.

Small interfering RNA knockdown in BMDMs

For Brd2, Brd3, and Brd4 knockdown, 20 nM Dharmacon ON-TARGETplus SMARTpool small interfering RNA (siRNA) with DharmaFECT formulation 4 reagent (Dharmacon) was used. Cells were used for experiments 48 h post transfection. Knockdown was validated by quantitative RT-PCR (qRT-PCR).

qRT-PCR

RNA from macrophages and RAW264.7 cells was isolated and quantified as published using the 7500 Fast Real-Time PCR System (Applied Biosystems), Power SYBR Green PCR Master Mix (Applied Biosystems), and Quantitech primers for mouse Brd2, Brd3, Brd4, TNF-α, IL-6, MCP-1, eGFP, and GAPDH (Qiagen). For whole blood, 50–100 μl mouse blood was obtained from the tail vein, and RNA was purified with the Mouse RiboPure-Blood RNA Kit (Ambion). For 28S rRNA amplification, the following primers were used: 5′-GCGAAATACCGGCACGAGACCGATAG-3′ and 5′-GGTTTCACGCCCTCTTGAACTCTCTC-3′. Melt curve analysis indicated formation of a single product in all cases. Relative mRNA expression levels were determined using ΔCt values and were normalized to GAPDH or 28S rRNA levels, as indicated.

Chromatin immunoprecipitation

One hour after LPS stimulation and/or JQ1 treatment, BMDMs were fixed in 1% formaldehyde at 37°C for 10 min, then subjected to chromatin immunoprecipitation (ChIP) as previously published (35). Chromatin was precipitated with 2 μg α-acetylated histone H3 (Upstate), α-Brd2 (Bethyl

Labs), α-Brd4 (Bethyl Labs), or α-GST (Upstate). Then 2 ng of each sample was analyzed in duplicate or triplicate by qPCR. The fold difference was calculated as 2^[Ct(input) − Ct(ChIP)], and fold enrichment over an unrelated Ab (α-GST) was assessed. Oligonucleotides were as follows: IL-6: 5′-TGTGGGATTTTCCCATGAGT-3′ and 5′-TGCCTTCACTTACTTGCAGAGA-3′; TNF-α: 5′-AGCGAGGACAGCAAGGGA-3′ and 5′-TCTTTTCTGGAGGGAGTGTGG-3′.

LPS-induced shock in mice

Weight-matched 8-wk-old male mice were injected i.p. with 50 mg/kg JQ1 (+) or JQ1(-). Two hours later, 20 mg/kg *E. coli* LPS was injected into the contralateral side of the abdomen. For survival experiments, mice were monitored for mortality. Serum cytokines were measured in tail vein sera just prior to LPS injection, then 20, 40, 60, 90, 120, and 240 min after LPS injection.

Statistical analysis

For comparison of treatment group differences, we used the unpaired, two-tailed Student *t* test or one-factor ANOVA in conjunction with the Dunnett or Tukey–Kramer multiple comparisons tests. Error bars indicate SEs of the mean (SEM), with significant differences ($p < 0.05$) indicated. Analyses were performed in Graphpad Prism 5, or, for survival curves, Kaplan–Meier analysis was performed with SigmaStat 3.1 software.

Results

Decreased Brd2 levels correspond to decreased macrophage inflammatory responses

Our previous demonstration that *brd2 lo* mice are protected from obesity-induced inflammation (13) predicted that Brd2 regulates inflammation in response to a broad array of proinflammatory stimuli. To begin testing this prediction, we first asked whether Brd2 hypomorphs (confirmed in Fig. 1A) resist obesity-associated inflammation owing to constitutive changes in numbers and/or ratios of immune cells, a possibility consistent with our demonstration that Brd2 short hairpin RNA (shRNA) expression in hematopoietic stem cells decreases their contribution to total BM (A.C. Belkina, W.P. Blanton, and G.V. Denis, manuscript in preparation). Table I shows similar percentages of B cells, T cells, and macrophages in spleen and BM of *brd2 lo* and WT mice. Absolute numbers of splenocytes and BM cells from *brd2 lo* were also similar to those in WT (not shown). To test the alternative possibility that Brd2 regulates proinflammatory responses rather than in vivo cell distribution, we stimulated BMDMs from *brd2 lo* mice with LPS and quantified production of inflammatory cytokines. The *brd2 lo* BMDMs produced lower levels of proinflammatory TNF-α, IL-1β, IL-6, and MCP-1/CCL2, compared with WT macrophages (Fig. 1B). Importantly, the number of $CD11b^+$ BMDMs differentiated from BM of *brd2 lo* mice was similar to that from BM of WT mice, consistent with lower cytokine production by *brd2 lo* macrophages on a per cell basis (data not shown), and in agreement with a lack of developmental defects, as suggested by the fresh ex vivo immunophenotyping data (Table I). We conclude that *brd2 lo* macrophages have a cell-intrinsic impairment in proinflammatory responses.

BET knockdown ablates cytokine expression in BMDMs

To verify independently a role for Brd2 in primary macrophage inflammation, absent any remaining concern about differentiation defects in *brd2 lo* cells, we tested the effect of Brd2 knockdown on cytokine responses of WT BMDMs. We transfected WT BMDMs with Brd2-specific siRNA and confirmed gene knockdown by qPCR 48 h post transfection. Of note, downregulation of any single BET protein did not alter the levels of expression of the other two members of the BET family (Supplemental Fig. 1). We then stimulated knockdown BMDMs with LPS and quantified inflammatory cytokine mRNA by qRT-PCR. TNF-α, IL-6, and

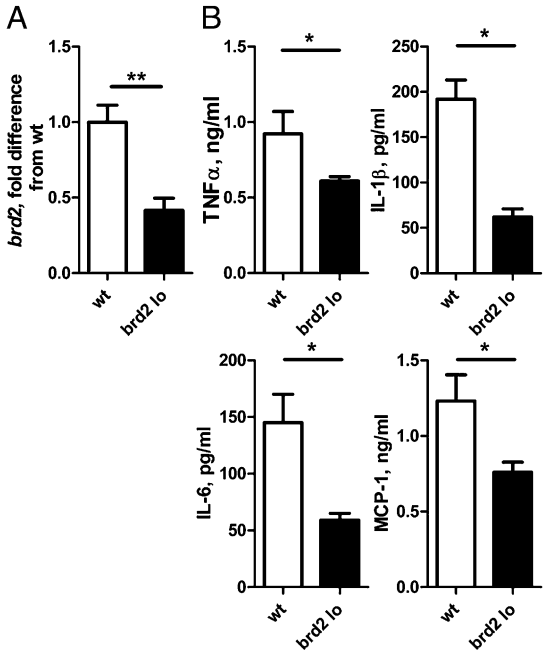

FIGURE 1. *brd2 lo* macrophages demonstrate a diminished inflammatory response. (**A**) Brd2 mRNA levels in leukocytes from WT (white bar) compared with *brd2 lo* (black bar) mice, as measured by qRT-PCR. Relative mRNA expression was normalized to GAPDH. $n = 4$. $^{**}p < 0.01$ as calculated by Student *t* test. (**B**) Production of proinflammatory cytokines by *brd2 lo* and WT BMDMs stimulated for 24 h with 50 ng/ml *E. coli* LPS. All bars in all figures show mean values and SEM. $n = 4$. $^{*}p < 0.05$ as calculated by Student *t* test.

MCP-1 mRNA levels were significantly lower in cells transfected with the siBrd2 pool compared with the nontargeting siRNA pool control (Fig. 2A). Taken together with decreased inflammatory cytokine production by *brd2 lo* macrophages, these data indicate that Brd2 promotes proinflammatory cytokine production in macrophages. Functional significance of BET protein knockdown was evidenced by decreased TNF-α, IL-6, and MCP-1 protein levels in Brd2, Brd3, and Brd4 knockdown macrophages (Fig. 2B). Interestingly, the effects of BET protein ablation were somewhat selective, as evidenced by failure of BET knockdown to affect protein levels of keratinocyte chemoattractant (KC), a mouse ortholog of IL-8 and a potent proinflammatory chemokine (36) (Fig. 2B). We conclude that Brd2, Brd3, and Brd4 have nonredundant and selective roles in the macrophage inflammatory response through their ability to regulate cytokine production.

Table I. Major immune cell subsets in *brd2 lo* mice spleen and BM

	WT	*brd2 lo*
Spleen		
$B220^+$	52.7 ± 2.1	50.4 ± 1.1
$CD3^+$	31.1 ± 1.9	29.9 ± 0.9
$CD3^+CD4^+CD8^-$ (of $CD3^+$ gate)	44.7 ± 2.2	40.8 ± 3.3
$CD3^+CD4^-CD8^+$ (of $CD3^+$ gate)	26.5 ± 1.4	28.7 ± 2.4
$CD11b^+$	9.3 ± 0.9	8.8 ± 1.4
$CD11b^+$ low (of $CD11b^+$ gate)	77.6 ± 2.5	80.6 ± 3.1
$CD11b^+$ high (of $CD11b^+$ gate)	6.7 ± 3.6	4.7 ± 0.6
BM		
$B220^+$	23.6 ± 4.9	22.0 ± 2.4
$CD11b^+$	29.1 ± 2.5	29.9 ± 2.8
$Gr\text{-}1^+$	33.3 ± 3.6	33.7 ± 1.5

Major immune cell subsets in spleen and BM of *brd2 lo* mice, compared with WT. Shown are mean percentages of the live cell gate or parent gate ± SEM. $n = 4$.

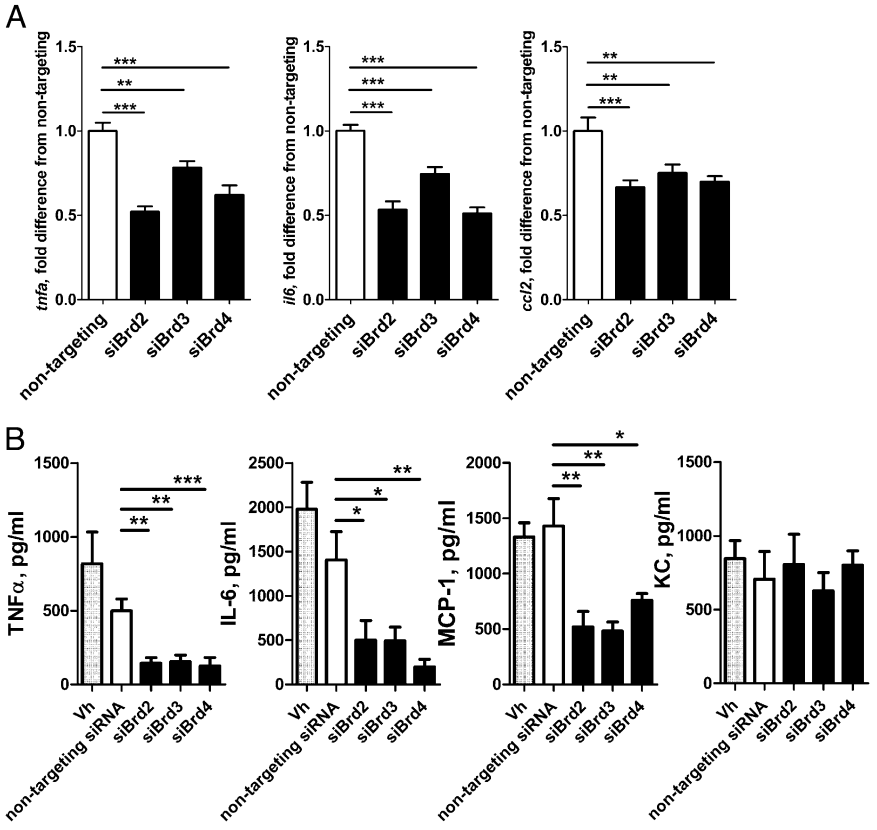

FIGURE 2. In vitro knockdown of BET protein genes reduces the inflammatory response. (**A**) WT BMDMs were transfected with ON-TARGETplus siRNA pools specific for knockdown of Brd2, Brd3, or Brd4, or nontargeting control pools, then stimulated with 50 ng/ml *E. coli* LPS for 2 h before quantitation of the indicated cytokine mRNAs by qRT-PCR. Relative mRNA expression was normalized to GAPDH. $n = 5$. $^{**}p < 0.01$, $^{***}p < 0.001$ as calculated by ANOVA with Dunnett multiple comparison. (**B**) Cytokine protein concentrations in supernatants from LPS-stimulated (24 h, 50 ng/ml) BMDMs after individual BET knockdown. $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as calculated by ANOVA with Dunnett multiple comparison.

Brd2 regulates NF-κB activity

Given that all of the cytokines affected by decreased Brd2 levels are targets of NF-κB, a master proinflammatory transcription factor, we tested the possibility that Brd2 regulates cytokine production by altering NF-κB activity. We modeled *brd2 lo* macrophages in vitro with siRNA-mediated Brd2 knockdown in a RAW264.7 macrophage cell line. We then transiently transfected Brd2 knockdown macrophages with an NF-κB luciferase reporter gene and stimulated cells with LPS. NF-κB reporter activity was significantly decreased in Brd2 knockdown cells (Fig. 3), supporting the conclusion that Brd2 regulates proinflammatory cytokines through its ability to support NF-κB–regulated transcription.

BET inhibitor JQ1 ablates inflammatory responses in LPS-stimulated BMDMs

Given that downregulation of multiple individual BET proteins is anti-inflammatory (Fig. 2), we tested the efficacy of JQ1, a broad-spectrum BET protein inhibitor, as an anti-inflammatory therapeutic drug that could counter macrophage-mediated inflammation. We exposed RAW264.7 macrophage-like cells to LPS and concomitantly treated them with either JQ1(+), the biologically active enantiomer, or JQ1(−), the inactive enantiomer. Levels of secreted TNF-α and MCP-1 were dramatically lower in JQ1 (+)-treated RAW 264.7 macrophages than in JQ1(−)-treated macrophages (Fig. 4A; >50% and >80% decrease, respectively). Furthermore, IL-6 production was 90% lower in the presence of JQ1(+) (Fig. 4A). Protein data were consistent with mRNA analyses. For example, TNF-α mRNA was reduced to < 25% of the levels produced by cells treated with JQ1(−) (Supplemental Fig. 2A). Intracellular TNF-α stain independently confirmed lower protein levels in JQ1(+)-treated cells (Supplemental Fig. 2B). Quantitatively similar dose-dependent decreases in TNF-α, IL-6, and MCP-1 were produced by JQ1(+)-treated primary BMDMs (Fig. 4B), independently confirming that the BET protein inhibitor JQ1 had relatively broad anti-inflammatory effects on macrophages. KC was produced at similar concentrations by LPS-stimulated BMDMs in the presence of JQ1(+) and JQ1(-) (Fig. 4B). This observation is in concordance with the demonstration that KC levels are unaffected by knockdown of BET proteins with siRNA (Fig. 2B) and may reflect the fact that LPS-induced elevation of KC transcription depends on the TLR4-AP1 axis rather than NF-κB (37).

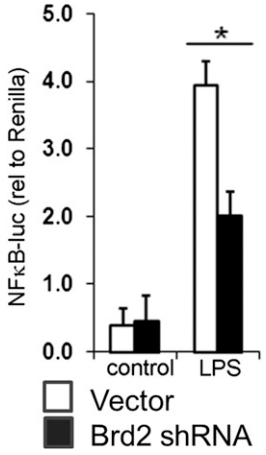

FIGURE 3. In vitro knockdown of Brd2 decreases NF-κB luciferase activity. RAW264.7 cells were transfected with Brd2-specific shRNA (black bars) or empty vector (white bars) plus NF-κB luciferase reporter plasmid. At 48 h after transfection, cells were stimulated with *E. coli* LPS for 2 h. NF-κB–driven luciferase activity is shown (mean and SEM). $n = 3$. $^{*}p < 0.05$ as calculated by Student *t* test comparing empty vector and Brd2 shRNA-containing plasmids.

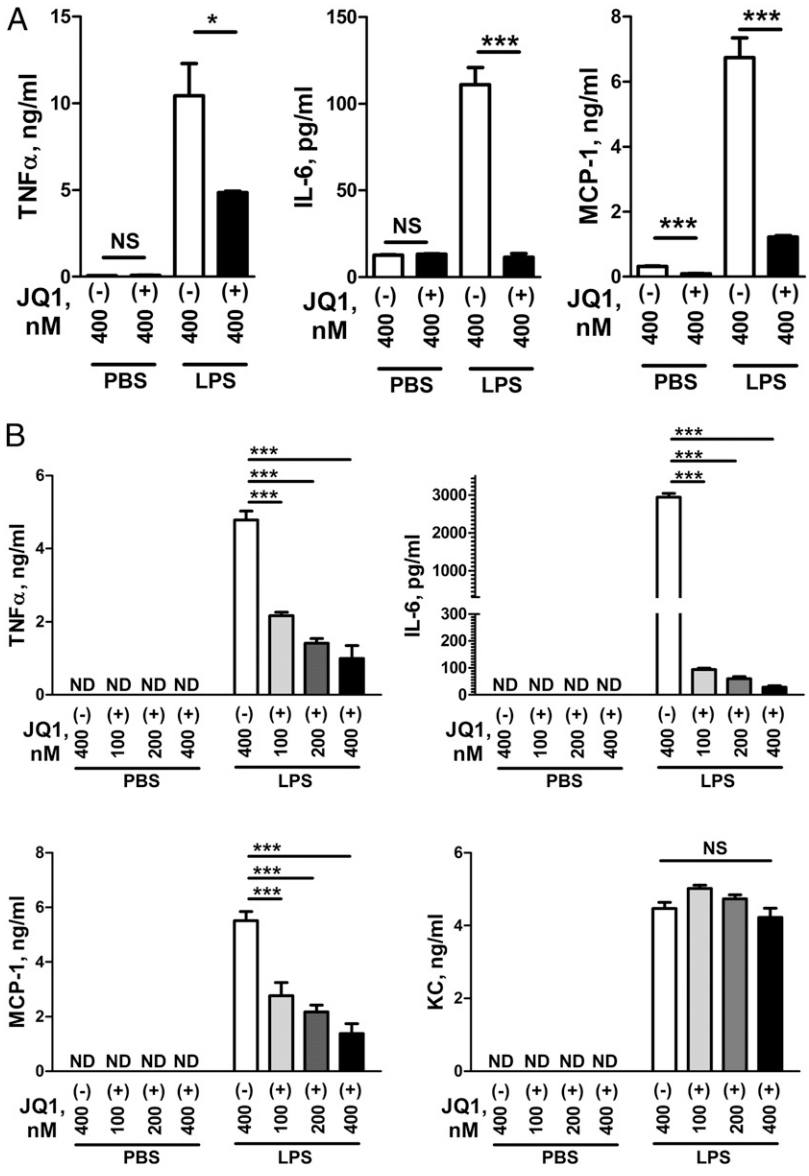

FIGURE 4. The BET family bromodomain inhibitor JQ1 reduces cytokine production in vitro. (**A**) RAW 264.7 cells were exposed simultaneously to 50 ng/ml *E. coli* LPS plus 400 nM JQ1(+) or JQ1(−) for 24 h. Mean value and SEM for three determinations are shown. JQ1 enantiomers are abbreviated by (+) or (−), respectively. $*p < 0.05$, $***p < 0.001$ as calculated by Student *t* test comparing JQ1(+)- and JQ1(−)-treated samples. The chemokine KC was not detectable in RAW 264.1 macrophages under these conditions. (**B**) BMDMs were exposed simultaneously to 50 ng/ml *E. coli* LPS or PBS and different concentrations of JQ1(+) or JQ1(−) for 24 h. Supernatant cytokines were quantified in multiplex protein assays. $n = 6$. $***p < 0.001$ as calculated by ANOVA with Dunnett multiple comparison. ND, Not detected.

Anti-inflammatory effect of JQ1 is not strain specific and cannot be overcome by IFN-γ priming

Genetic background is known to influence the patterns of innate immune responses in mice. In particular, C57BL/6 mice are often referred to as an "M1" strain, whereas BALB/c mice tend to have an "M2" type of response (38, 39). To test whether the anti-inflammatory effects of JQ1 are influenced by genetic background, we exposed primary macrophages derived from C57BL/6 or BALB/c BM to JQ1(+) and stimulated the cells with LPS. Overall, in BALB/c BMDMs the effect of JQ1(+) was very similar to what we observed in C57BL/6, and included blockade of TNF-α, IL-6, and MCP-1 (but not KC; Fig. 5A). Furthermore, IL-10, produced at detectable levels by macrophages from BALB/c mice, but not C57BL/6 mice, was inhibited by JQ1(+) treatment (Fig. 5A). These data indicate that the anti-inflammatory effect of JQ1 (+) is independent of IL-10 upregulation. Overall, we conclude the anti-inflammatory effect of JQ1(+) is not genotype specific.

IFN-γ priming is a first event in the classical cascade of macrophage activation. The effect of the subsequent trigger stimulus, like LPS, bacterial DNA, or TNF-α, is vastly amplified and expanded by IFN-γ priming (40, 41). To test whether IFN-γ priming overcomes the effect of JQ1(+) on macrophages, we exposed cells to IFN-γ prior to LPS stimulation. As previously reported, the effect of IFN-γ priming on cytokine production is modest in this model (40). We found that pretreatment with IFN-γ does not prevent the inhibition of cytokine production by subsequent exposure to JQ1(+) and LPS. Interestingly, addition of JQ1(+) simultaneously with IFN-γ significantly potentiated inhibition of TNF-α, IL-6, and MCP-1 production in BALB/c macrophages, (Fig. 5A) suggesting that BET proteins may also be effectors of IFN-γ signaling. As expected from previous results, KC production was not robustly inhibited.

JQ1 treatment disrupts association of Brd2 and Brd4 with the regulatory chromatin of proinflammatory cytokine promoters

We have demonstrated that Brd2 regulates NF-κB activity outside the context of naturally assembled chromatin (Fig. 3). To assess the effect of blocking BET protein function on NF-κB activity within chromosomal DNA, we generated BMDMs from male NF-κB^{EGFP} knockin mice (34) and stimulated cells with LPS in the presence of JQ1(+) or JQ1(−). Like other myeloid immune cells originating from these mice (34), the BMDMs demonstrated high

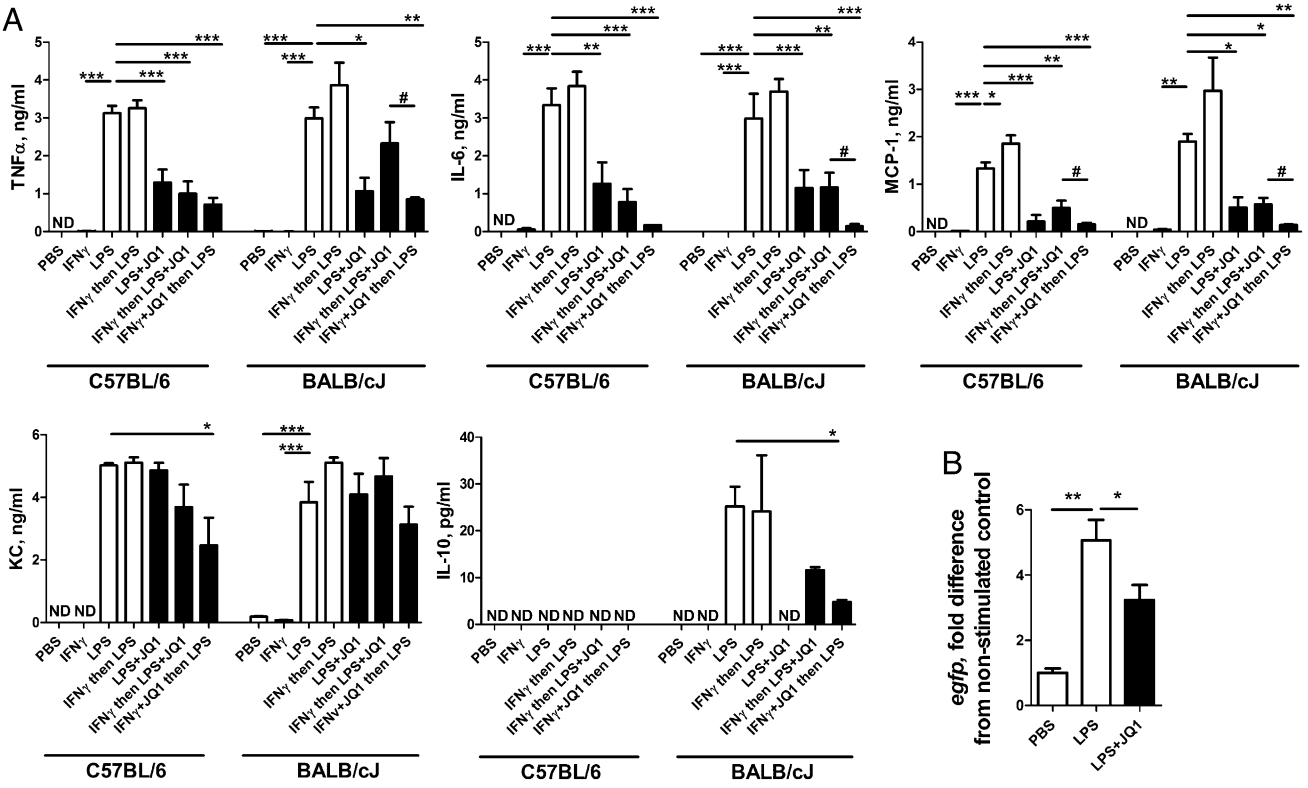

FIGURE 5. JQ1 effect on cytokine production is strain independent and is not overcome by IFN-γ priming of macrophages. (**A**) Open bars show C57BL/6 and BALB/c BMDMs exposed to PBS, 100 U/ml IFN-γ; 50 ng/ml *E. coli* LPS; 50 ng/ml *E. coli* LPS with 400 nM JQ1(+); or primed with 100 U/ml IFN-γ and stimulated with LPS 4 h later, as indicated below graph. Black bars show BMDMs primed with IFN-γ or PBS and stimulated with LPS 4 h later, with JQ1(+) being added either simultaneously with LPS (4 h after priming with IFN-γ) or simultaneously with IFN-γ. Cytokine production was measured 24 h after LPS stimulation. Supernatant cytokines were quantified in multiplex protein assays. $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as calculated by ANOVA with Dunnett multiple comparison. $^{\#}p < 0.05$ as calculated by Student t test. (**B**) eGFP NF-κB BMDMs were stimulated with PBS, 50 ng/ml LPS or LPS + JQ1(+), as indicated. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ as calculated by ANOVA with Dunnett multiple comparison. ND, Not detected.

background eGFP fluorescence even when not stimulated with LPS, probably mirroring the basal NF-κB activation. We therefore quantified the LPS response by eGFP mRNA. eGFP mRNA increased ~ 5-fold in response to LPS stimulation, and was significantly reduced when macrophages were simultaneously exposed to LPS and JQ1(+) (Fig. 5B).

Findings thus far agree with the current understanding that BET proteins regulate acetylated histone–packaged genes through physical association with modified histones, the molecular target of JQ1(+) (9, 42). Because active cytokine promoters are packaged by acetylated histone proteins, we reasoned that JQ1(+) functions as an anti-inflammatory compound by altering BET association with acetylated histone–packaged cytokine promoters (4, 5). To test this possibility, we quantified BET protein/cytokine promoter interactions by ChIP. For these studies, we used anti-Brd2 or anti-Brd4 Abs to precipitate sheared chromosomal DNA from BMDMs stimulated with LPS in the presence of either JQ1(+) or JQ1(−). Both Brd2 and Brd4 modestly bound the IL-6 promoter in nonstimulated cells (Fig. 6A); however, when cells were activated with LPS, we observed significant amounts of IL-6 promoter DNA precipitated with anti-Brd2 and anti-Brd4 Abs (Fig. 6A). Importantly, JQ1(+), but not JQ1(−), treatment prevented association of both Brd2 and Brd4 with the IL-6 promoter. An alternative, but functionally equivalent, interpretation is that JQ1(+) promoted dissociation of BET proteins from the IL-6 promoter. These data show that the LPS-inducible association of BET proteins with the IL-6 promoter is significantly decreased in the presence of JQ1(+), which explains the reduced production of proinflammatory cytokines in macrophages.

To measure the effect of JQ1(+) on BET protein/promoter interactions more universally, we reamplified ChIP samples from Fig. 6A to quantify Brd2 and Brd4 association with the TNF-α promoter. As expected, LPS induced Brd2 association with the TNF-α promoter in the absence of JQ1(+) (Fig. 6B, *left panel*). Furthermore, Brd2 was not associated with the TNF-α promoter in LPS/JQ1(+)-treated cells. Interestingly, we did not find significant Brd4/TNF-α promoter association in BMDMs under any of the conditions tested (Fig. 6B, *right panel*). This negative result may reflect bona fide differences between the molecular mechanisms that regulate IL-6 and TNF-α. Alternatively, the Ab-binding Brd4 epitope may be masked by other transcriptional proteins present only on the TNF-α promoter.

BET protein inhibition with JQ1 protects mice from an LPS-induced cytokine storm and death

To test the efficacy of JQ1 to ablate macrophage cytokine production in vivo, we asked whether JQ1 administration could ameliorate the LPS-induced "cytokine storm," a severe, systemic production of inflammatory cytokines dominated by macrophages (and their monocyte precursors) in response to endotoxemic shock (43). We administered 50 mg/kg of JQ1(+) or JQ1(−) (a dose well tolerated in daily chemotherapy regimens; Ref. 9) 2 h before challenging the mice with a lethal i.p. dose of *E. coli* LPS (20 mg/kg). Although both JQ1(+)- and JQ1(−)-pretreated mice demonstrated clinical signs of endotoxemia (reduced movement, hunched posture, diarrhea), JQ1(+) treatment dramatically improved

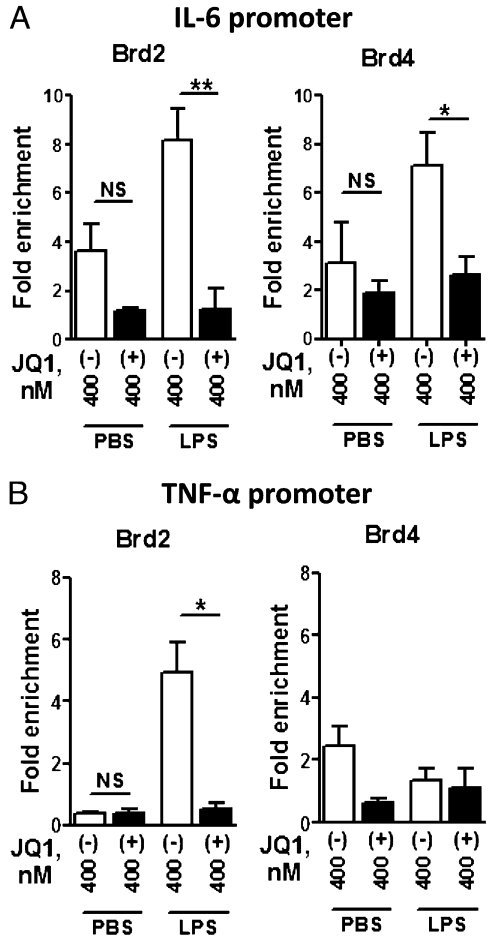

FIGURE 6. JQ1(+) displaces BET proteins Brd2 and Brd4 from inflammatory cytokine promoters. Mouse BMDMs were treated with PBS or 50 ng/ml *E. coli* LPS in the presence of either 400 mM JQ1(−) or 400 nM JQ1(+), as indicated, then harvested for ChIP 1 h later. (**A**) Brd2 or Brd4 association with the IL-6 promoter. (**B**) Brd2 or Brd4 association with the TNF-α promoter. Mean values and SEM are shown; $n = 3$ for all data points except Brd4-precipitated chromatin treated with PBS+JQ1(−), where $n = 2$. $^{*}p < 0.05$, $^{**}p < 0.01$ as calculated by Student t test comparing JQ1(+) and JQ1(−) results.

survival: By 48 h post LPS, 100% of JQ1(−) mice were dead, whereas > 80% of JQ1(+) mice survived, with almost complete resolution of clinical symptoms (Fig. 7A). Furthermore, all JQ1 mice that survived for 48 h post LPS recovered completely, as documented ≥ 14 d following LPS challenge.

To monitor the dynamics of the cytokine response in the endotoxemic mice, we measured concentrations of proinflammatory cytokines in serum every 20 min after LPS administration. Concentrations of key LPS-responsive inflammatory cytokines (IL-6, MCP-1, TNF-α) were significantly reduced in mice pretreated with JQ1(+) (Fig. 7B), compared with JQ1(−) controls. However (and as predicted by Figs. 2B, 4B, and 5A), KC levels were similar in JQ1(+) and JQ1(−) pretreated mice (Fig. 7B). Of interest, serum IL-10, a known anti-inflammatory cytokine, was also reduced in JQ1(+)-treated animals. The ability of JQ1(+) to decrease IL-10 in vivo agrees with the ability of JQ1(+) to block IL-10 production in vitro (Fig.5A). JQ1(+) treatment did not significantly impair early production of SAA, an acute-phase, liver-derived protein that is a signature of endotoxemia in both LPS-treated mice and human sepsis patients (Fig. 7C). Given that serum SAA levels largely reflect hepatocyte function, lack of a JQ1(+) effect on SAA levels supports the interpretation that anti-inflammatory effects of JQ1(+) on macrophages are cell type limited. Taken together, these observations therefore indicate that JQ1(+) treatment specifically targets production of proinflammatory cytokines and protects mice from the systemic proinflammatory response elicited by endotoxemic shock, without altering acute-phase proteins, but this protective mechanism does not work through elevated IL-10. Although the in vivo data cannot attribute effects of JQ1(+)-mediated ablation of cytokines to a specific cell type, the effects are consistent with the interpretation that JQ1(+) blunts inflammatory responses, at least in part, through its ability to block macrophage proinflammatory responses to bacterial stimuli.

Discussion

Our data support the conclusion that BET proteins play critical roles in proinflammatory cytokine responses. We used multiple approaches that include an in vitro model of *brd2 lo* RAW macrophages, *brd2 lo* primary BMDMs and siBrd2-treated WT BMDMs. In the experiments with siRNA knockdown of BET family proteins, we expanded our initial focus on Brd2 and found that all three members of the BET family were essential for proinflammatory cytokine expression. These data raise an interesting question about the redundancy of BET proteins: Despite high homology, their functions do not overlap enough for intact BET proteins to rescue $brd2^{null}$ or $brd4^{null}$ knockout mice, both of which are embryonic lethal (13, 29–31). Notwithstanding possible untested mechanistic differences, the similar effects of Brd2, Brd3, and Brd4 knockdown on inflammatory cytokine expression indicate that developmental functions of BET proteins may be highly specific and distinct from inflammation-associated functions. Taken together, the data predict that Brd3 and Brd4 deficiency may prove as promising as Brd2 for protection from inflammatory conditions beyond Brd2-regulated IR in obesity (13), if the problems of lethality associated with whole-body ablation of these proteins can be addressed.

JQ1 treatment of BMDMs qualitatively recapitulated the phenotype of BET protein knockdown through siRNA; however, the JQ1 effect is stronger than the siRNA effect. This result may occur because JQ1 simultaneously acts on the bromodomains of all three BET proteins. In contrast, each siRNA provides only 60–70% knockdown of the targeted mRNA. We attempted to expose the WT BMDMs to the combination of all three siRNAs, but this treatment appears to be highly toxic for the cells (data not shown) as opposed to the well-tolerated JQ1 treatment. These data suggest that BET proteins likely harbor functions that are crucial for cell survival but that are bromodomain independent, and thus not targeted by JQ1.

Although the BET bromodomain is known to associate with chromatin through protein–protein interaction with acetylated histones, our data support the unexpected possibility that histone interaction is not the only mechanism underlying BET-mediated regulation of inflammatory genes. Rather, we demonstrate that Brd2 knockdown macrophages have a limited ability to activate NF-κB–dependent transcription in the context of a transfected plasmid (i.e., outside the constraints of a complex chromatin structure). Chromatin-independent action of BET proteins may be explained by data showing that Brd2, like Brd4, may bind and activate the NF-κB protein RelA through association with an acetylated RelA K310 residue (44). Because this interaction would occur through the Brd2 bromodomain, the data are consistent with the interpretation that JQ1(+) may function through disrupting the RelA K310/BET protein tether. The possibility that BET proteins can be tethered to genes through NF-κB rather than through histone acetylation is consistent with the demonstration

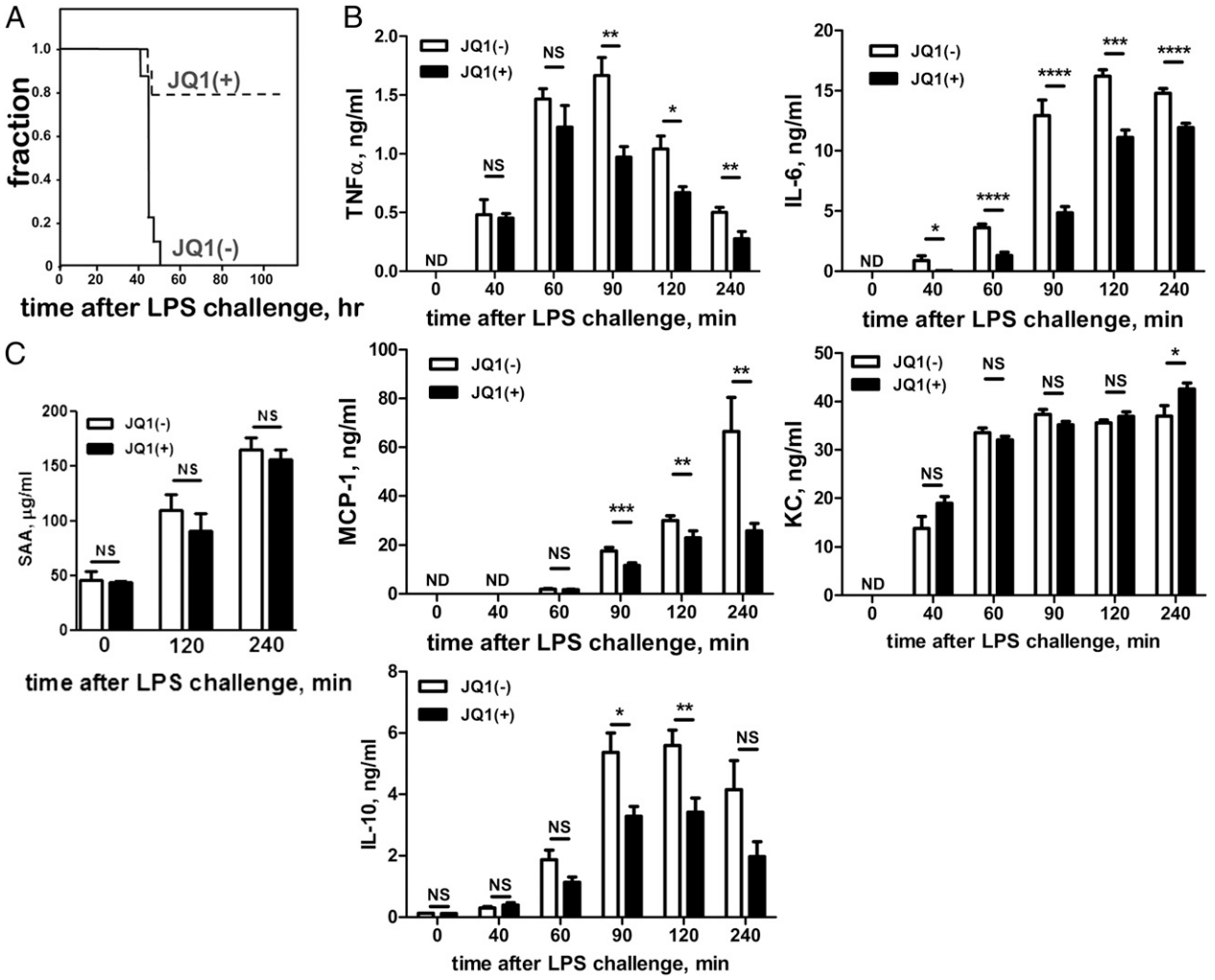

FIGURE 7. JQ1 protects mice from LPS-induced death. Mice were injected with a lethal dose of *E. coli* LPS (20 mg/kg, i.p.). At 2 h before and 24 h after LPS injection, mice also received JQ1 (50 mg/kg, i.p.) of one or the other enantiomer, as indicated. (**A**) Survival curves, Kaplan–Meier analysis. $n = 9$ per arm, $p = 0.001$. (**B**) Serum levels of TNF-α, IL-6, MCP-1, KC, and IL-10 in LPS-challenged mice. Cytokines in serum were determined 40, 60, 90, 120, and 240 min after challenge. $n = 7$–9. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ as calculated by Student t test and comparison of JQ1(+) and JQ1 (−). (**C**) Serum SAA levels in mice challenged with lethal dose of LPS 2 h and 4 h after challenge. $n = 6$. A p value >0.05 was considered significant as calculated by Student t test; ND, Not detected.

that BET proteins can regulate both transfected NF-κB reporter plasmids and naturally chromatinized promoters. Interruption of direct BET protein/NF-κB interactions may also regulate chromatinized genes independently from mechanisms involving BET/acetylated histone interactions.

Another benzodiazepine-based small-molecule BET protein inhibitor, I-BET, was described recently to block IL-6 in mice (45). However, the I-BET compound, although reported to act upon both Brd2 and Brd4 bromodomains, failed to affect TNF-α and MCP-1, both of which are major predictors of survival in murine models of systemic inflammatory response syndrome and septic shock (46, 47). The authors of that report furthermore showed that TNF-α expression was downregulated with siRNAs to BET proteins, although surprisingly, I-BET had no effect on TNF-α protein production. Our JQ1(+) results therefore demonstrate a previously unappreciated, widespread potency of BET inhibition as a more comprehensive, anti-inflammatory strategy that may have efficacy in TNF-α–, IL-6–, and/or MCP-1–mediated inflammatory conditions. Our demonstration by ChIP that JQ1(+) inhibits association between Brd2 and both TNF-α and IL-6 promoters further supports this interpretation.

The potency of BET inhibition with JQ1(+) in a standard in vivo model of endotoxemia (48) specifically links the identification of BET proteins as proinflammatory cytokine regulators to inflammatory disease. LPS-induced endotoxemia is associated with rapid, dramatic elevation of inflammatory cytokines (46), which, as predicted by our in vitro observations, respond to in vivo JQ1(+) therapy. Given that serum IL-6 and TNF-α are major predictors of outcome in septic and LPS-induced shock (46, 49), and that both of these cytokines were successfully targeted by JQ1, the data justify clinical tests of JQ1 in this disease category, which thus far has been dismayingly resistant to clinical intervention. The vast improvement in survival, likely a direct consequence of diminished "cytokine storm" in the challenged mice, also raises the possibility of JQ1 efficacy in pathogen-mediated cytokine storms characteristic of, for example, hemorrhagic fever viruses (48, 50, 51). Success may hinge on therapeutics developed in mice for well-defined or limited patient cohorts. Our success with JQ1(+) and its reasonable safety profile may justify efficacy tests of JQ1(+) in other inflammatory diseases, including human systemic inflammatory response syndrome and sepsis.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Clapier, C. R., and B. R. Cairns. 2009. The biology of chromatin remodeling complexes. *Annu. Rev. Biochem.* 78: 273–304.
2. Natoli, G. 2009. Control of NF-kappaB-dependent transcriptional responses by chromatin organization. *Cold Spring Harb. Perspect. Biol.* 1: a000224.
3. Shahbazian, M. D., and M. Grunstein. 2007. Functions of site-specific histone acetylation and deacetylation. *Annu. Rev. Biochem.* 76: 75–100.
4. Saccani, S., S. Pantano, and G. Natoli. 2001. Two waves of nuclear factor kappaB recruitment to target promoters. *J. Exp. Med.* 193: 1351–1359.
5. Sullivan, K. E., A. B. M. Reddy, K. Dietzmann, A. R. Suriano, V. P. Kocieda, M. Stewart, and M. Bhatia. 2007. Epigenetic regulation of tumor necrosis factor alpha. *Mol. Cell. Biol.* 27: 5147–5160.
6. Dhalluin, C., J. E. Carlson, L. Zeng, C. He, A. K. Aggarwal, and M. M. Zhou. 1999. Structure and ligand of a histone acetyltransferase bromodomain. *Nature* 399: 491–496.
7. Owen, D. J., P. Ornaghi, J. C. Yang, N. Lowe, P. R. Evans, P. Ballario, D. Neuhaus, P. Filetici, and A. A. Travers. 2000. The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p. *EMBO J.* 19: 6141–6149.
8. Sanchez, R., and M.-M. Zhou. 2009. The role of human bromodomains in chromatin biology and gene transcription. *Curr. Opin. Drug Discov. Devel.* 12: 659–665.
9. Filippakopoulos, P., J. Qi, S. Picaud, Y. Shen, W. B. Smith, O. Fedorov, E. M. Morse, T. Keates, T. T. Hickman, I. Felletar, et al. 2010. Selective inhibition of BET bromodomains. *Nature* 468: 1067–1073.
10. Thorpe, K. L., P. Gorman, C. Thomas, D. Sheer, J. Trowsdale, and S. Beck. 1997. Chromosomal localization, gene structure and transcription pattern of the ORFX gene, a homologue of the MHC-linked RING3 gene. *Gene* 200: 177–183.
11. Rhee, K., M. Brunori, V. Besset, R. Trousdale, and D. J. Wolgemuth. 1998. Expression and potential role of Fsrg1, a murine bromodomain-containing homologue of the Drosophila gene female sterile homeotic. *J. Cell Sci.* 111: 3541–3550.
12. Dey, A., J. Ellenberg, A. Farina, A. E. Coleman, T. Maruyama, S. Sciortino, J. Lippincott-Schwartz, and K. Ozato. 2000. A bromodomain protein, MCAP, associates with mitotic chromosomes and affects G(2)-to-M transition. *Mol. Cell. Biol.* 20: 6537–6549.
13. Wang, F., H. Liu, W. P. Blanton, A. Belkina, N. K. Lebrasseur, and G. V. Denis. 2010. Brd2 disruption in mice causes severe obesity without Type 2 diabetes. *Biochem. J.* 425: 71–83.
14. Guo, N., D. V. Faller, and G. V. Denis. 2000. Activation-induced nuclear translocation of RING3. *J. Cell Sci.* 113: 3085–3091.
15. Denis, G. V., C. Vaziri, N. Guo, and D. V. Faller. 2000. RING3 kinase transactivates promoters of cell cycle regulatory genes through E2F. *Cell Growth Differ.* 11: 417–424.
16. Dey, A., F. Chitsaz, A. Abbasi, T. Misteli, and K. Ozato. 2003. The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. *Proc. Natl. Acad. Sci. USA* 100: 8758–8763.
17. Mochizuki, K., A. Nishiyama, M. K. Jang, A. Dey, A. Ghosh, T. Tamura, H. Natsume, H. Yao, and K. Ozato. 2008. The bromodomain protein Brd4 stimulates G1 gene transcription and promotes progression to S phase. *J. Biol. Chem.* 283: 9040–9048.
18. Sinha, A., D. V. Faller, and G. V. Denis. 2005. Bromodomain analysis of Brd2-dependent transcriptional activation of cyclin A. *Biochem. J.* 387: 257–269.
19. Brès, V., S. M. Yoh, and K. A. Jones. 2008. The multi-tasking P-TEFb complex. *Curr. Opin. Cell Biol.* 20: 334–340.
20. Jang, M. K., K. Mochizuki, M. Zhou, H.-S. Jeong, J. N. Brady, and K. Ozato. 2005. The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. *Mol. Cell* 19: 523–534.
21. Yang, Z., J. H. N. Yik, R. Chen, N. He, M. K. Jang, K. Ozato, and Q. Zhou. 2005. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. *Mol. Cell* 19: 535–545.
22. Hewings, D. S., M. Wang, M. Philpott, O. Fedorov, S. Uttarkar, P. Filippakopoulos, S. Picaud, C. Vuppusetty, B. Marsden, S. Knapp, S. J. Conway, and T. D. Heightman. 2011. 3,5-dimethylisoxazoles act as acetyl-lysine-mimetic bromodomain ligands. *J. Med. Chem.* 54: 6761–70.
23. French, C. A., C. L. Ramirez, J. Kolmakova, T. T. Hickman, M. J. Cameron, M. E. Thyne, J. L. Kutok, J. A. Toretsky, A. K. Tadavarthy, U. R. Kees, et al. 2008. BRD-NUT oncoproteins: a family of closely related nuclear proteins that block epithelial differentiation and maintain the growth of carcinoma cells. *Oncogene* 27: 2237–2242.
24. French, C. A. 2010. Demystified molecular pathology of NUT midline carcinomas. *J. Clin. Pathol.* 63: 492–496.
25. Delmore, J. E., G. C. Issa, M. E. Lemieux, P. B. Rahl, J. Shi, H. M. Jacobs, E. Kastritis, T. Gilpatrick, R. M. Paranal, J. Qi, et al. 2011. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* 146: 904–917.
26. Zuber, J., J. Shi, E. Wang, A. R. Rappaport, H. Herrmann, E. A. Sison, D. Magoon, J. Qi, K. Blatt, M. Wunderlich, et al. 2011. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* 478: 524–528.
27. Mertz, J. A., A. R. Conery, B. M. Bryant, P. Sandy, S. Balasubramanian, D. A. Mele, L. Bergeron, and R. J. Sims, III. 2011. Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc. Natl. Acad. Sci. USA* 108: 16669–16674.
28. Belkina, A. C., and G. V. Denis. 2012. BET domain co-regulators in obesity, inflammation and cancer. *Nat. Rev. Cancer* 12: 465–477.
29. Gyuris, A., D. J. Donovan, K. A. Seymour, L. A. Lovasco, N. R. Smilowitz, A. L. P. Halperin, J. E. Klysik, and R. N. Freiman. 2009. The chromatin-targeting protein Brd2 is required for neural tube closure and embryogenesis. *Biochim. Biophys. Acta* 1789: 413–421.
30. Shang, E., X. Wang, D. Wen, D. A. Greenberg, and D. J. Wolgemuth. 2009. Double bromodomain-containing gene Brd2 is essential for embryonic development in mouse. *Dev. Dyn.* 238: 908–917.
31. Houzelstein, D., S. L. Bullock, D. E. Lynch, E. F. Grigorieva, V. A. Wilson, and R. S. P. Beddington. 2002. Growth and early postimplantation defects in mice deficient for the bromodomain-containing protein Brd4. *Mol. Cell. Biol.* 22: 3794–3802.
32. Xu, H., G. T. Barnes, Q. Yang, G. Tan, D. Yang, C. J. Chou, J. Sole, A. Nichols, J. S. Ross, L. A. Tartaglia, and H. Chen. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* 112: 1821–1830.
33. Shoelson, S. E., J. Lee, and A. B. Goldfine. 2006. Inflammation and insulin resistance. *J. Clin. Invest.* 116: 1793–1801.
34. Magness, S. T., H. Jijon, N. Van Houten Fisher, N. E. Sharpless, D. A. Brenner, and C. Jobin. 2004. In vivo pattern of lipopolysaccharide and anti-CD3-induced NF-kappa B activation using a novel gene-targeted enhanced GFP reporter gene mouse. *J. Immunol.* 173: 1561–1570.
35. McCarthy, K. M., D. McDevit, A. Andreucci, R. Reeves, and B. S. Nikolajczyk. 2003. HMGA1 co-activates transcription in B cells through indirect association with DNA. *J. Biol. Chem.* 278: 42106–42114.
36. De Filippo, K., R. B. Henderson, M. Laschinger, and N. Hogg. 2008. Neutrophil chemokines KC and macrophage-inflammatory protein-2 are newly synthesized by tissue macrophages using distinct TLR signaling pathways. *J. Immunol.* 180: 4308–4315.
37. Kim, H. Y., H. K. Kim, J. R. Kim, and H. S. Kim. 2005. Upregulation of LPS-induced chemokine KC expression by 15-deoxy-delta12,14-prostaglandin J2 in mouse peritoneal macrophages. *Immunol. Cell Biol.* 83: 286–293.
38. Watanabe, H., K. Numata, T. Ito, K. Takagi, and A. Matsukawa. 2004. Innate immune response in Th1- and Th2-dominant mouse strains. *Shock* 22: 460–466.
39. Mills, C. D., K. Kincaid, J. M. Alt, M. J. Heilman, and A. M. Hill. 2000. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J. Immunol.* 164: 6166–6173.
40. El Chartouni, C., and M. Rehli. 2010. Comprehensive analysis of TLR4-induced transcriptional responses in interleukin 4-primed mouse macrophages. *Immunobiology* 215: 780–787.
41. Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* 75: 163–189.
42. Nakamura, Y., T. Umehara, K. Nakano, M. K. Jang, M. Shirouzu, S. Morita, H. Uda-Tochio, H. Hamana, T. Terada, N. Adachi, et al. 2007. Crystal structure of the human BRD2 bromodomain: insights into dimerization and recognition of acetylated histone H4. *J. Biol. Chem.* 282: 4193–4201.
43. López-Bojórquez, L. N., A. Z. Dehesa, and G. Reyes-Terán. 2004. Molecular mechanisms involved in the pathogenesis of septic shock. *Arch. Med. Res.* 35: 465–479.
44. Huang, B., X.-D. Yang, M.-M. Zhou, K. Ozato, and L.-F. Chen. 2009. Brd4 coactivates transcriptional activation of NF-kappaB via specific binding to acetylated RelA. *Mol. Cell. Biol.* 29: 1375–1387.
45. Nicodeme, E., K. L. Jeffrey, U. Schaefer, S. Beinke, S. Dewell, C.-W. Chung, R. Chandwani, I. Marazzi, P. Wilson, H. Coste, et al. 2010. Suppression of inflammation by a synthetic histone mimic. *Nature* 468: 1119–1123.
46. Remick, D. G., D. E. Newcomb, G. L. Bolgos, and D. R. Call. 2000. Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture. *Shock* 13: 110–116.
47. Juskewitch, J. E., B. E. Knudsen, J. L. Platt, K. A. Nath, K. L. Knutson, G. J. Brunn, and J. P. Grande. 2012. LPS-induced murine systemic inflammation is driven by parenchymal cell activation and exclusively predicted by early MCP-1 plasma levels. *Am. J. Pathol.* 180: 32–40.
48. Rittirsch, D., L. M. Hoesel, and P. A. Ward. 2007. The disconnect between animal models of sepsis and human sepsis. *J. Leukoc. Biol.* 81: 137–143.
49. Oberholzer, A., S. M. Souza, S. K. Tschoeke, C. Oberholzer, A. Abouhamze, J. P. Pribble, and L. L. Moldawer. 2005. Plasma cytokine measurements augment prognostic scores as indicators of outcome in patients with severe sepsis. *Shock* 23: 488–493.
50. Marshall, J. C. 2008. Sepsis: rethinking the approach to clinical research. *J. Leukoc. Biol.* 83: 471–482.
51. Ward, P. A., and M. Bosmann. 2012. A historical perspective on sepsis. *Am. J. Pathol.* 181: 2–7.

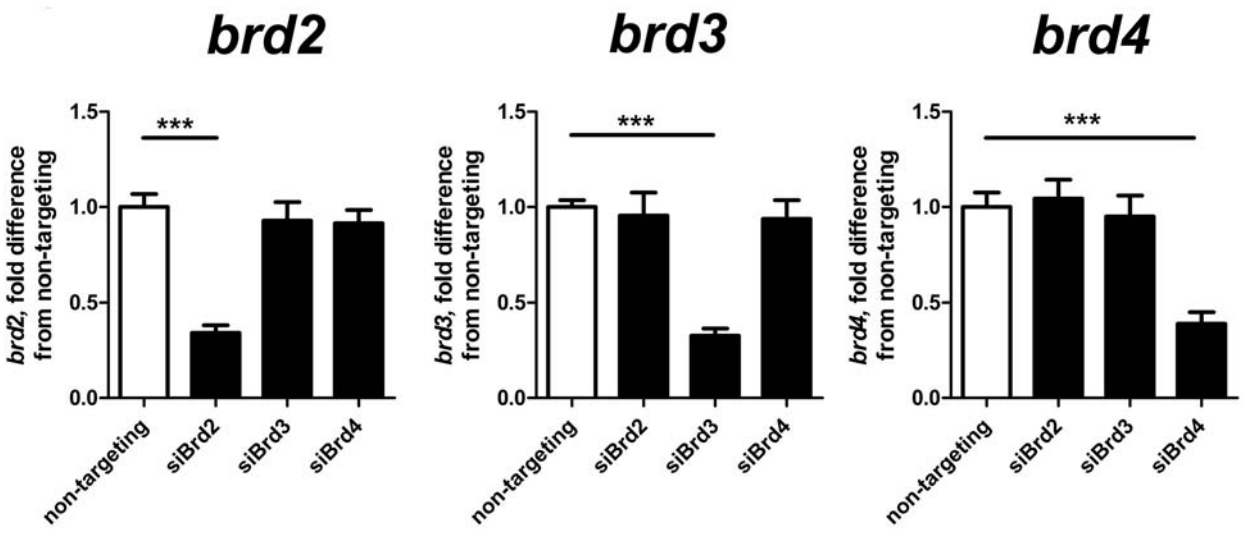

Figure S1. siRNA knockdown of BET protein genes in BMDMs.

Knockdown efficacy as assessed by RT-qPCR 48 hours after transfection. N = 5; *brd2, brd3, brd4* levels were calculated relative to *gapdh,* ***, $P < 0.001$ as calculated by ANOVA with Dunnett's multiple comparison.

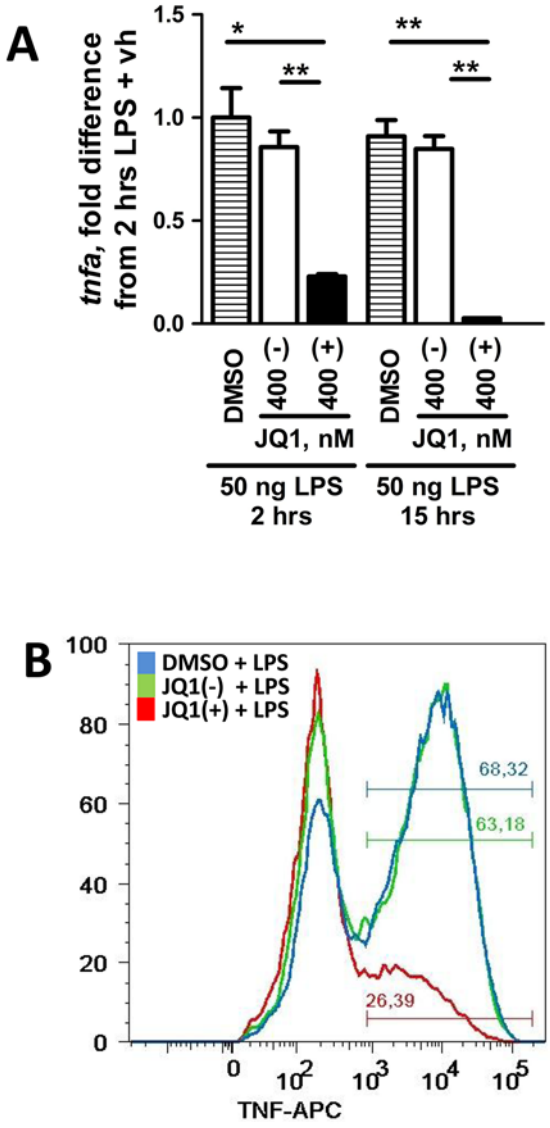

Figure S2. JQ1 reduces TNF-α mRNA and protein levels *in vitro*.

A. RAW 264.7 cells were exposed simultaneously to 50 ng/ml *E. coli* LPS and 400 nM JQ1(+) (black bars), 400 nM JQ1(-) (white bars) or DMSO vehicle (striped bars) for 2 or 15 hours before harvest as indicated. *gapdh*-normalized mRNA expression is shown relative to TNF-α mRNA at 2 hrs post LPS in the presence of DMSO, which was arbitrarily given value of 1. N = 4; *, $P < 0.05$, **, $P < 0.01$ as calculated by ANOVA with Dunnett's multiple comparison. **B**. RAW

264.7 cells were exposed simultaneously to 50 ng/ml *E. coli* LPS and 400 nM JQ1(+), 400 nM JQ1(-) or DMSO for 2 or 15 hours, harvested, fixed, permeabilized and stained intracellularly with APC-TNF-α. Shown is one of three independent determinations, with percentage of TNF-α positive cells indicated about the histograms.