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BET protein function is required for inflammation: Brd2 genetic disruption and BET inhibitor JQ1 impair mouse macrophage inflammatory responses¹

Anna C. Belkina^{*,†}, Barbara S. Nikolajczyk[†], and Gerald V. Denis^{*,‡}

^{*}Cancer Research Center, Boston University School of Medicine, Boston, Massachusetts, United States of America

[†]Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts, United States of America

Abstract

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 pro-inflammatory cytokine response remain poorly characterized. Bromodomain extra terminal (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 pro-inflammatory cytokine production in macrophages. Studies that utilize siRNA 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 hyper-inflammatory conditions associated with high levels of cytokine production.

Introduction

Regulation of inflammatory gene expression is tightly controlled through chromatin “readers” that specifically bind histone post-translational 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, multi-protein complexes include both transcriptional co-activation and co-repression of inflammatory genes in differentiated adult cells (2), thus chromatin

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[‡]Corresponding author. Gerald V. Denis, Boston University School of Medicine, Room K520, 72 East Concord Street, Boston, Massachusetts, United States of America 02118. Phone: 617-414-1371. Fax: 617-638-5673, gdenis@bu.edu.

readers play critical roles in exquisitely tuned inflammatory responses to a variety of immune system stimuli.

Pro-inflammatory 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 lysines 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 forty bromodomain proteins (8, 9), which include histone acetyltransferases (HATs), helicases, scaffolding proteins and other co-factors 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 pro-inflammatory stimuli.

The bromodomain and extra-terminal (BET) family is distinct group of bromodomain proteins that in mammals includes Brd2, Brd3, 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 been explored poorly. Establishing links between BET proteins and inflammation has become clinically critical due 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 (NMC) xenografts in mice (9), and was proven efficient to block growth of various leukemic cells (25–27). However, other possible applications of JQ1, including those targeting BET protein functions in non-malignant cells, are untested (Reviewed in 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 genes 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 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, amongst other inflammatory diseases (Reviewed in 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 pro-inflammatory 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. *brd2 lo* mice were generated as previously reported (13). NF- κ B^{EGFP} mice (34) were obtained from Christian Jobin, University of North Carolina, Chapel Hill, North Carolina, USA. In these mice, a single copy of eGFP reporter was placed under control of *cis*-NF- κ B^{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

Dulbecco's Minimum Essential Medium (DMEM) plus glutamine and 25mM glucose was from Mediatech. Penicillin, streptomycin, and fungizone were purchased from Gibco. 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). 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 μ m cell strainer (BD Biosciences), followed by treatment with 1X red blood cell lysis buffer (eBioscience). Bone marrow (BM) cells were isolated as described below. Cells were counted and stained with directly conjugated monoclonal antibodies 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 – 100,000 gated events using FlowJo 8.7 (TreeStar).

Isolation, culture and stimulation of bone marrow-derived macrophages

Femurs, 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 RPMI1640 medium buffered with 20 mM HEPES pH 7.4 and supplemented with 10% fetal bovine serum, penicillin, streptomycin, fungizone and 50 μ M 2-mercaptoethanol. Bone fragments were removed by sterile filtration with 70 μ m 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 medium supplemented as above. To initiate differentiation, the medium was supplemented with 50 ng/ml of recombinant M-CSF (eBioscience) for 5–7 days. 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). 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- κ B luciferase reporter assay

RAW264.7 cells were transfected using Lipofectamine 2000 (Invitrogen) and components of the Cignal NF- κ B Luc Reporter 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/permeabilization buffer kit (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 (APC)-anti-TNF- α (eBioscience). Labeled cells were analyzed by flow cytometry as described above.

siRNA knockdown in BMDMs

Twenty nM Dharmacon ON-TARGETplus SMARTpool siRNA with DharmaFECT formulation #4 reagent (Dharmacon) was used for Brd2, Brd3 and Brd4 knockdown. Cells were used for experiments 48 hrs post-transfection. Knockdown was validated by RT-qPCR.

RT-qPCR

RNA from macrophages and RAW264.7 cells was isolated and quantified as published using 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 μ l of mouse blood was obtained from the tail vein and RNA was purified with Mouse RiboPure Blood RNA kit (Ambion). For 28S rRNA amplification, the following primers were used: 5'-GCGAAATACCGGCACGAGACCGATAG-3' and 5'-GGTTTCACGCCCTCTTGAACCTCTCTC-3'. Melt curve analysis indicated formation of a single product in all cases. Relative mRNA expression levels were determined using Δ Ct 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 μ g of α -acetylated histone H3 (Upstate), α -Brd2 (Bethyl Labs), α -Brd4 (Bethyl Labs) or α -GST (Upstate). Two nanograms of each sample were then analyzed in duplicate or triplicate by qPCR. Fold difference was calculated as $2^{-(Ct(input)-Ct(ChIP))}$, then fold enrichment over an unrelated antibody (α -GST) was assessed. Oligonucleotides were: 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-week-old male mice were injected *i.p.* with 50 mg/kg of JQ1 (+) or JQ1(-). Two hours later, 20 mg/kg of *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's or Tukey-Kramer multiple comparisons tests. Error bars indicate standard errors 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 in 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 pro-inflammatory stimuli. To begin testing this prediction, we first asked whether Brd2 hypomorphs (confirmed in Fig. 1A) resist obesity-associated inflammation due to constitutive changes in numbers and/or ratios of immune cells, a possibility consistent with our demonstration that Brd2 shRNA expression in hematopoietic stem cells decreases their contribution to total bone marrow (Belkina et al, 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 compared to WT (not shown). To test the alternative possibility that Brd2 regulates pro-inflammatory responses rather than *in vivo* cell distribution, we stimulated bone marrow derived macrophages (BMDMs) from *brd2 lo* mice with LPS and quantified production of inflammatory cytokines. *brd2 lo* BMDMs produced lower levels of pro-inflammatory TNF- α , IL-1 β , IL-6 and MCP-1/CCL2, compared to WT macrophages (Fig. 1B). Importantly, the number of CD11b+ BMDMs differentiated from BM of *brd2 lo* mice was similar to 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 pro-inflammatory 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 hrs post-transfection. Importantly, down regulation of any single BET protein did not alter the levels of expression of the other two members of the BET family (Fig. S1). We then stimulated knockdown BMDMs with LPS and quantified inflammatory cytokine mRNA by RT-qPCR. TNF- α , IL-6 and MCP-1 mRNA levels were significantly lower in cells transfected with the siBrd2 pool compared to the non-targeting siRNA pool control (Fig. 2A). Taken together with decreased inflammatory cytokine production by *brd2 lo* macrophages, these data indicate that Brd2 promotes pro-inflammatory 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 pro-inflammatory chemokine (36) (Fig. 2B-you need to mention the result here). We conclude that Brd2, Brd3 and Brd4 have non-redundant and selective roles in the macrophage inflammatory response through their ability to regulate cytokine production.

Brd2 regulates NF- κ B activity

Given that all of the cytokines affected by decreased Brd2 levels are targets of NF- κ B, a master pro-inflammatory transcription factor, we tested the possibility that Brd2 regulates cytokine production by altering NF- κ B 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- κ B luciferase reporter gene, and stimulated cells with LPS. NF- κ B reporter activity was significantly decreased in Brd2 knockdown cells (Fig. 3), supporting the conclusion that Brd2 regulates pro-inflammatory cytokines through its ability to support NF- κ B-regulated transcription.

BET inhibitor JQ1 ablates inflammatory responses in LPS-stimulated BMDMs

Given that down regulation 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 compared to 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 less than 25% of the levels produced by cells treated with JQ1(-) (Fig. S2A). Intracellular TNF- α stain independently confirmed lower protein levels in JQ1(+)-treated cells (Fig. S2B). Quantitatively similar dose-dependent decreases in TNF- α , IL-6 and MCP-1 were produced by JQ1(+) treated primary BMDMs (Fig. 4B), independently confirming 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 be due to the fact that LPS-induced elevation of KC transcription depends on the TLR4-AP1 axis rather than NF- κ B (37).

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 "M1" strain, while Balb/c mice tend to "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 bone marrow 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 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 up regulation. 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 pre-treatment 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). As expected from previous results, KC production was not robustly inhibited.

JQ1 treatment disrupts association of Brd2 and Brd4 with the regulatory chromatin of pro-inflammatory cytokine promoters

We have demonstrated that Brd2 regulates NF- κ B activity outside the context of naturally assembled chromatin (Fig. 3). To assess the effect of blocking BET protein function on NF- κ B activity within chromosomal DNA, we generated BMDMs from male NF- κ B^{EGFP} knockin mice (34) and stimulated cells with LPS in presence of JQ1(+) or JQ1(-). Like other myeloid immune cells originating from these mice (34), the BMDMs demonstrated high background eGFP fluorescence even when not stimulated with LPS, probably mirroring the basal NF- κ B activation. We therefore quantified LPS response by eGFP mRNA. eGFP mRNA increased approximately 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 antibodies 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 the non-stimulated 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 antibodies (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 the LPS-inducible association of BET proteins with the IL-6 promoter is significantly decreased in the presence of JQ1(+), which explains the decreased production of pro-inflammatory cytokines in macrophages.

To measure the effect of JQ1(+) on BET protein/promoter interactions more universally, we re-amplified 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). 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). This negative result may reflect *bona fide* differences between the molecular mechanisms that regulate IL-6 and TNF- α . Alternatively, the antibody-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 (9)) 2 hours before challenging the mice with a lethal intraperitoneal 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 survival: by 48 hours post-LPS, 100% of JQ1(-) mice were dead, while more than 80% of JQ1(+) mice survived with almost complete resolution of clinical symptoms (Fig. 7A). Furthermore, all JQ1 mice that survived for 48 hrs post-LPS recovered completely, as documented 14 days following LPS challenge.

To monitor the dynamics of the cytokine response in the endotoxemic mice, we measured concentrations of pro-inflammatory 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 pre-treated with JQ1(+) (Fig. 7B) compared to JQ1(-) controls. However (and as predicted by Fig. 2B, 4B and 5A), KC levels were similar in JQ1(+) and JQ1(-) pre-treated mice (Fig. 7B). Interestingly, 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 ability of JQ1(+) to block IL-10 production *in vitro* (Fig. 5A). JQ1(+) treatment did not significantly impair early production of serum amyloid A (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 pro-inflammatory cytokines and protects mice from the systemic pro-inflammatory 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 pro-inflammatory responses to bacterial stimuli.

Discussion

Our data support the conclusion that BET proteins play critical roles in pro-inflammatory 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 pro-inflammatory 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 insulin resistance 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 be due to the fact that 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- κ B 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- κ B 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- κ B rather than through histone acetylation is consistent with the demonstration that BET proteins can regulate both transfected NF- κ B reporter plasmids and naturally chromatinized promoters. Interruption of direct BET protein/NF- κ B 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, while reported to act upon both Brd2 and Brd4 bromodomains, failed to affect TNF- α and MCP-1, although both of which are major predictors of survival in murine models of systemic inflammatory response syndrome (SIRS) and sepsis shock (46, 47). The authors of that report furthermore showed that TNF- α expression was down regulated 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 pro-inflammatory 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 SIRS or sepsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

BET	bromodomain and extraterminal domain
BM	bone marrow

BMDM	bone marrow derived macrophage
ChIP	chromatin immunoprecipitation
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HAT	histone acetyltransferase
KC	keratinocyte chemoattractant
shRNA	short hairpin RNA
siRNA	small interfering RNA
WT	wildtype

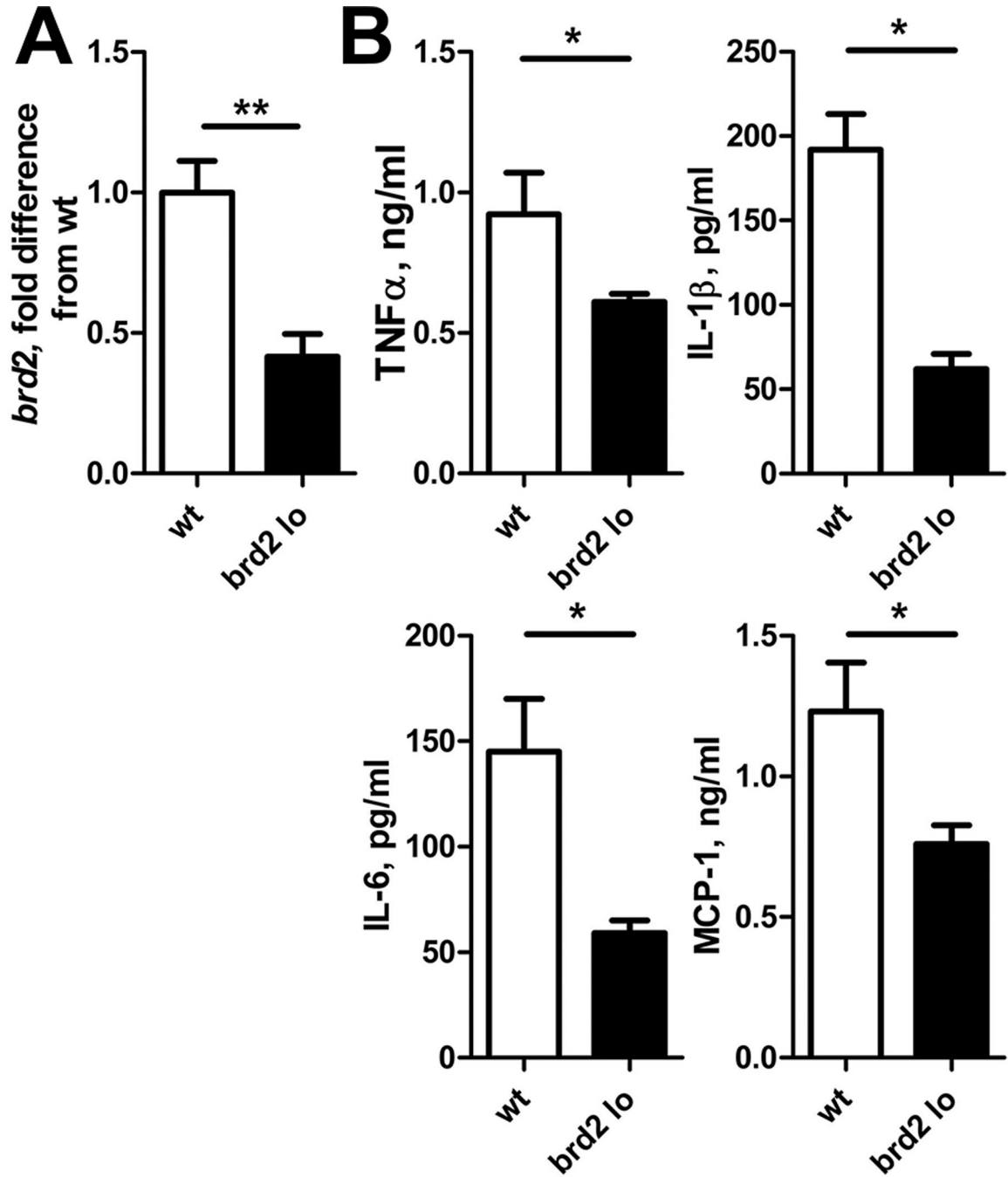


Figure 1. *brd2 lo* macrophages demonstrate a diminished inflammatory response

A. *Brd2* mRNA levels in leukocytes from WT (white bar) compared to *brd2 lo* (black bar) mice as measured by RT-qPCR. Relative mRNA expression was normalized to GAPDH. N = 4; **, $P < 0.01$, as calculated by Student's t test. B. Production of pro-inflammatory cytokines by *brd2 lo* and WT BMDMs stimulated for 24 hours 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's t test.

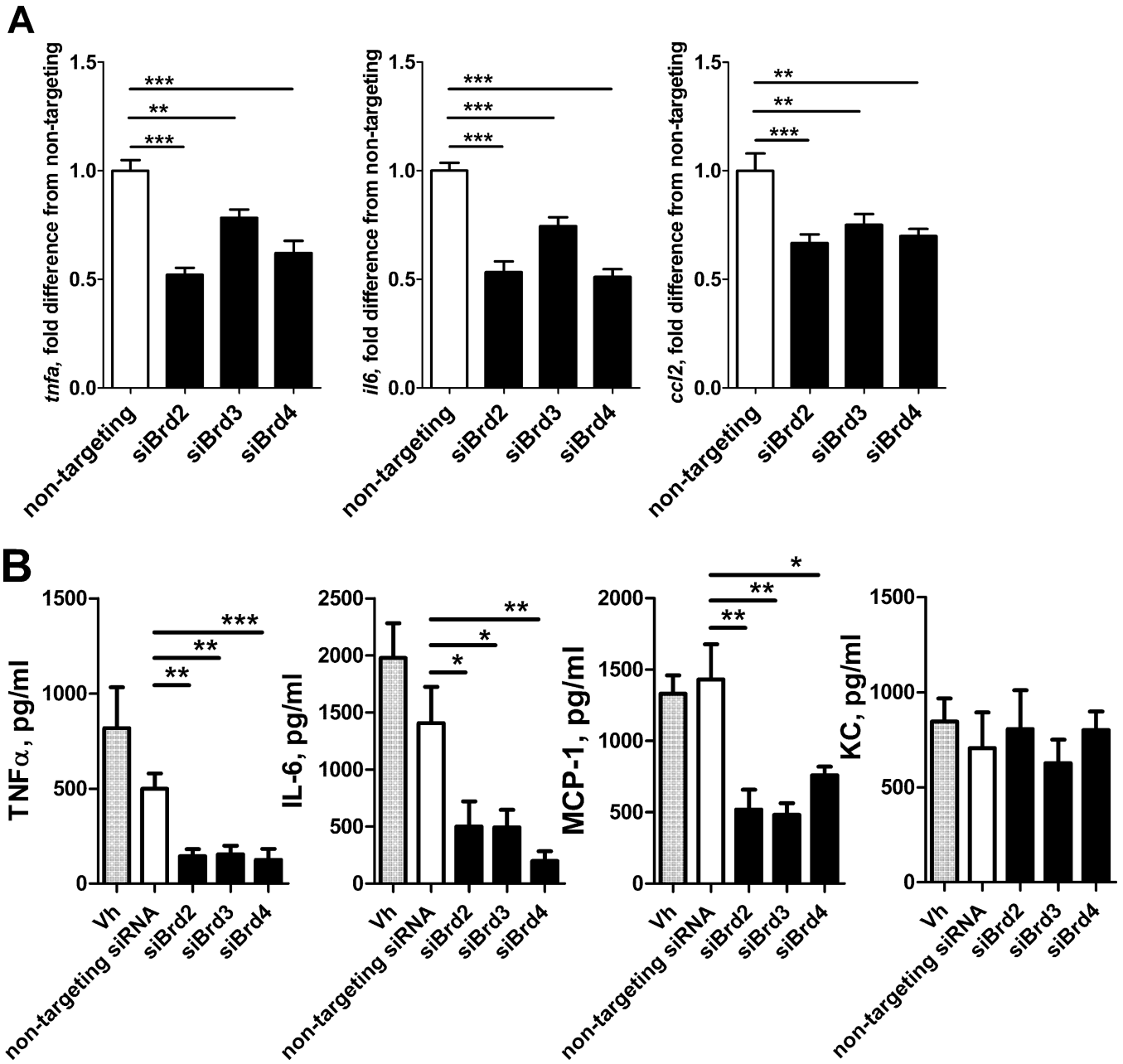


Figure 2. *In vitro* knockdown of BET protein genes reduces the inflammatory response
A. WT BMDMs were transfected with ON-TARGET Plus siRNA pools specific for knockdown of Brd2, Brd3 or Brd4, or non-targeting control pools, then stimulated with 50 ng/ml *E. coli* LPS for 2 hrs before quantitation of the indicated cytokine mRNAs by RT-qPCR. Relative mRNA expression was normalized to GAPDH. N = 5; **, $P < 0.01$, ***, $P < 0.001$ as calculated by ANOVA with Dunnett's multiple comparison. **B.** Cytokine protein concentrations in supernatants from LPS-stimulated (24 hours, 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's multiple comparison.

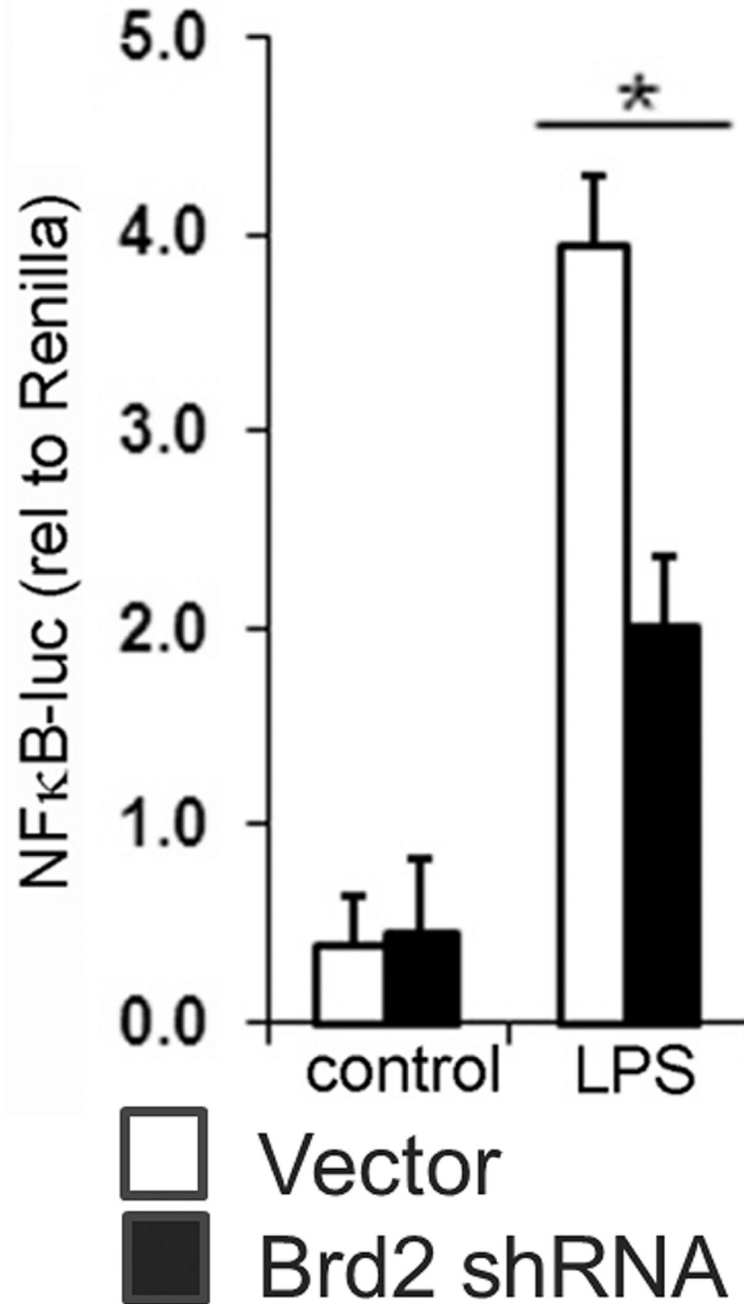


Figure 3. *In vitro* knockdown of Brd2 decreases NF-κB-luciferase activity

RAW264.7 cells were transfected with Brd2-specific shRNA (black bars) or empty vector (white bars) plus NF-κB luciferase reporter plasmid. Forty-eight hours after transfection, cells were stimulated with *E. coli* LPS for 2 hrs. NF-κB-driven luciferase activity is shown (mean and SEM). N = 3. *, $P < 0.05$ as calculated by Student's t test comparing empty vector and Brd2 shRNA-containing plasmids.

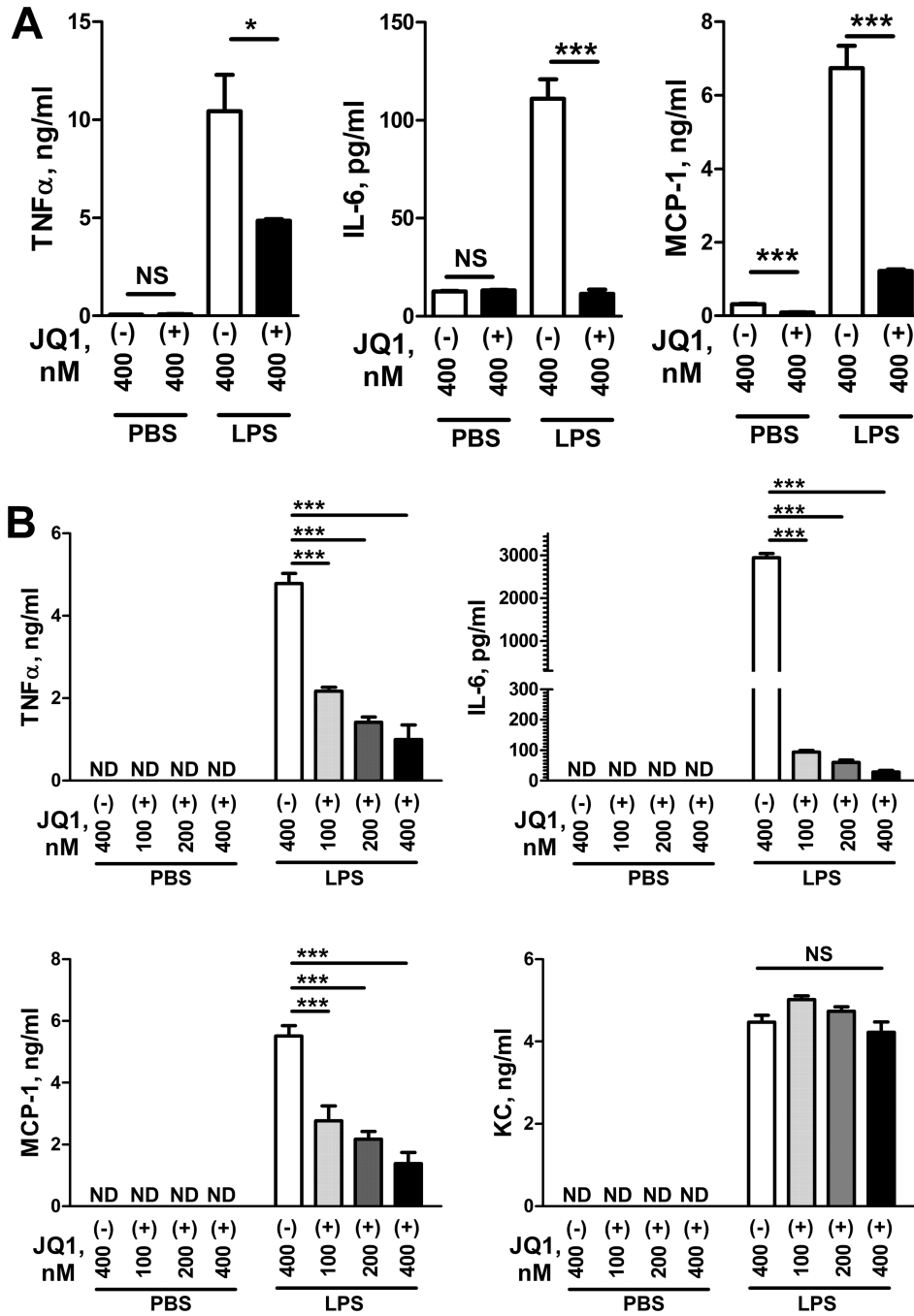


Figure 4. The BET family bromodomain inhibitor JQ1 reduces cytokine production *in vitro*
A. RAW 264.7 cells were exposed simultaneously to 50 ng/ml *E. coli* LPS plus 400 nM JQ1(+) or JQ1(-) for 24 hrs. Mean value and SEM for 3 determinations are shown. JQ1 enantiomers are abbreviated by (+) or (-), respectively. *, $P < 0.05$, ***, $P < 0.001$ as calculated by Student's 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 hrs. Supernatant cytokines were quantified in multiplex protein

assays. N = 6; ***, $P < 0.001$ as calculated by ANOVA with Dunnett's multiple comparison. ND – not detected.

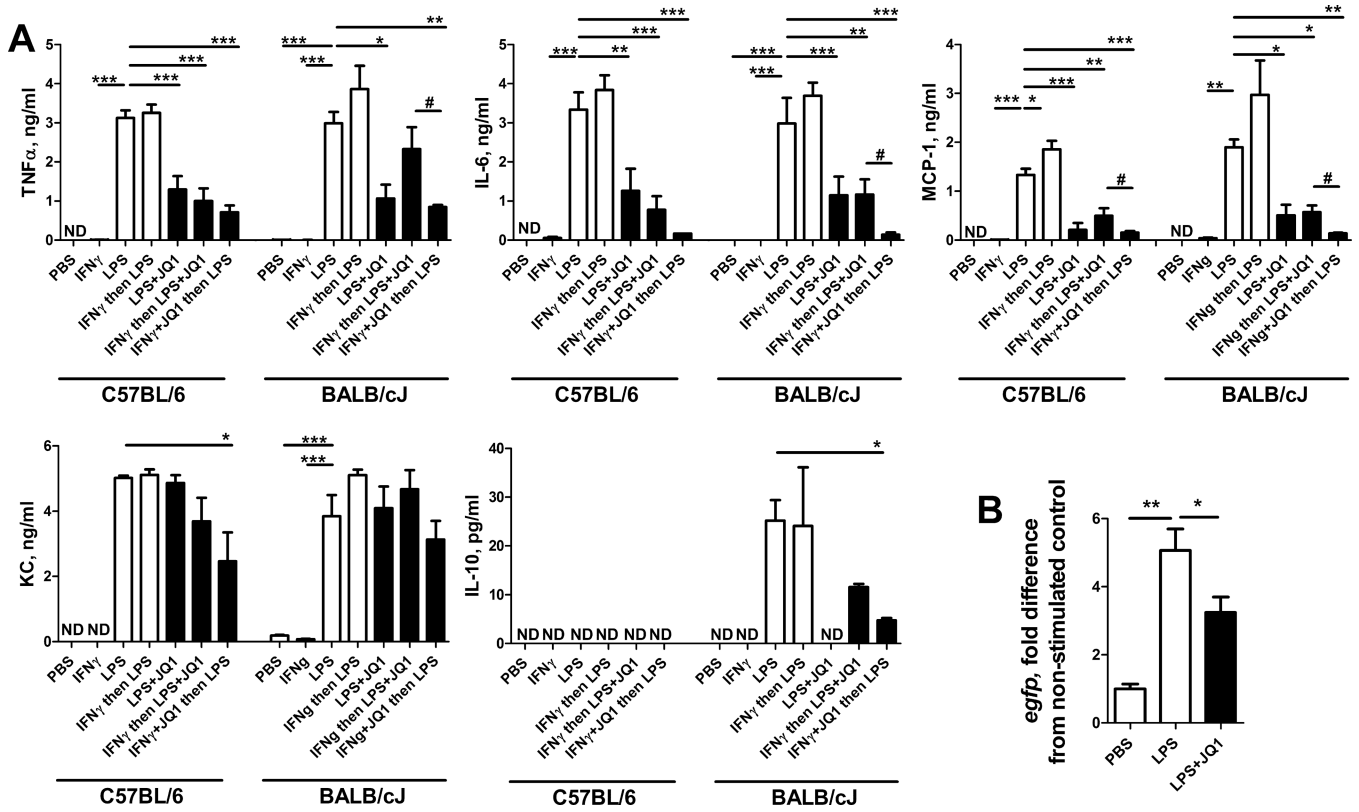


Figure 5. JQ1 effect on cytokine production is strain-independent and is not overcome by IFN γ priming of macrophages

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 hours later as indicated below graph. Black bars show BMDMs primed with IFN γ or PBS and stimulated with LPS 4 hours later, with JQ1(+), being added either simultaneously with LPS (4 hours after priming with IFN γ) or simultaneously with IFN γ . Cytokine production was measured 24 hours after LPS stimulation. Supernatant cytokines were quantified in multiplex protein assays. N = 5; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ as calculated by ANOVA with Dunnett's multiple comparison. #, $P < 0.05$ as calculated by Student's t test; ND – not detected. **B.** eGFP NF- κ B BMDMs were stimulated with PBS, 50 ng/ml LPS or LPS + JQ1(+) as indicated. N = 3 ***, $P < 0.01$; *, $P < 0.05$ as calculated by ANOVA with Dunnett's multiple comparison.

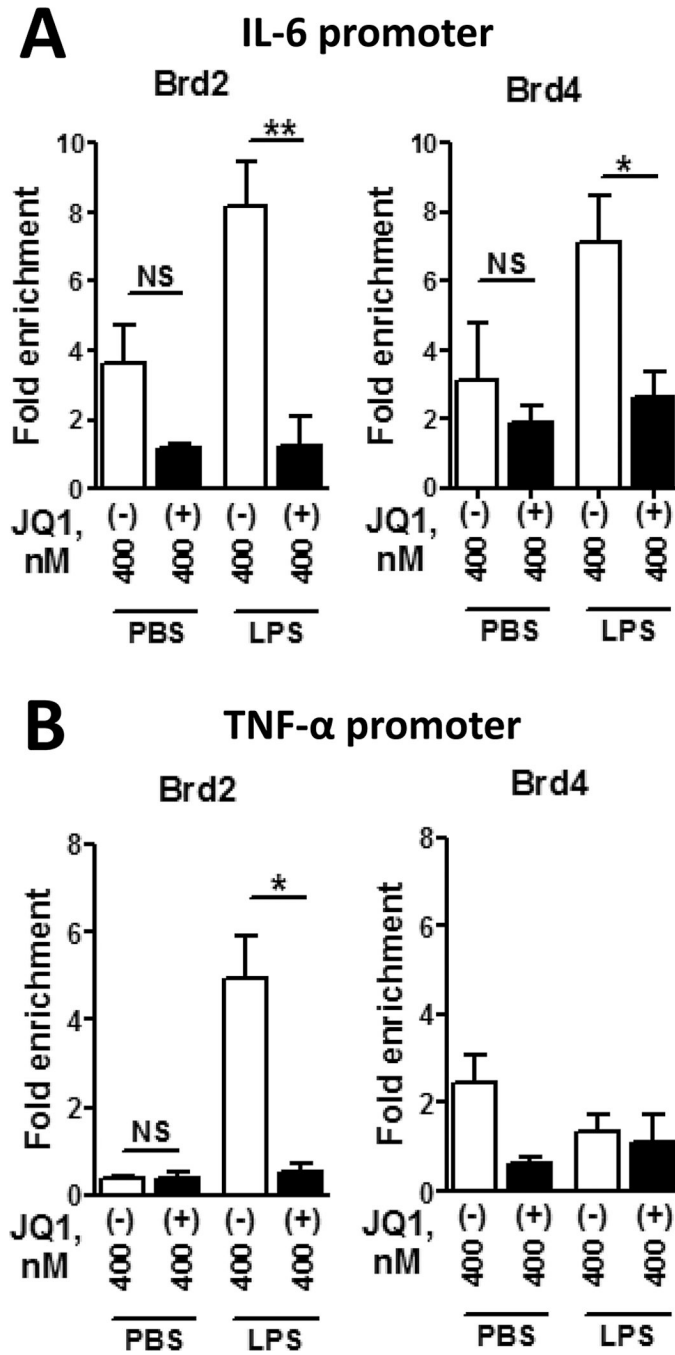


Figure 6. JQ1(+) displaces BET proteins Brd2 and Brd4 from inflammatory cytokine promoters
 Mouse BMDMs were treated with PBS or 50 ng/ml *E. coli* LPS in presence of either 400 nM JQ1(-) or 400 nM JQ1(+) as indicated, then harvested for ChIP 1 hour later. **A.** Brd2 or Brd4 association with the IL-6 promoter. **B.** Brd2 or Brd4 association with the TNF- α promoter. Mean values and SEM are shown; N=3 for all datapoints except for Brd4-precipitated chromatin treated with PBS+JQ1(-), where N=2. *, $P < 0.05$; **, $P < 0.01$ as calculated by Student's t test comparing JQ1(+) and JQ1(-) results.

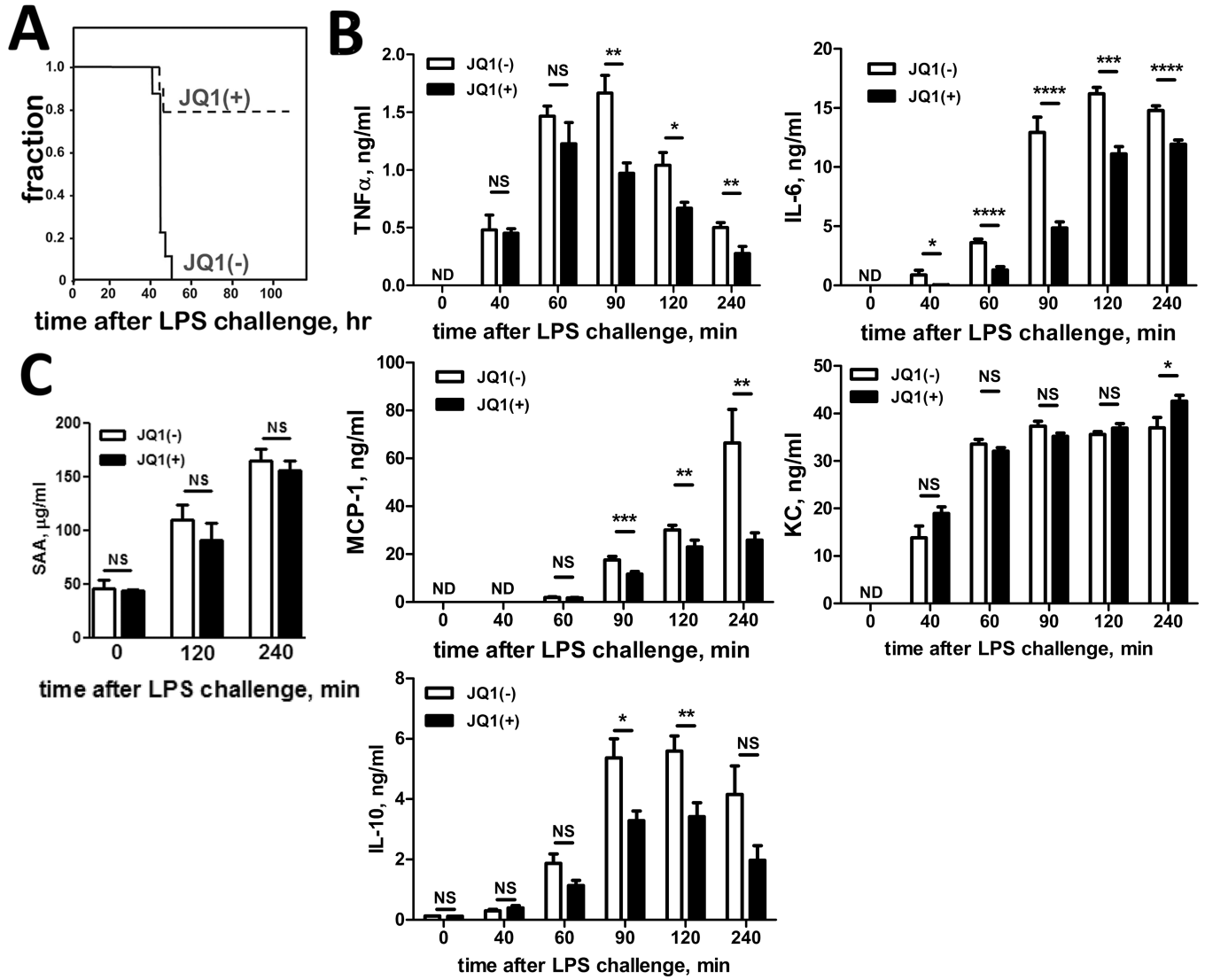


Figure 7. JQ1 protects mice from LPS-induced death

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's t test and comparison of JQ1(+) and JQ1(-). **C.** Serum SAA levels in mice challenged with lethal dose of LPS 2 hours and 4 hours after challenge. N = 6. NS indicates insignificant difference ($P > 0.05$) as calculated by Student's t test.

Table IMajor immune cells 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
Bone marrow		
B220+	23.6 ± 4.9	22.0 ± 2.4
CD11b+	29.1 ± 2.5	29.9 ± 2.8
Gr-1+	33.3 ± 3.6	33.7 ± 1.5

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