# Inhibition of Bromodomain and Extraterminal Domain Family Proteins Ameliorates Experimental Renal Damage

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## ABSTRACT

Renal inflammation has a key role in the onset and progression of immune– and nonimmune–mediated renal diseases. Therefore, the search for novel anti–inflammatory pharmacologic targets is of great interest in renal pathology. JQ1, a small molecule inhibitor of bromodomain and extraterminal (BET) proteins, was previously found to preserve renal function in experimental polycystic kidney disease. We report here that JQ1–induced BET inhibition modulated the *in vitro* expression of genes involved in several biologic processes, including inflammation and immune responses. Gene silencing of BRD4, an important BET protein, and chromatin immunoprecipitation assays showed that JQ1 alters the direct association of BRD4 with acetylated histone–packaged promoters and reduces the transcription of proinflammatory genes (*IL-6*, *CCL-2*, and *CCL-5*). *In vivo*, JQ1 abrogated experimental renal inflammation in murine models of unilateral ureteral obstruction, antimembrane basal GN, and infusion of Angiotensin II. Notably, JQ1 downregulated the expression of several genes controlled by the NF-$\kappa$B pathway, a key inflammatory signaling pathway. The RelA NF-$\kappa$B subunit is activated by acetylation of lysine 310. In damaged kidneys and cytokine–stimulated renal cells, JQ1 reduced the nuclear levels of RelA NF-$\kappa$B. Additionally, JQ1 dampened the activation of the Th17 immune response in experimental renal damage. Our results show that inhibition of BET proteins reduces renal inflammation by several mechanisms: chromatin remodeling in promoter regions of specific genes, blockade of NF-$\kappa$B pathway activation, and modulation of the Th17 immune response. These results suggest that inhibitors of BET proteins could have important therapeutic applications in inflammatory renal diseases.

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All cells in an organism contain the same genetic information, but the gene expression pattern varies between cell types. Epigenetics involves heritable changes in gene expression that are not associated with DNA sequence modifications. Epigenetic mechanisms, especially DNA methylation and histone modifications, are dynamic processes and mediate the diversified gene expression profiles in normal and diseased states. Some of these epigenetic marks are being characterized in acute and chronic renal disease and could help explain gene expression regulation patterns in injured kidney. Oxidative stress, proinflammatory cytokines, and uremic toxins

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might induce epigenetic modifications involved in alterations of immune response, fibrosis, and cardiovascular disease in patients with renal disease.[1–4] These modifications are potentially reversible, presenting an opportunity for identifying novel therapeutic targets.

The bromodomain and extraterminal (BET) protein family (BRD2, BRD3, BRD4, and BRDT) has two conserved N–terminal bromodomains (BD1 and BD2) and participates in tumor development, infections, autoimmunity, and inflammation.[5–7] Bromodomains in BET proteins bind to acetylated lysine residues on histones and other nuclear proteins to regulate the transcriptional program.[8] The extraterminal domain in BET proteins interacts with histone modifiers, such as the arginine demethylase JMJD6 and the lysine methyltransferase NSD3, or ATP–dependent nucleosome–remodeling enzymes SWI/SNF, causing changes in chromatin structure.[9,10] Additionally, BET proteins form part of the mammalian mediator complex that facilitates binding between transcription factors and the transcriptional machinery.[11,12]

Among BET proteins, BRD4 is the best studied and participates in several steps of the transcription hierarchy. BRD4 recruits the positive transcription elongation factor b (P-TEFb) complex (heterodimer of CDK9 and cyclin T1, T2, or K) to the promoter region and stimulates RNA polymerase II–dependent transcription through its kinase activity.[13,14] The oncogene *c-myc* was the first BRD4 target gene to be described.[15,16] Subsequent studies reported that BRD4 binds to acetylated histones in the enhancers or promoter regions of inflammatory genes.[17] BRD4 interacts with specific acetylated residues of transcription factors. One of the best characterized is NF-$\kappa$B, a key player in regulating immune and inflammatory responses.[18] BRD4, through its two bromodomains, specifically recognizes the acetylated lysine-310 residue of the RelA NF-$\kappa$B subunit.[19] In tumor cells, BRD4/RelA binding avoids RelA ubiquitinization and its proteasome-mediated degradation, thereby maintaining persistent activation of the nuclear NF-$\kappa$B complex and promoting cancer cell proliferation.[20,21]

Two potent BET inhibitors, I-BET and JQ1, have recently been developed.[5,22] These small molecules compete for the bromodomain binding pocket and displace BET proteins from binding to acetylated lysine residues. Several studies have shown that these compounds strongly inhibit malignant cell growth by targeting cancer-promoting genes.[23–27] Initial studies in midline carcinoma and hematologic malignances showed that BET inhibitors induce release of the BRD4-NUT oncoprotein and the transcriptional suppression of *MYC* and *BCL2* proto-oncogenes.[28] Two human phase 1 clinical trials in patients with cancer are currently in progress.[8,29] *In vitro* evidence in nonmalignant cells suggests that BET inhibitors could be a potential therapeutic option for inflammatory diseases. In activated macrophages, JQ1 and I-BET reduced inflammatory gene production in response to LPS.[22,30] In synovial fibroblasts of patients with rheumatoid arthritis, I-BET suppressed proinflammatory factors and diminished their capacity to recruit immune cells.[31] In a murine model of collagen-induced arthritis, JQ1 reduced inflammation and joint damage.[32] JQ1 also ameliorated bleomycin–induced murine lung fibrosis[33] and cardiac damage in a mouse model of pressure overload–induced left ventricular hypertrophy.[34] A recent study reported that JQ1 delayed cyst growth and preserved renal function in a murine model of polycystic kidney disease by inhibiting *c-Myc* gene and cystic epithelial cell proliferation.[35] Our aim was to investigate whether BET inhibition could modulate experimental inflammation in the kidney, investigating potential mechanisms involved.

## RESULTS

### JQ1 Modulates Inflammatory-Related Genes in Cultured Renal Cells

The anti-inflammatory properties of the BET inhibitor JQ1 were evaluated *in vitro* in human tubular epithelial cells (HK2 cell line) after TNF-$\alpha$ stimulation. This well characterized inflammatory cytokine regulates many proinflammatory genes and is involved in experimental and human kidney diseases.[36] In HK2 cells, TNF-$\alpha$ stimulation upregulated gene expression of the proinflammatory factors *CCL-2*, *CCL-5*, and *IL-6* for up to 6 hours, peaking around 3 hours. JQ1 dose dependently diminished overexpression of these genes (Figure 1A). The enantiomer (−)JQ1 had no effect, indicating the specificity of the response to active JQ1 (Figure 1A). Interestingly, *BRD4* gene or protein levels were not altered in cells treated with JQ1 compounds, suggesting that BET proteins activity was independent of changes in their expression (Supplemental Figure 1A and B). In addition, similar results were observed with another BET inhibitor (I-BET 762) (Supplemental Figure 2).

Next, we investigated the transcriptional pathways altered by BET inhibition using a whole–genome gene expression array. Global gene expression analysis showed that 210 probes (corresponding to 193 genes) were regulated after TNF-$\alpha$ stimulation, of which 155 (73.8%; 139 genes) were upregulated and 55 were downregulated (26.2%; 54 genes) (Figure 1B). Among TNF-$\alpha$–inducible genes, 74 probes (35.2%; 62 genes) were diminished by JQ1, whereas 81 probes (38.6%; 77 genes) were unaffected (Figure 1B). Functional pathway analysis revealed that JQ1 modulated the expression of genes related to cell activation (*CSF2*, *IL-6*, *IL-8*, *LTB*, and *STAT5A*), cell proliferation (*SMAD3*, *CCND1*, and *ICOSLG*), apoptosis (*BIRC3*, *BCL3*, and *TNFSF10*), and immune response (*CCL-2*, *CCL-20*, and *IL-7R*) (Figure 1, C and D). Conversely, the expression of some chemokines (*CXCL-2*), adhesion molecules (*ICAM1*), and immune-associated genes (*CD40* and *TNF-$\alpha$*) was unchanged after JQ1 treatment (Figure 1E). In contrast to observations in cancer cells and cystic epithelial cells,[15,35,37] c-Myc expression was not changed by JQ1 in renal cells as described in the heart or the hematopoietic differentiation of stem cells.[38,39] JQ1 did not modify certain genes of the NF-$\kappa$B pathway (*NF-$\kappa$B1A*,

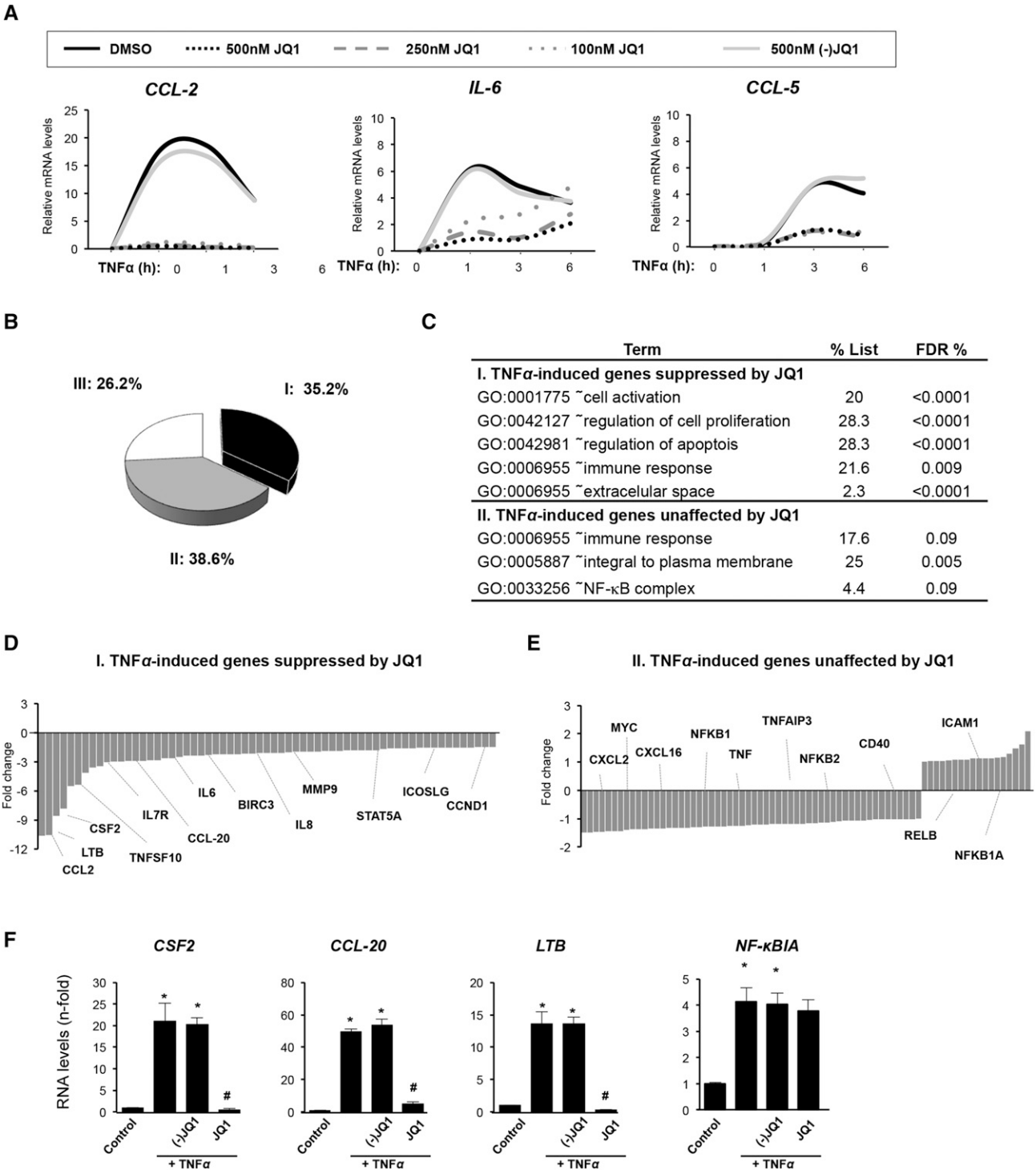


**Figure 1.** JQ1 downregulates proinflammatory gene expression in cultured renal cells. (A) Renal tubular epithelial cells (HK2 cell line) were preincubated with vehicle (DMSO), different concentrations of the BET proteins inhibitor JQ1 (100, 250, or 500 nM), or the nonactive control (−)JQ1 (500 nM) for 1 hour, and then, cells were stimulated with TNF-$\alpha$ (5 ng/ml) for the indicated times. Gene expression levels of CCL-2, CCL-5, and IL-6 were analyzed by real–time quantitative PCR. Results of six independent experiments are presented. In vehicle-treated cells, there were no changes in gene expression compared with untreated cells (considered as control). (B–F) BET bromodomain inhibition regulates the transcriptional program in renal tubular epithelial cells under proinflammatory conditions. HK2 cells were stimulated with TNF-$\alpha$ (5 ng/ml) for 3 hours in the presence of JQ1 or the nonactive control (−)JQ1 (500 nM) and analyzed by (B–E) whole–genome gene expression arrays or (F) real–time quantitative PCR. (B) Pie chart of the percentages of differentially expressed probes in each cluster: I, upregulated probes in TNF-$\alpha$–stimulated cells versus untreated cells showing only

*RelB*, *NF-κB2*, and *NF-κB1*). Some of these data were corroborated by quantitative RT-PCR (Figure 1, A and F).

### BRD4, through Bromodomains, Binds to Inflammatory Gene Promoters and Regulates Transcription

The role of BRD4 in TNF-α–mediated proinflammatory gene regulation was evaluated by gene silencing. Transfection of HK2 cells with small interfering RNA (siRNA) specifically targeting BRD4 but not with scramble siRNA significantly reduced gene upregulation of *IL-6*, *CCL-2*, and *CCL-5* caused by TNF-α (Figure 2).

To determine whether JQ1 alters the direct association of BRD4 with acetylated histone-packaged promoters, chromatin immunoprecipitation (ChIP) assays with an anti-BRD4 antibody were performed. In the presence of the nonactive control (−)JQ1, BRD4 is robustly associated with the *CCL-2*, *CCL-5*, and *IL-6* promoters in TNF-α–stimulated cells (Figure 3A). However, in the presence of JQ1, the binding of BRD4 to the regulatory regions was significantly reduced, indicating that BRD4 directly regulates the expression of these genes.

BET proteins recognize acetylated residues in histones, facilitating recruitment of the transcriptional activation machinery.[28] Because active promoters are closely related to the enrichment of acetylated histones, the presence of acetylated histone H3 (AcH3) or AcH4 in the promoter region of these proinflammatory genes was investigated. AcH3 was more enriched in TNF-α–treated cells, consistent with the active transcription of these genes, and AcH3 enrichment was significantly reduced by JQ1 (Figure 3B). However, AcH4 levels were not enhanced in TNF-α–treated cells and were unchanged by JQ1 (Figure 3C).

### JQ1 Ameliorates Experimental Renal Inflammation by the Inhibition of BRD4 Binding to Proinflammatory Genes Promoters

The JQ1 effect in renal inflammation was investigated in the unilateral ureteral obstruction (UUO) model. Inflammatory cell infiltration is one of the earliest characteristics of renal damage after UUO.[40,41] The presence of infiltrating of monocytes/macrophages in obstructed kidneys was observed after 2 days and increased at 5 days (Figure 4). Treatment with JQ1 diminished the presence of infiltrating monocytes/macrophages, but only the dose of 100 mg/kg per day exerted a significant inhibitory effect (Figure 4, A and C). The immunohistochemical characterization of immune cells revealed that JQ1 significantly reduced the number of monocytes/macrophages, neutrophils, dendritic cells, and T lymphocytes in obstructed kidneys to levels similar to those of controls (Figure 4, B and D). In obstructed kidneys, renal lesions and gene upregulation of *Ngal* and *Kim1*, two biomarkers of kidney injury,[40,42] were diminished by JQ1 (Supplemental Figure 3).

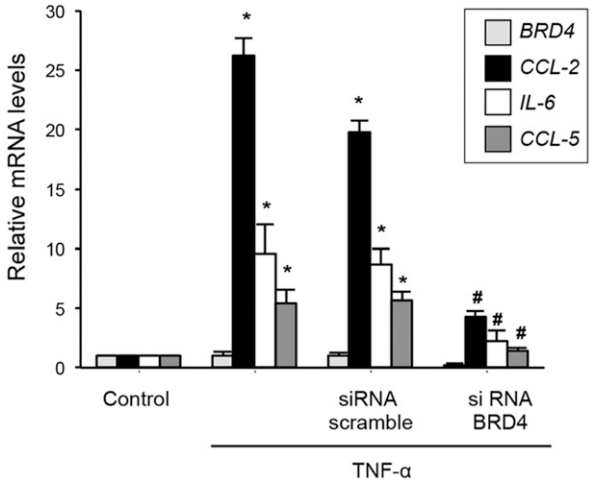


**Figure 2.** BRD4 disruption inhibits TNF-α–induced upregulation of proinflammatory genes in renal tubular epithelial cells. HK2 cells were transfected with a pool of BRD4 siRNA or nonspecific scramble siRNA (40 nM) for 48 hours. Cells were then treated with TNF-α (5 ng/ml) for 3 hours more. Gene expression of BRD4 and the proinflammatory genes *CCL-2, CCL-5* and *IL-6* was measured by real–time quantitative PCR. Data are representative of four independent experiments, and results are expressed as the means± SEM. *$P<0.05$ versus control untransfected cells; #$P<0.05$ versus nonspecific scramble siRNA.

The recruitment of inflammatory cells into the kidney is mediated by proinflammatory mediators.[43] Two days after UUO, gene upregulation of *Ccl-2*, *Ccl-5*, and *Il-6* was found. JQ1 treatment at 100 mg/kg per day significantly diminished proinflammatory gene overexpression (Figure 5, A and B) and protein levels as exemplified in CCL-2 (Figure 5C). Other inflammation/immune-related genes upregulated in the obstructed kidneys were also inhibited by JQ1, including chemokines (*Ccl-20* and *Cxcl-16*), adhesion molecules (*Icam-1*), and components of the NF-κB pathway (*NF-κB1a*) (Figure 5D). ChIP assays showed that BRD4 binding to the promoter regions of *Ccl-2*, *Ccl-5*, and *Il-6* genes was significantly greater in obstructed than in contralateral kidneys. However, this BRD4 enrichment was significantly less in obstructed kidneys from JQ1-treated mice (Figure 5E). Our results show that direct binding of BRD4 to proinflammatory gene promoters and the consequent chromatin remodeling comprise a potential mechanism explaining the renal anti–inflammatory effects of BET inhibition.

those suppressed by JQ1; II, upregulated probes in TNF-α that were unchanged after JQ1 treatment; and III, downregulated probes in TNF-α–stimulated cells. (C) Functional analysis pathways (gene ontology [GO] analysis) of genes differentially expressed in clusters I and II. D and E show representative genes of I and II clusters. Genes with >1.5-fold change were considered to be differentially expressed. (F) Data validation by real–time quantitative PCR. Data are expressed as means±SEM of three independent experiments. *$P<0.05$ versus control (vehicle-treated cells); #$P<0.05$ versus TNF-α–treated cells.

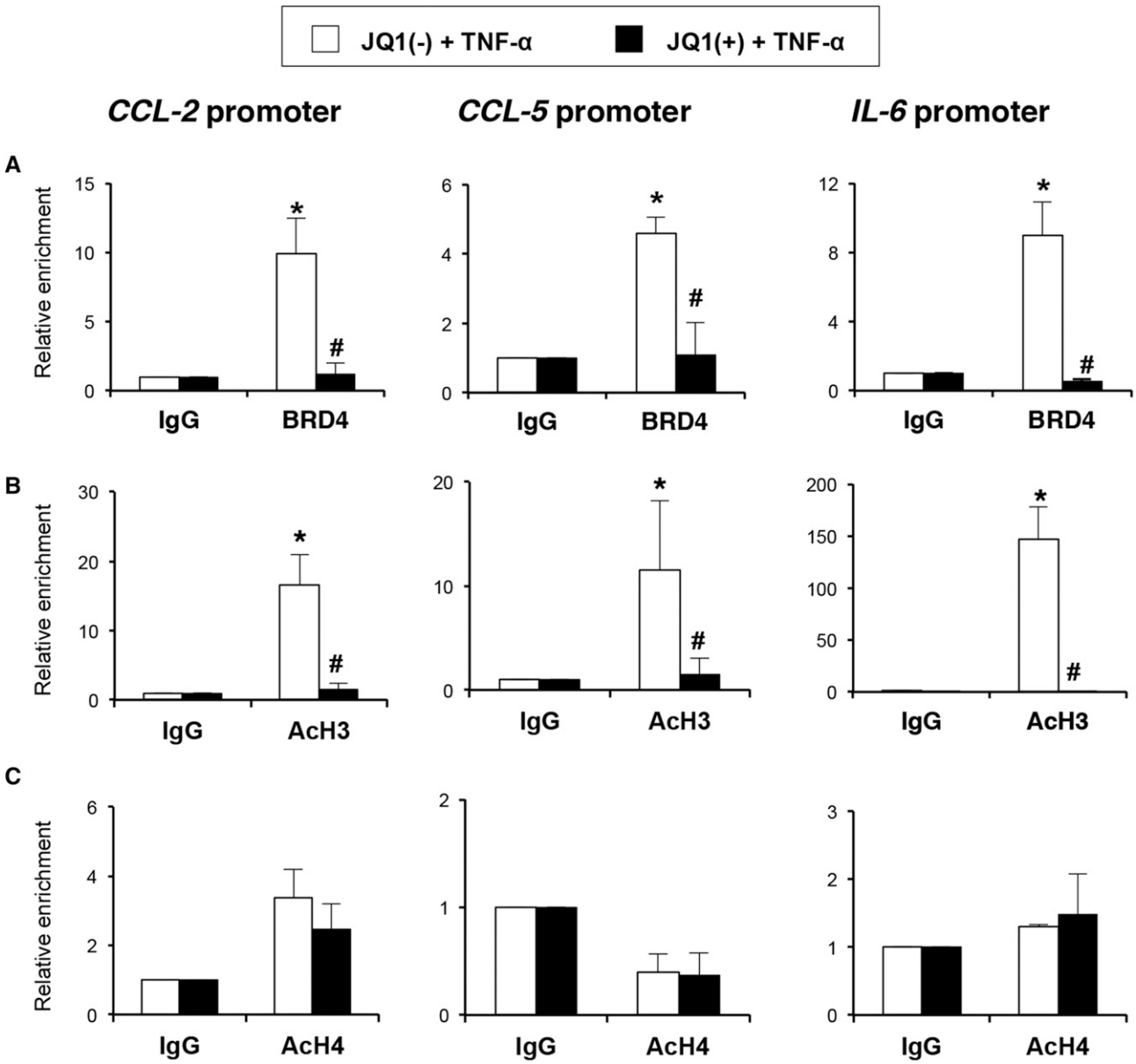


**Figure 3.** JQ1 inhibits the direct binding of BRD4 at the *CCL-2*, *CCL-5*, and *IL-6* promoters through histone acetylation in renal tubular epithelial cells. HK2 cells were pretreated with (−)JQ1 or JQ1 (500 nM) for 1 hour and further stimulated with TNF-$\alpha$ (5 ng/ml) for 3 hours more. ChIP assays were performed using specific antibodies against (A) BRD4, (B) AcH3, and (C) AcH4. Normal rabbit IgG was used as a negative control. Enrichment of BRD4–specific DNA sequences around the transcriptional sites of the human *CCL-2*, *CCL-5*, and *IL-6* promoters was measured by real–time quantitative PCR using specific primers. Data from three independent experiments are shown, in which each quantitative PCR was run in triplicate. Results are represented as the relative enrichment of BRD4 binding compared with the negative control antibody (IgG). *$P<0.05$ versus corresponding IgG; #$P<0.05$ versus (−)JQ1-treated cells.

### JQ1 Ameliorates Inflammation and Kidney Injury in the Antimembrane Basement Membrane GN Mice Model

The experimental antiglomerular basement membrane nephritis induced by nephrotoxic serum (NTS) administration is a model commonly used to study mechanisms of human crescentic GN.[44] In NTS-challenged mice, JQ1 diminished inflammatory cell infiltration (Figure 6, A and B), overexpression of proinflammatory genes (*Ccl-2*, *Ccl-5*, and *Il-6*) (Figure 6C), and biomarkers of renal injury (*Ngal* and *Kim1*) (Figure 6D). NTS-injected mice presented proliferation of intrinsic glomerular cells in the Bowman's space, resulting in the rapid deterioration of renal function, resembling human pathology.[44] JQ1 restored changes in renal function (as serum creatinine and urinary albumin) (Figure 6, E and F) and ameliorated glomerular lesions (Figure 6, G and H).

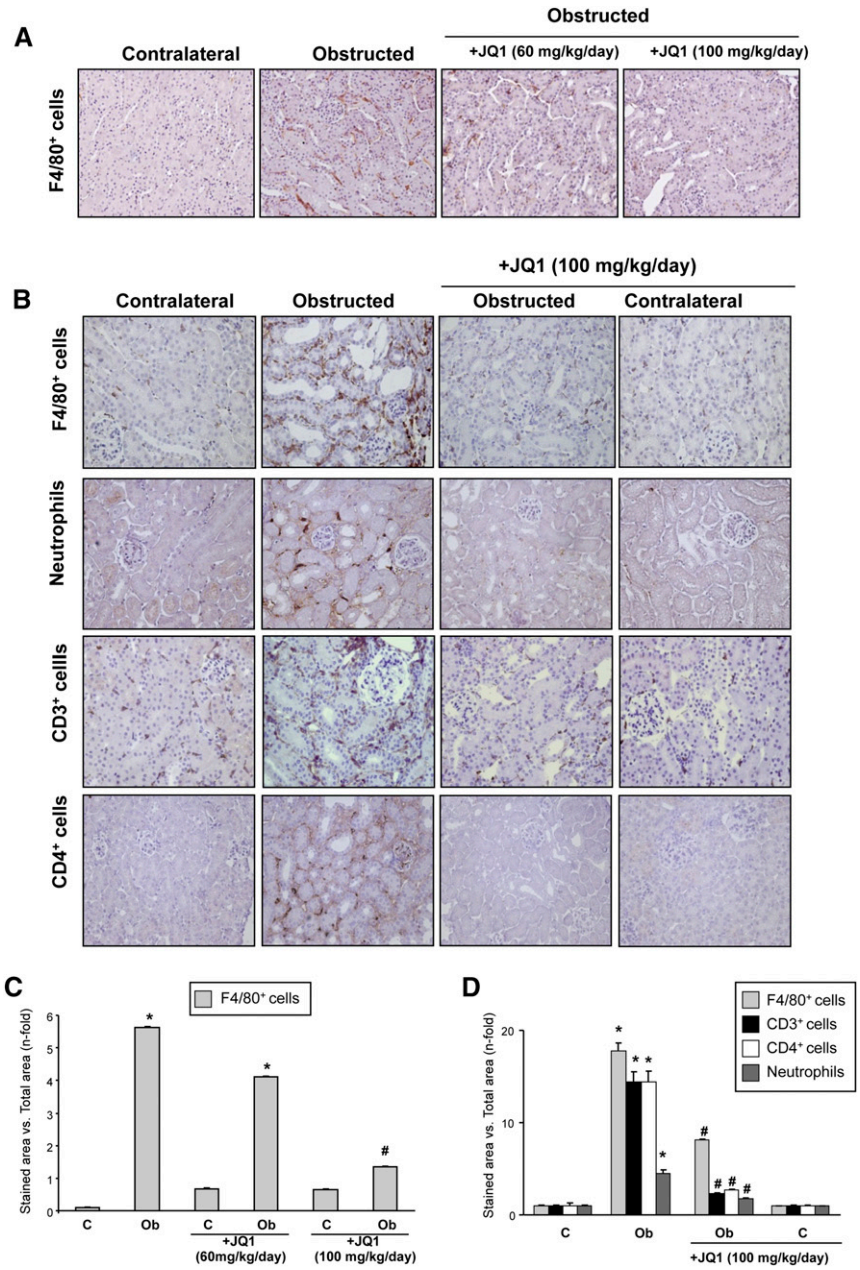


**Figure 4.** JQ1 reduces renal inflammation in the experimental models of UUO. The UUO model was done in mice and studied after (A) 2 or (B) 5 days. Some mice were treated with JQ1 (60 or 100 mg/kg per day intraperitoneally as indicated) or vehicle (10% hydroxypropyl $\beta$-cyclodextrin) starting 24 hours before UUO. In paraffin–embedded kidney sections, inflammatory cell infiltration was evaluated using antibodies against F4/80 (monocytes/macrophages/dendritic cells), myeloperoxidase (neutrophils), CD3 (T lymphocytes), and CD4 (T helper lymphocytes). A representative animal from each group is shown. Magnification, ×200×. C and D show the quantification of immunohistochemistry. Data are expressed as the means±SEM of six to eight animals per group. *$P<0.05$ versus contralateral (C); #$P<0.05$ versus vehicle–treated obstructed (Ob) kidneys.

## JQ1 Diminishes Renal Inflammation in the Angiotensin II Infusion Model

The model of systemic administration of Angiotensin II (AngII) is a nonimmune model of renal damage.[42,45] As described, AngII induced an early inflammatory response; at 3 days, increased infiltrating interstitial monocytes/macrophages and proinflammatory mediators were found that were diminished in JQ1-treated mice (Figure 7, A–C). In AngII-infused mice, there was no change in renal function (evaluated by serum creatinine levels) (Figure 7D) when inflammation appeared. In contrast, *Ngal* gene expression and protein levels in the kidney were significantly increased in AngII-infused mice and normalized by JQ1 treatment (Figure 7, E and F).

## JQ1 Modulates the Canonical NF-$\kappa$B Pathway Activation in the Kidney *In Vivo* and *In Vitro*

BRD4 recognizes and binds to the acetylated lysine-310 of the RelA NF-$\kappa$B subunit.[19,20] We hypothesized that blockade of this interaction by JQ1 might suppress the inflammatory response mediated by NF-$\kappa$B pathway activation in kidney damage.

NF-$\kappa$B remains in the cytosol of resting cells as an inactive complex formed by p50 and RelA/p65 subunits bound to the inhibitory subunit I$\kappa$B$\alpha$. One of the earliest steps in NF-$\kappa$B activation is the phosphorylation of I$\kappa$B$\alpha$ and RelA.[18] In HK2 cells, TNF-$\alpha$ increased RelA and I$\kappa$B$\alpha$ phosphorylation, but in the presence of JQ1, there were no changes in their phosphorylation levels (Figure 8, A and B). Interestingly, the nuclear levels of RelA were reduced in JQ1–treated TNF-$\alpha$–stimulated cells (Figure 8C).

Previous work by our group showed that activation of NF-$\kappa$B contributes to the progression of renal damage in UUO and AngII infusion models.[40,42,45] Blockade of RelA NF-$\kappa$B activation diminished renal inflammation in those models, reducing inflammatory cell infiltration mainly by downregulating proinflammatory genes. Treatment with JQ1 produced significantly lower renal RelA nuclear levels in obstructed kidneys than in untreated obstructed ones (Figure 8D). Similarly, JQ1 diminished renal RelA nuclear levels in the injured kidneys by NTS (Figure 8E) or AngII infusion (Figure 8F). These data show that BET inhibition blocks NF-$\kappa$B activation by lowering nuclear RelA levels and subsequent functional activation.

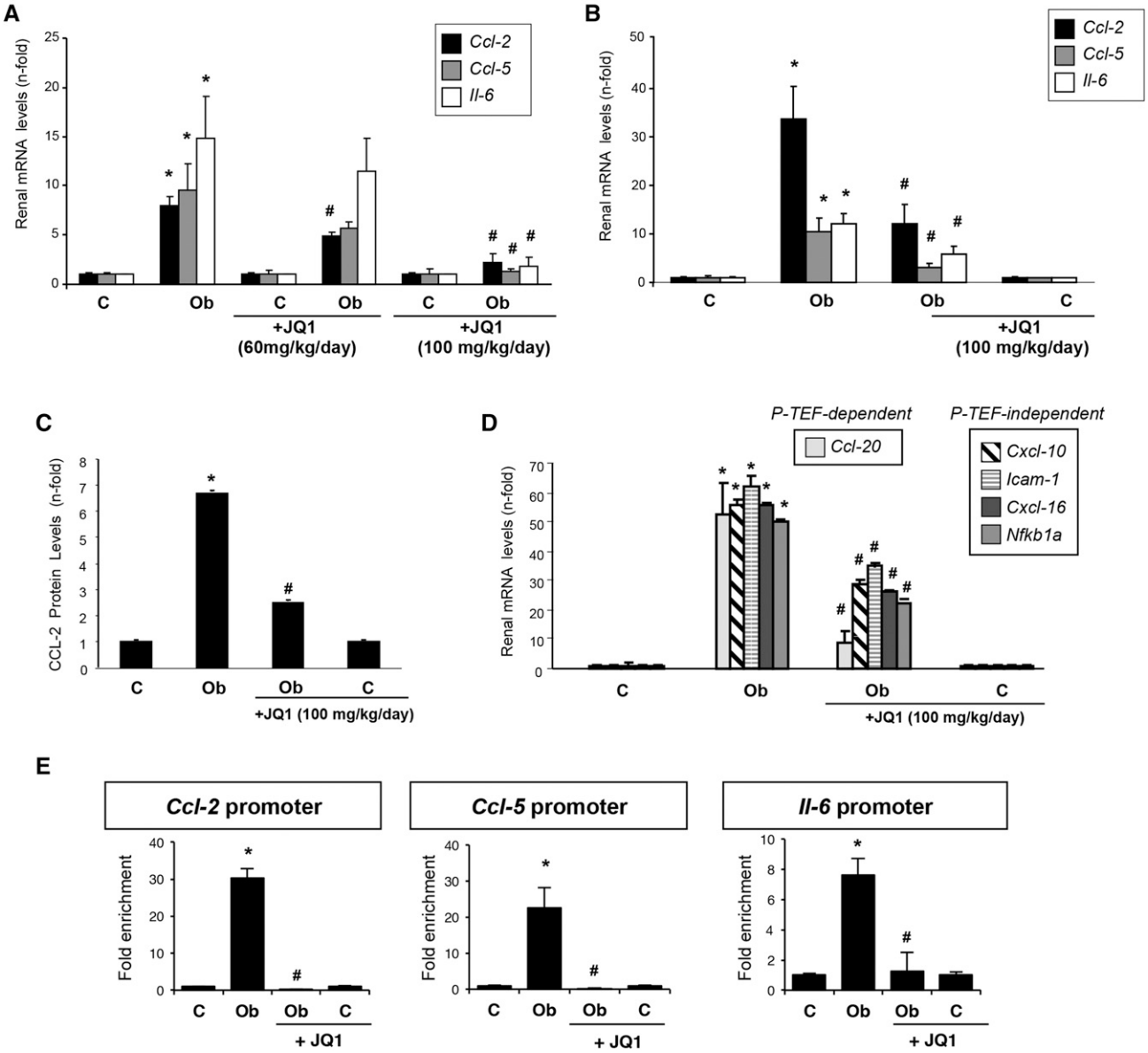


**Figure 5.** JQ1 attenuates gene expression of proinflammatory factors in obstructed kidneys. Mice were treated with JQ1 (100 or 60 mg/kg per day) or vehicle (10% cyclodextran solution) starting 24 hours before UUO and studied after 2 or 5 days. (A and B) RNA was isolated from frozen samples of whole kidney, and gene expression levels were evaluated by real–time quantitative PCR. (C) CCL-2 protein levels were evaluated by ELISA. Results are means±SEM of six to eight animals per group. Data are normalized versus contralateral untreated kidney (considered as one). (D) BRD4 downregulates the proinflammatory gene expression in the damaged kidney. (E) A ChIP assay was carried out in renal samples from UUO mice treated or not treated with JQ1 using an antibody specific for BRD4 or normal rabbit IgG, the latter being a negative control. Enrichment of BRD4 binding regions in the promoters of mouse *Ccl-2*, *Ccl-5*, and *Il-6* genes was quantified by quantitative PCR using specific primers. Data are from two independent experiments, and each quantitative PCR was run in triplicate. Results are expressed as the *n*-fold enrichment of anti-BRD4 antibody relative to a negative control antibody (normal rabbit IgG) and further normalized versus contralateral kidney (considered as one). *$P<0.05$ versus contralateral (C); #$P<0.05$ versus vehicle–treated obstructed (Ob) kidneys.

### JQ1 Inhibits Th17 Immune Response in Experimental Renal Inflammation

Growing evidence suggests that the Th17 immune response and its effector cytokine IL-17A contribute to the pathogenesis of renal diseases, including immune and nonimmune nephropathies.[46–48] In UUO kidneys, IL-17A gene and protein levels were increased and significantly downregulated by JQ1 (Figure 9, A–C, Supplemental Figure 4). Similarly, JQ1 diminished renal IL-17A production in the injured kidneys by NTS (Figure 9D).

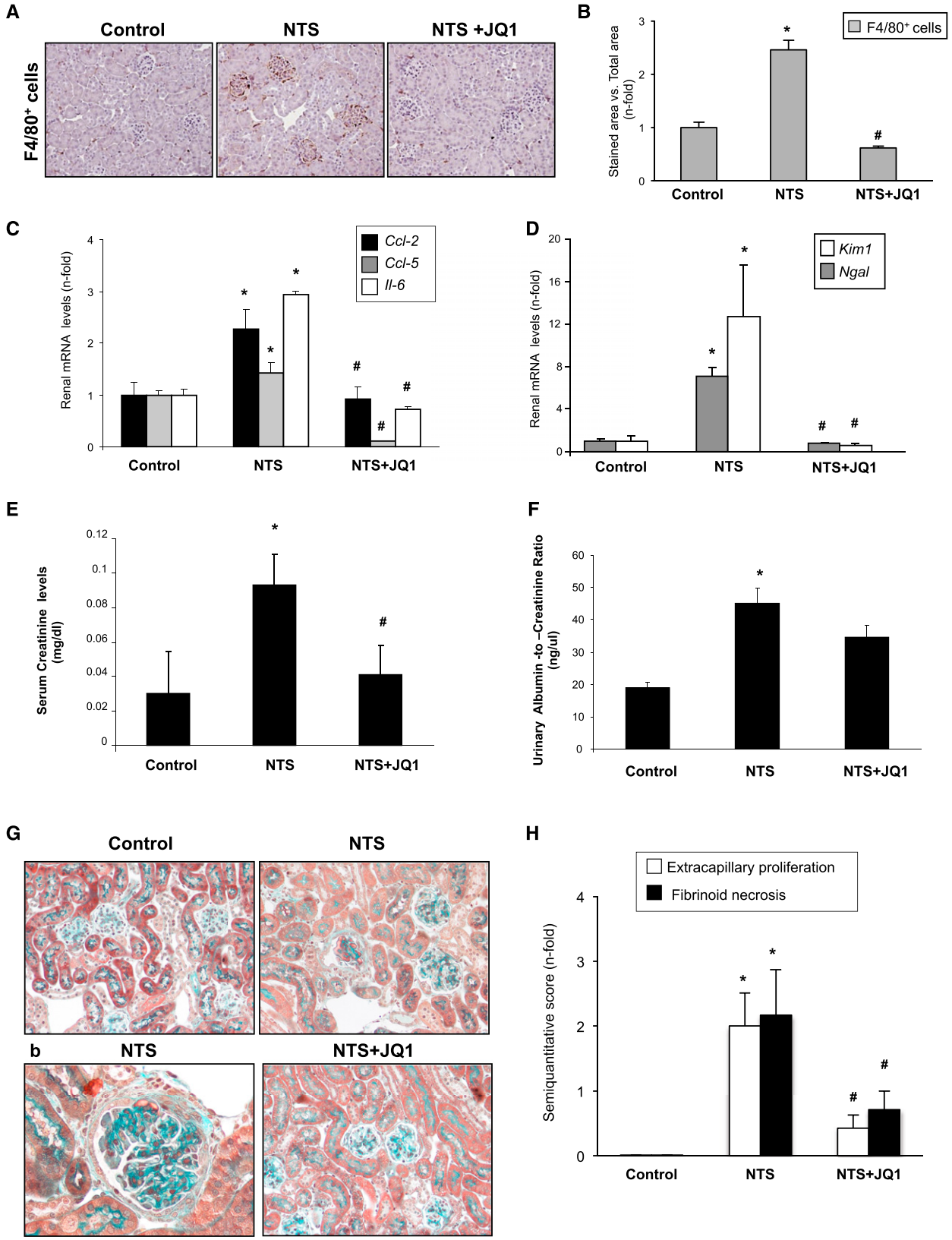


**Figure 6.** JQ1 diminishes renal inflammation in the NTS nephritis. Accelerated NTS nephritis was done in mice by administration of an antimurine glomerular basement membrane rabbit antiserum, and mice were studied 10 days after immunization. Mice were treated or not treated with JQ1 (100 mg/kg per day) starting before the first NTS injection. (A) In paraffin–embedded kidney sections of NTS mice,

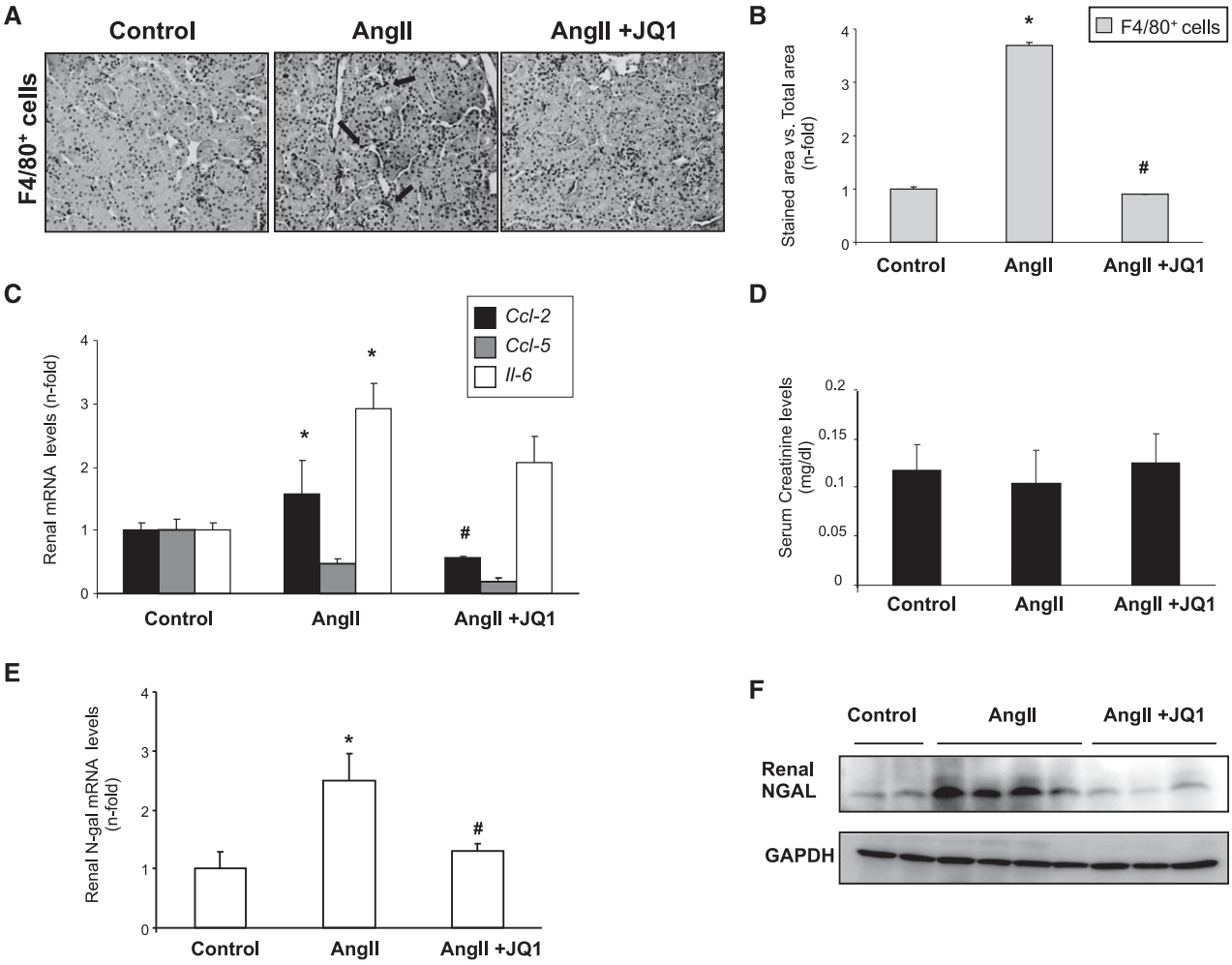


**Figure 7.** JQ1 diminishes renal inflammation in the model of AngII infusion in mice. Systemic infusion of AngII (1000 ng/kg per minute; subcutaneous osmotic mini-pumps) was done for 3 days. Mice were treated or not treated with JQ1 (100 mg/kg per day) starting 24 hours before infusion. (A) In paraffin–embedded kidney sections of AngII-infused mice, some interstitial infiltrating monocytes/macrophages were found (detected using an antibody against F4/80; marked by arrows). A representative animal from each group is shown. (B) Shows the quantification of immunohistochemistry. (C) RNA was isolated from frozen samples of whole kidney, and proinflammatory gene expression levels were evaluated by real–time quantitative PCR. (D) Data of serum creatinine levels. (E) Renal *Ngal* gene expression and (F) protein levels are shown. Data are expressed as the means±SEM of four to six animals per group. *$P<0.05$ versus control; #$P<0.05$ versus AngII-infused mice.

## DISCUSSION

Our studies show that the specific BET inhibitor JQ1 reduces renal inflammation in different models of renal damage, including UUO and NTS–and AngII–induced renal damage. We have described that JQ1 reduces renal inflammation by several mechanisms: (*1*) direct binding of BET proteins to acetylated residues of H3 histone in the promoter regions of proinflammatory genes (shown in *CCL-2*, *IL-6*, and *CCL-5*), (*2*) blockade of NF-$\kappa$B pathway activation by reducing RelA

glomerular infiltration of monocytes/macrophages was found (detected using an antibody against F4/80). B shows the quantification of immunohistochemistry. (C and D) RNA was isolated from frozen samples of whole kidney, and gene expression levels were evaluated by real–time quantitative PCR. (E) JQ1 ameliorates renal damage in the NTS nephritis. Data on (E) serum creatinine levels and (F) the ratio of albumin to creatinine in the urine are shown. Data are expressed as the means±SEM of five to seven animals per group. Morphologic lesions were evaluated by Masson trichrome staining. Treated mice with JQ1 (100 mg/kg per day) displayed fewer and less severe glomerular lesions. G shows a representative animal from each group, and the quantification of the lesion is shown in H. Magnification, ×200 in G; ×400 in G, b. *$P<0.05$ versus control; #$P<0.05$ versus NTS.

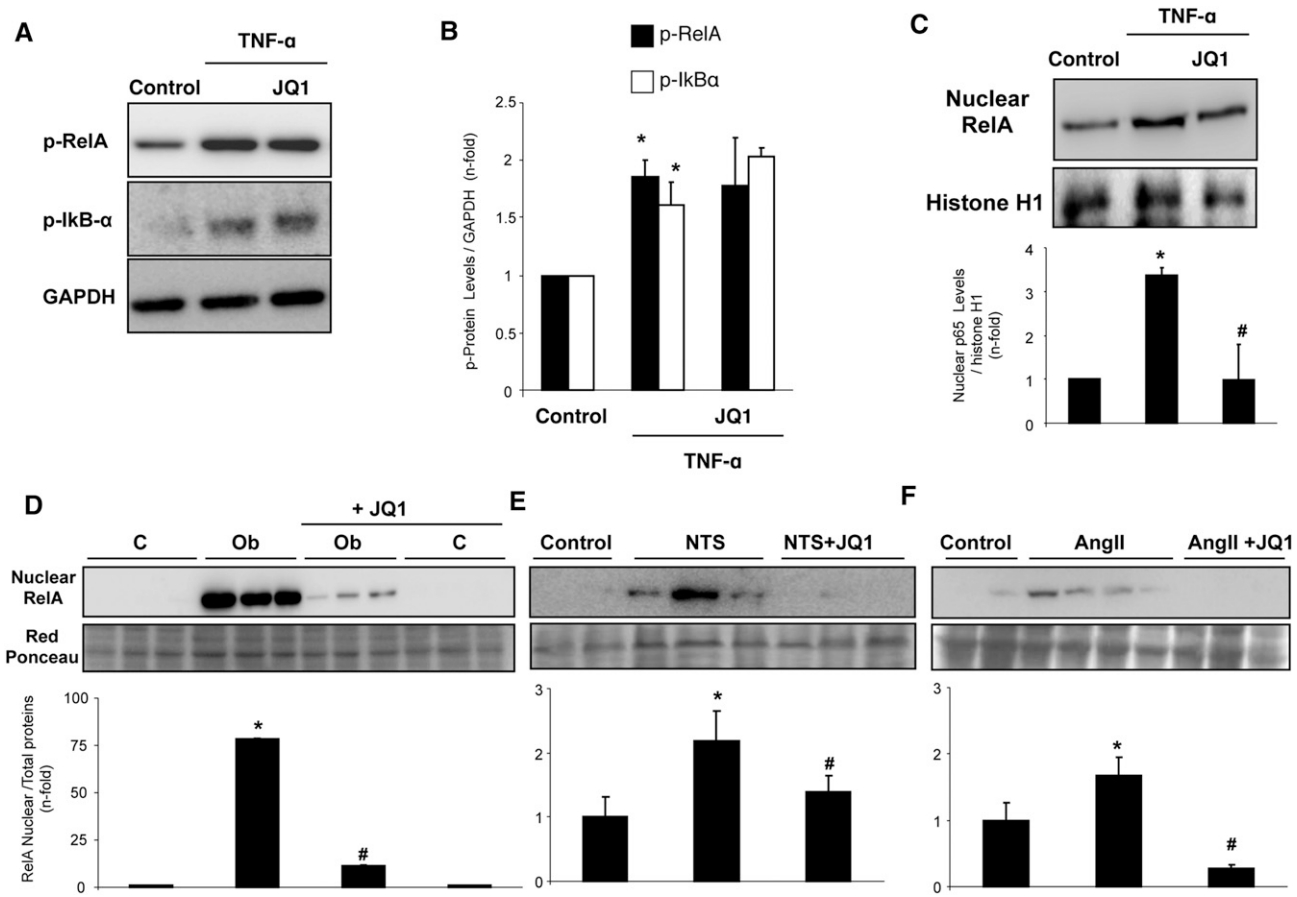


**Figure 8.** JQ1 inhibits NF-κB pathway activation in renal cells and murine damaged kidney. HK2 cells were pretreated with 500 nM JQ1 for 1 hour and stimulated with 5 ng/ml TNF-α for 20 minutes. (A) Activation of NF-κB was evaluated in terms of changes in phosphorylation levels of IκBα (p-IκBα) and the p65 NF-κB subunit (p-RelA) in total protein extracts by Western blot. GAPDH was used as the loading control. (B) Quantification. Means±SEM of three independent experiments. *$P<0.05$ versus control; #$P<0.05$ versus TNF-α. (C) Activation of NF-κB was evaluated by p65 NF-κB subunit (RelA) in nuclear extracts by Western blot. Histone H1 was used as the loading control. Means±SEM of three independent experiments. *$P<0.05$ versus control; #$P<0.05$ versus TNF-α. (D–F) In nuclear proteins isolated from different mice models treated or not treated with JQ1, p65 NF-κB levels were evaluated by Western blot. Ponceau Red was used as the loading control. *$P<0.05$ versus control mice; #$P<0.05$ versus injured kidneys (obstructed;NTS;AngII respectively). Data are expressed as the means±SEM of six to eight animals per group.

nuclear levels, and (*3*) modulation of the Th17 immune response. These results suggest that BET inhibitors could have important therapeutic applications in inflammatory renal diseases.

Evidence is emerging that epigenetic mechanisms control acute inflammation. Histone acetylation and/or deacetylation are involved in NF-κB–mediated inflammation.[49] Decreased H3 lysine-9 trimethylation in inflammatory gene promoters upregulates inflammatory gene expression in cells under hyperglycemic conditions.[50] Several studies suggest that BET proteins regulate the inflammatory process. In activated macrophages, BET inhibitors suppress the expression of cytokines (TNF-α and IL-6) and chemokines (CCL-2), protecting against endotoxic shock.[22] Moreover, BRD2 gene disruption in bone marrow–derived macrophages blunts proinflammatory cytokine (IL-6 and TNF-α) production.[30] In experimental renal damage and cultured tubular epithelial cells under inflammatory conditions, we found that JQ1 reduces the expression of many proinflammatory mediators by modulating BRD4 actions. Gene silencing and ChIP assays show a key role for BRD4 in regulating the proinflammatory genes *CCL-2*, *IL-6*, and *CCL-5* at the promoter level. JQ1 targeting the interaction between BRD4 and acetylated histones reduces proinflammatory gene transcription, showing that chromatin remodeling is a mechanism by which BET inhibition might impair the renal inflammatory response. JQ1 treatment reduced histone H3 acetylation without affecting histone H4, suggesting that BRD4 binding to the promoter region prevents recruitment of H3–specific histone deacetylases.

Many experimental studies have shown that NF-κB blockade attenuates renal inflammation and ameliorates disease progression, including our studies in the UUO and AngII

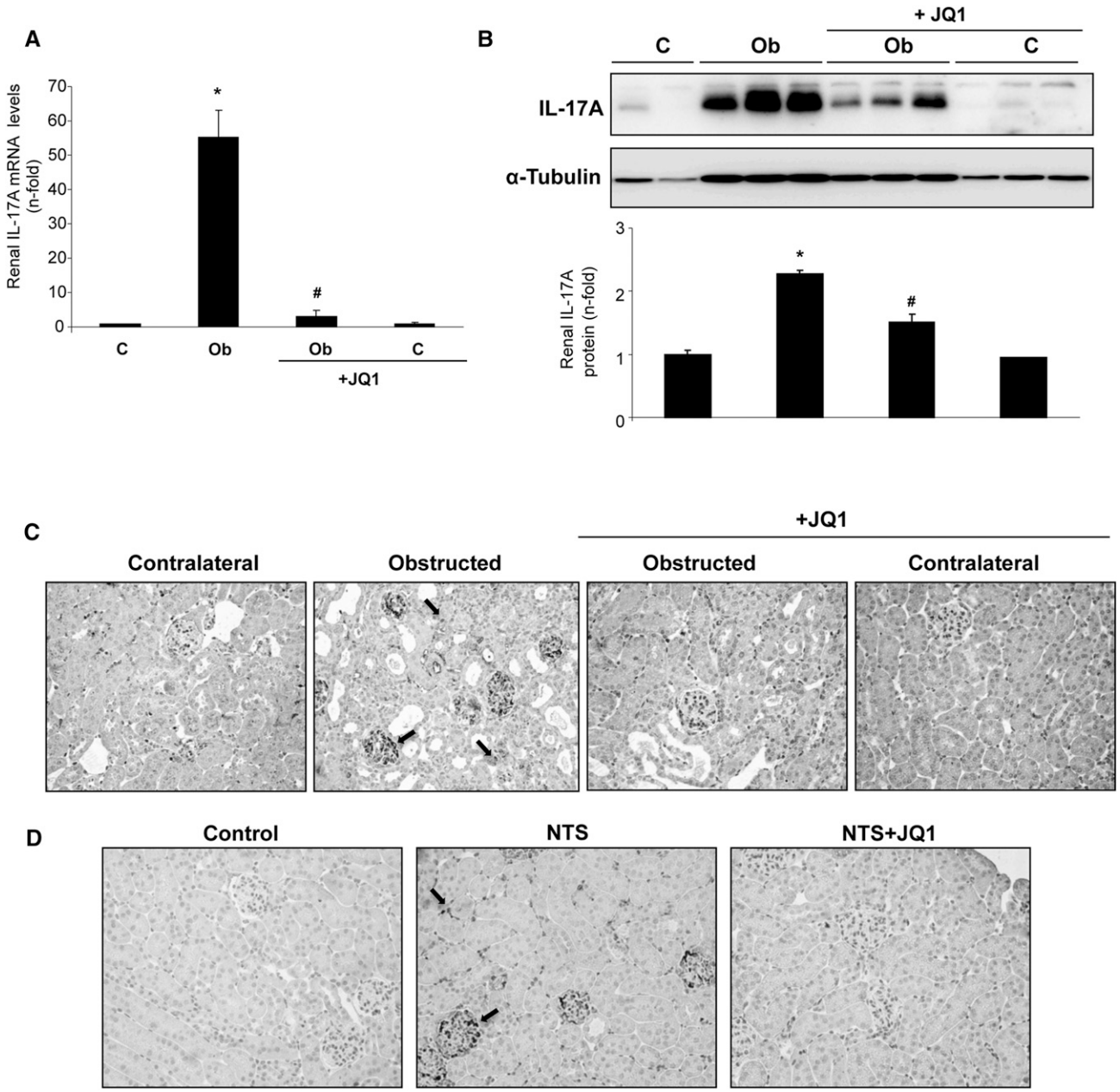


**Figure 9.** JQ1 diminishes the Th17 immune response in experimental renal damage. Renal IL-17A (A) gene or (B) protein levels were evaluated in the UUO model by real time or Western blot, respectively; GAPDH or Tubulin was used as the loading control, respectively. Data are expressed as the means±SEM of six to eight animals per group. C, control; Ob, obstructed. *$P<0.05$ versus control mice; #$P<0.05$ versus injured kidney. Immunolocalization of IL-17A in (C) UUO or (D) NTS mice treated or not treated with JQ1. The figure shows a representative image of five mice per group.

infusion model.[18,41] We found *in vivo* and *in vitro* that JQ1 reduced RelA nuclear levels and thereby, NF-κB transcriptional activation, revealing a chromatin-independent mechanism involved in the anti-inflammatory effect of BET inhibitors in injured kidneys. Intensive research has unraveled the role of BET proteins in NF-κB pathway regulation, showing that BRD4 is a coactivator of NF-κB.[20,51,52] BRD4 binding to acetylated lysine-310 of RelA is essential for recruiting BRD4 and CDK9 to the promoters of specific NF-κB target genes.[19] Our data suggest that inhibition of BRD4 binding to acetylated RelA and subsequent NF-κB–mediated proinflammatory gene expression downregulation are other mechanisms contributing to the anti-inflammatory effects of JQ1 in renal damage.

Whole–genome expression arrays in TNF-α–stimulated tubular epithelial cells showed that some NF-κB–regulated

genes were downregulated by JQ1, whereas others were unchanged. Many components of the NF-κB pathway, including NF-κB1/p50, NF-κB2/RelA/p65, NF-κBIA/IκBα, RelB, A20/TNFAIP3, and TNIP1, were among the genes unaffected by this inhibitor. Previous studies have shown that JQ1 displaces BRD4 and consequently, P-TEFb from promoter regions, leading to a decrease in Pol II elongation at active genes.[17] P-TEFb participates in the transcriptional elongation of a subset of NF-κB genes, whereas others are P-TEFb independent.[52–54] Amir-Zilberstein *et al.*[52] reported that NF-κB–controlled genes lacking a TATA-box sequence in their core promoters are regulated independently of the P-TEFb elongation factor. Some of our results support this hypothesis; all of the aforementioned JQ1–unaffected, NF-κB–regulated genes are TATA less and therefore, not regulated by P-TEFb and BRD4. Amir-Zilberstein *et al.*[52] also suggested that a TATA box is a prerequisite for P-TEFb to bind to NF-κB–regulated gene promoters. However, we observed that, although several JQ1–downregulated, NF-κB–regulated genes (*e.g.*, *CCL-5*, *CCL-20*, and *IL-8*) have a TATA–box core promoter, many others are TATA less (*e.g.*, *LTB*, *CSF2*, *MMP9*, and *CCND1*). Our data suggest that JQ1 inhibited those P-TEFb–dependent, NF-κB–controlled genes by a chromatin-independent process but mediated them by direct effect on RelA/p65 levels.

Our *in vitro* data showed that JQ1 only reduces RelA nuclear levels, without affecting the basal or phosphorylated levels of cytosolic NF-κB proteins. Moreover, JQ1 preferentially inhibits the upregulation of proinflammatory genes under NF-κB control, whereas key NF-κB pathway components, such as NF-κB1/p50, NF-κB2/RelA/p65, NF-κBIA/IκBα, RelB, and A20/TNFAIP3, were unaffected. These data are consistent with the observation that TNF-α stimulation induced acetylated lysine-310–dependent recruitment of BRD4 to the promoter of proinflammatory genes, with no effect on A20/TNFAIP3 expression.[19] Our results suggest that inhibiting the acetylated RelA/Brd4 interaction with BET-bromodomain inhibitors may provide a novel pharmacologic strategy to block NF-κB activation in inflammatory renal disease. This strategy may have more selective anti–inflammatory effects than using NF-κB kinase inhibitors or proteasome-targeted drugs.

Studies of genetically modified mice or using neutralizing antibodies against IL-17A or its receptor have shown the involvement of Th17 immune responses in chronic inflammatory diseases,[55–60] including immune and nonimmune experimental renal damage.[47,48] The presence of Th17 cells in UUO-damaged kidneys has been described.[47,48] We have confirmed this observation, detecting IL-17A$^+$ cells by immunohistochemistry and upregulation of IL-17A mRNA and protein. Treatment with JQ1 practically abolished kidney IL-17A levels in UUO and NTS-induced nephritis. BRD4 and BRD2 bind directly to the chromatin in the CNS2 regulatory region of the *IL-17a* gene controlling its expression.[59] *In vitro*, other Th17–related cytokines, such as CCL-20 and CSF-1, were downregulated by JQ1. Moreover, BET inhibition suppresses Th17 differentiation from human and mouse naive CD4$^+$ T cells and the activation of previously differentiated Th17 cells.[16] Accordingly, BET inhibition protects against development of collagen-induced arthritis and experimental autoimmune encephalomyelitis caused by the impaired generation and function of Th17 cells.[59] Our results expand the strategies for neutralizing IL-17A–mediated inflammatory effects in the kidney by using bromodomain inhibitors.

During renal damage, various extracellular signals promote rapid induction of proinflammatory gene expression by activating and recruiting transcription factors and/or transcriptional coactivators to the promoter regions. Our findings highlight the importance of BET proteins in regulating the inflammatory response in the kidney, where they act as epigenetic readers that recognize acetylation marks in histones and other proteins, including transcription factors (as described for NF-κB). Additionally, BET proteins help regulate the mammalian transcription complex that initiates the transcription of proinflammatory genes. Our data show that blocking BET protein bromodomains with small molecule inhibitors, such as JQ1, might be a powerful therapeutic strategy for inflammatory renal diseases. These findings identify BRD4 as a critical genomic regulator of renal inflammation and provide mechanistic insights into the intrinsic epigenetic vulnerabilities of inflammatory disease that can be exploited for pharmacologic intervention.

## CONCISE METHODS

### Experimental Models

All animal procedures were performed according to the guidelines of animal research for the European Community and with prior approval by the Ethics Committee of the Health Research of the IIS-Fundación Jiménez Díaz. The BET bromodomain inhibitor JQ1, a thieno-triazolo-1,4-diazepine, was synthesized and provided collaboratively by James Bradner (Dana-Farber Cancer Institute, Boston, MA).[5] For *in vivo* studies, JQ1 was dissolved in 10% hydroxypropyl β-cyclodextrin and used at a therapeutic dose (100 or 60 mg/kg per day intraperitoneally) as previously described.[31]

The model of UUO was established in male C57BL/6 mice under isoflurane-induced anesthesia as described; the left ureter was ligated with silk (5/0) at two locations and cut between ligatures to prevent urinary tract infection (obstructed kidney).[40,47] Some animals were treated daily with JQ1 from 1 day before UUO and studied after 2 and 5 days ($n$=6–8 mice per group). At the time of euthanasia, animals were anesthetized with 5 mg/kg xylazine (Rompun; Bayer HealthCare, Whippany, NJ) and 35 mg/kg ketamine (Ketolar; Pfizer), and the kidneys were perfused *in situ* with cold saline before removal. Studies compared both kidneys (contralateral versus obstructed) in each mouse. Kidneys were further processed for immunohistochemistry (fixed and paraffin embedded) or ChIP assays (fixed in 1% formaldehyde followed by quenching with 0.125 M glycine) or snap frozen in liquid nitrogen for RNA and protein studies.

**Table 1.** Gene assays

| Gene | Mouse or Human Assay Identification |
|---|---|
| Mouse assay identification (FAM) | |
| *Ccl-2* | Mm00441242_m1 |
| *Ccl-5* | Mm01302428_m1 |
| *Il-6* | Mm00446190_m1 |
| *Ngal* | Mm01324470_m1 |
| *Kim-1* | Mm00506686_m1 |
| Mouse assay identification (VIC) | |
| *gapdh* | Mm99999915_g1 |
| Human assay identification (FAM) | |
| *CCL-2* | Hs00234140_m1 |
| *CCL-5* | Hs00174575_m1 |
| *IL-6* | Hs00174131_m1 |
| *BRD2* | Hs01121986_g1 |
| *BRD4* | Hs04188087_m1 |
| *LTB* | Hs00242739_m1 |
| *CSF2* | Hs00929873_m1 |
| *CCL-20* | Hs01011368_m1 |
| *NFKB1A* (*IκBα*) | Hs00355671_g1 |
| *CXCL-16* | Hs00222859_m1 |
| Human assay identification (VIC) | |
| *18s eukaryotic ribosomal* | 4210893E |
| *GAPDH* | Hs02758991_g1 |

The model of systemic infusion of AngII was done in C57BL/6 mice subcutaneously (ALZET Osmotic mini-pumps Model 2001; Alza Corp., Palo Alto, CA) at the dose of 1000 ng/kg per minute for 3 days as described.[42,45] Some animals were also daily treated with JQ1 from 1 day before AngII infusion. As control, saline infusion was done.

The accelerated NTS nephritis was produced in male C57BL/6 mice using antimurine glomerular basement membrane rabbit antiserum as described.[44] Mice were injected with 50 $\mu$l NTS diluted 1:10 in sterile saline on day 1. Then, mice were injected with 4 $\mu$l/g body wt on days 2 and 3, and they were studied 10 days later. Some animals were also daily treated with JQ1 starting 1 day before the first NTS administration. The renal lesions were evaluated by Masson staining. Extracapillary proliferation ratio was calculated by counting injury and normal glomeruli (60–70 glomeruli for each renal sample). The proportion of pathologic glomeruli was evaluated by examination of at least 50 glomeruli per section by an examiner masked to the experimental conditions.

## Cell Cultures and Treatments

Human renal proximal tubular epithelial cells (HK2 cell line; ATCC CRL-2190; American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium (Gibco, Carlsbad, CA) as described elsewhere.[42] JQ1 and its enantiomer (−)JQ1 (Selleckchem), the latter used as the nonactive control, were dissolved in DMSO (0.25%). Cells were maintained in serum-free medium for 24–48 hours before experiments.

## Cell Transfection and Gene Silencing

Subconfluent HK2 cells were transfected with 40 nM Dharmacon ON-TARGETplus Human BRD4 (L-004937) SMARTpool siRNA using DharmaFECT 1 Transfection Reagent (Dharmacon). As control, a nonspecific scrambled siRNA was used. Cells were then incubated in serum-free medium for 48 hours before stimulation. Knockdown of BRD4 was validated by quantitative RT-PCR.

## Histology and Immunohistochemistry

Paraffin–embedded kidney sections (5 $\mu$m) were stained using standard histology procedures as described elsewhere.[47] Briefly, sections were incubated for antigen retrieval (PTlink System; Dako) followed by blockade of nonspecific bindings, incubated with primary antibodies and biotinylated secondary antibodies, and developed by standard procedures. The specificity was checked by omission of primary antibodies. The positive-stained areas were assessed in 10 randomly chosen fields (×200 magnification) using Image-Pro Plus software. Data are expressed as the positive-stained area relative to the total area. The primary antibodies used were myeloperoxidase, CD3, CD4 (ready to use; Dako), and F4/80 (1:50; Serotec).

## Protein Studies

Total protein samples for frozen renal tissue were isolated in lysis buffer as described[45] or NE-PER Reagent (Pierce, Rockford, IL) to obtain nuclear and cytoplasmic fractions. Proteins (20–100 $\mu$g per lane; quantified using a BCA protein assay kit) were separated on 8%–12% polyacrylamide-SDS gels under reducing conditions as described.[47] The quality of proteins and the efficacy of protein transfer were evaluated by Ponceau Red staining (not shown). The following primary antibodies were used: p-p65 (Ser536; 1:500; Cell Signaling Technology, Danvers, MA), p65 (Sc-372) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), p-I$\kappa$B$\alpha$ (Sc-8404) (Ser32; 1:500; Santa Cruz Biotechnology), BRD4 (1:2000; A301–985A; Bethyl), tubulin (1:5000; Sigma-Aldrich, St. Louis, MO), histone H1 (Sc-8030)

**Table 2.** ChIP primers

| Gene | Forward primers (5′-3′) | Reverse primers (5′-3′) |
|---|---|---|
| **Human** | | |
| *CCL-2* | CCCTCTGCCCGCTTTCAATA | CTTGGGGAATGAAGGTGGCT |
| *CCL-5* | ATACCGGCCAATGCTTGGTT | TCCACGTGCTGTCTTGATCC |
| *IL-6* | CACCCTCACCCTCCAACAAA | TTCTCTTTCGTTCCCGGTGG |
| **Mouse** | | |
| *Ccl-2* | TGATGCTACTCCTTGGCACC | GGCTTCAGTGAGAGTTGGCT |
| *Ccl-5* | CTGCTACCCTGGCTCCCTAT | TGAGGATGATGGTGAGGGCA |
| *Il-6* | TGTGGGATTTTCCCATGAGT | TGCCTTCACTTACTTGCAGAGA |

(1:500; Santa Cruz Biotechnology), $\beta$-actin (1:10,000; A5441; Sigma-Aldrich), and GAPDH (1:5000; Chemicon International).

### Gene Expression Studies

Total RNA from cells or frozen renal tissue was isolated with TriPure Reagent (Roche, Basel, Switzerland). cDNA was synthesized with a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using 2 $\mu$g total RNA primed with random hexamer primers following the manufacturer's instructions. Next, quantitative gene expression analysis was performed by real-time PCR on an AB7500 Fast Real-Time PCR System (Applied Biosystems) using fluorogenic TaqMan MGB Probes and primers designed by Assay-on-Demand gene expression products (Table 1). Data were normalized to GAPDH RNA expression. The mRNA copy numbers were calculated for each sample by the instrument software using the Ct value (arithmetic fit point analysis for the lightcycler).

### ChIP

Cells ($10^7$) were fixed with 1% formaldehyde (Sigma-Aldrich) followed by quenching. DNA fragments 500–1000 bp long were generated by shearing chromatin on a BioRuptor (Diagenode). The shared chromatin was incubated with BRD4 antibody (A301–985A; Bethyl) or normal rabbit IgG (negative control; Upstate Biotechnologies Inc.) and recovered with protein A-agarose/salmon sperm DNA (Upstate Biotechnologies Inc.). After reversal, crosslinked, and proteinase K treatment, DNA was extracted and analyzed by quantitative RT-PCR with specific primers (Table 2). Relative enrichment was calculated as the percentage of input DNA for each sample using the formula % input $=2\ \exp[(\text{Ct [unbound]} - \log_2 \text{[unbound dilution factor]}) - \text{Ct (bound)}] \times 100$ and normalized to normal rabbit IgG antibody (considered as one).

ChIP assays in kidney samples were produced using the High Cell ChIP Kit (Diagenode) following the manufacturer's instructions using specific primers (Table 2). Results were shown as the *n*-fold enrichment of anti-BRD4 antibody over a negative control antibody (normal rabbit IgG).

### Human Gene Expression Arrays/Profiles

Total RNA was isolated using the RNAqueous Micro Kit (Ambion) according to the manufacturer's instructions. RNA quality and quantity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and a fluorometric Qubit 2.0 Assay (Invitrogen, Carlsbad, CA), respectively. RNA was hybridized to an Illumina Expression Array HumanHT-12, version 3.

Expression data were analyzed using R packages from the Bioconductor project (www.bioconductor.org) and lumi packages.[61] For the detection of differentially expressed genes, a linear model was fitted to the microarray data, and empiric Bayes moderated *t* statistics were calculated using the limma package of Bioconductor.[62] Genes with a $>1.5$-fold change and adjusted $P<0.05$ were considered significant according to the technical specifications of Illumina. For gene functional analysis of the differentially expressed genes, we used the DAVID Go Web–based tool.[63] The raw gene expression data have been submitted to the NCBI Gene Expression Omnibus under accession number GSE71602.

### Biochemical Analyses

Serum creatinine levels were determined by the Biochemical Analysis Service at Fundacion Jimenez Diaz Hospital, and data are expressed as milligrams per deciliter. Urinary concentrations of albumin were normalized by creatinine concentrations in individual mice samples. Urinary creatinine levels were determined by the Creatinine Assay Kit (ab65340; Abcam, Inc., Cambridge, MA). Albumin excretion levels were determined by using a mouse Albumin ELISA Kit (ab108792; Abcam, Inc.). Albumin-to-creatinine ratio was expressed as nanograms per microliter.

### Statistical Analyses

Results are expressed as the *n*-fold increase with respect to the control as the mean$\pm$SEM. In mouse studies, data were obtained comparing both kidneys (contralateral versus obstructed) in each mouse. Mean results from untreated contralateral kidneys were taken as reference values (control). Differences between groups were assessed by the Mann–Whitney test. Statistical significance was concluded for values of $P<0.05$. These analyses were done using SPSS, version 16.0 (IBM SPSS, Chicago, IL).

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## DISCLOSURES

None.

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