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Regulation of NO Synthesis, Local Inflammation, and Innate Immunity to Pathogens by BET Family Proteins

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Transcriptional activation of the Nos2 gene, encoding inducible nitric oxide synthase (iNOS), during infection or inflammation requires coordinate assembly of an initiation complex by the transcription factors NF-κB and type I interferon-activated ISGF3. Here we show that infection of macrophages with the intracellular bacterial pathogen *Listeria monocytogenes* caused binding of the BET proteins Brd2, Brd3, and, most prominently, Brd4 to the Nos2 promoter and that a profound reduction of Nos2 expression occurred in the presence of the BET inhibitor JQ1. RNA polymerase activity at the Nos2 gene was regulated through Brd-mediated C-terminal domain (CTD) phosphorylation at serine 5. Underscoring the critical importance of Brd for the regulation of immune responses, application of JQ1 reduced NO production in mice infected with *L. monocytogenes*, as well as innate resistance to *L. monocytogenes* and influenza virus. In a murine model of inflammatory disease, JQ1 treatment increased the colitogenic activity of dextran sodium sulfate (DSS). The data presented in our study suggest that BET protein inhibition in a clinical setting poses the risk of altering the innate immune response to infectious or inflammatory challenge.

Innate immunity results from the rapid recognition of and response to invading microorganisms. Binding of pathogen-associated molecular patterns (PAMPs) and signaling by pattern recognition receptors (PRRs), located at the cell surface, endosomal membranes, or the cytoplasm, cause profound changes in host gene expression. This enables the innate immune system to mount an adequate antimicrobial response (1, 2). The bacterial pathogen *Listeria monocytogenes* is a well-studied example of a microbe replicating in the host cell cytoplasm (3, 4). Cellular uptake commences when the bacterium is recognized by cell surface receptors that cause formation of an *L. monocytogenes*-containing endo- or phagosomal compartment. The subsequent expression and release of the bacterial hemolysin listeriolysin O (LLO) allow *L. monocytogenes* to disrupt the vacuolar membrane and escape its confinement to move and replicate in the cytoplasm. In keeping with its mode of uptake, *L. monocytogenes* stimulates signaling by cell surface-associated Toll-like receptors (TLRs), endosomal TLRs, and various cytoplasmic receptors, including those recognizing cyclic dinucleotides or DNA (5–8). Together these receptors activate multiple signaling pathways, including those leading to NF-κB activation or the synthesis of type I interferons (IFN-I). Whereas NF-κB activation is a property shared by most *L. monocytogenes* pattern recognition receptors, irrespective of their cellular localization, activation of interferon regulatory factors (IRFs) as a prerequisite for IFN-I synthesis is an exclusive property, in most *L. monocytogenes*-infected cells, of signals generated in the cytoplasm (9, 10).

Activation of the IFN-I receptor complex (IFNAR) sets off Jak-Stat signal transduction to generate tyrosine-phosphorylated Stat1 and Stat2, which heterodimerize and associate with a third subunit, IRF9, to assemble the transcriptional activator ISGF3 (11). Through ISGF3, IFN-I influence a significant part of the antimicrobial gene signature (12, 13). The target genes fall into two main categories. The classical interferon-stimulated genes (ISGs) contain a large fraction of antiviral genes, and IFN-I and ISGF3 suffice to initiate their transcription. A second class of genes utilizes IFN-I–ISGF3 as a necessary signal but requires further input from other signaling pathways. A prominent member of this class is the Nos2 gene, encoding inducible nitric oxide synthase (iNOS) (1, 2, 14, 15). IFN-I produced by *L. monocytogenes*-infected cells activate the ISGF3 complex. ISGF3 synergizes with NF-κB in the synthesis of Nos2 mRNA (3, 4, 16). NO synthase converts arginine to citrulline and an NO radical. $Nos2^{-/-}$ mice show increased sensitivity to *L. monocytogenes* infection (17), but NO production is not generally correlated with bacterial replication (18). According to recent findings, NO reduces survival of *L. monocytogenes*-infected cells and increases pathogen spread (9, 10, 19, 20). The data suggest a complex role of NO during *L. monocytogenes* infection that may not be limited to direct cytotoxic action.

Transcriptional induction of genes during an innate immune response is regulated either by *de novo* formation of an initiation complex and the recruitment of RNA polymerase II (Pol II) or by enabling a promoter-bound, paused polymerase to commence with elongation (11–13, 21–24). Preformed initiation complexes include TFIIH and Pol II phosphorylated at S5 of multiple amino acid heptarepeats that constitute its carboxy-terminal domain (CTD) (12, 13, 25). To proceed to elongation, the stalled polymerase requires infection-borne signals that enable promoter binding of the p-TEFb complex and activate the associated cyclin-dependent kinase 9 (CDK9). CDK9 phosphorylates S2 contained within the Pol II CTD heptarepeats, thus triggering the CTD association of proteins necessary for elongation. CDK9-mediated phosphor-

Received 14 October 2013 Accepted 10 November 2013
Published ahead of print 18 November 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/MCB.01353-13.
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doi:10.1128/MCB.01353-13

ylation also removes an elongation block established by the DSIF/NELF proteins (22). The BET family protein Brd4 was shown to copurify with p-TEFb and to guide it to acetylated chromatin (26–28). Brd4 contains two bromodomains and an extraterminal (ET) domain (29). Deletion of the bromodomains disrupted the interaction between Brd4 and the p-TEFb subunit cycT in one study (28), whereas in a more recent report, a small portion of the ET, the PID, was found to contact p-TEFb via both its cycT and CDK9 subunits (30). Accordingly, pTEF-b may be recruited to promoter chromatin by simultaneous bromodomain-mediated interaction with acetylated histones and PID-mediated binding to pTEFb. Reportedly, this mechanism ensures a rapid response to lipopolysaccharide (LPS) of a large number of genes, and the NF-κB pathway acts as a regulator of the necessary chromatin modifications (31). While the BET proteins Brd2 and Brd3 appear to be less or not at all involved in p-TEFb binding, their bromodomains are of paramount importance for transcriptional regulation. Brd2 and Brd3 bind H4 at specific acetyllysine residues (32, 33). Effects on transcription result from the additional ability to contact transcriptional activators (34). In addition, both Brd2 and Brd3 act as histone chaperones for transcription from nucleosomal templates *in vitro* (33). Like Brd2, Brd4 is capable of direct interaction with transcription factors and mediator proteins (35). For example, NF-κB is contacted by the Brd4 bromodomains when its RelA/p65 subunit is acetylated at lysine-310 (36).

Whereas immediate early genes in the inflammatory response are regulated at the p-TEFb recruitment step from preassembled initiation complexes, target genes of ISGF3 require SWI/SNF-dependent chromatin remodeling and are regulated predominantly at the level of initiation complex formation (21, 37–39). This is indicated by the increase in TFIID and Pol II binding associated with the onset of IFN-I-induced transcriptional activation. Consistent with this, *de novo* formation of an initiation complex at the Nos2 promoter occurs when IFN-I are produced during infection with *L. monocytogenes*. Our recent work has clarified the mechanism underlying the cooperative activities of ISGF3 and NF-κB in forming a preinitiation complex (PIC) at the Nos2 gene (16). The NF-κB signal is established rapidly after infection and causes recruitment of the general transcription factor TFIIH and the associated Pol II kinase CDK7. Even though the first wave of NF-κB binding is transient, TFIIH-CDK7 persists at the promoter until ISGF3 binds, with a delay of several hours. ISGF3 brings about Pol II binding, and Pol II is now immediately targeted by CDK7 for phosphorylation of S5 within the CTD heptarepeats. This mechanism ensures transcriptional memory of the NF-κB signal at the Nos2 promoter that lasts through the delay caused by IFN-I synthesis and ISGF3 activation. CTD phosphorylation at S5 is essential for the ability of Pol II to clear the transcriptional start site (TSS). However, elongation of Nos2 transcription additionally requires pTEFb-mediated S2 phosphorylation.

The BET protein inhibitors JQ1 and IBET reduce the expression of numerous genes associated with inflammation (40, 41). BET inhibitors also support a role for the action of Brd4 at the promoters of ISGs, where it recruits pTEFb and stimulates transcriptional elongation (42, 43). In our study, we examined the impact of BET inhibition on promoters regulated by both ISGF3 and NF-κB. In contrast with our expectations, BET protein recruitment was dispensable for pTEFb/CDK9 association with the Nos2 TSS but necessary to maintain association with CDK7 and to stimulate phosphorylation of the Pol II CTD at S5. Inhibition of BET proteins by JQ1 treatment strongly reduced NO production and immunity of mice to *L. monocytogenes* and influenza virus. Furthermore, JQ1 exacerbated the colitogenic effect of dextran sodium sulfate (DSS) treatment.

MATERIALS AND METHODS

Reagents. Recombinant IFN-β was purchased from Biomedica (Nova Scotia, Canada) and added to culture medium for a final concentration of 250 U/ml. The IκB kinase β (IKKβ) inhibitor BI605906 (a kind gift of Phillip Cohen, Dundee, Scotland) was used at a final concentration of 10 μM. (+)-JQ1 or (−)-JQ1 (44) was used at a final concentration of 250 nM for cells. Mice were treated with 50 mg/kg of body weight. The histone deacetylase inhibitors MS-275 (Selleckchem) and Ex-527 (Sigma) were used at concentrations of 2 and 10 μM, respectively. All pharmacological inhibitors were dissolved in dimethyl sulfoxide (DMSO).

Bacteria and infection. The *Listeria monocytogenes* strain LO28 was grown in brain heart infusion (BHI) broth overnight at 37°C. Infection of cells at a multiplicity of infection (MOI) of 20 was performed as described previously (10). Heat-killed *Listeria* was generated by incubating a bacterial overnight culture for 20 min at 70°C.

Mice and cells. Mice were housed under specific-pathogen-free (SPF) conditions. Animal experiments were approved by the institutional ethics committee and carried out in accordance with Austrian law (permit number GZ 680 205/67-BrGt/2003). Wild-type (wt) C57BL/6 mice were sacrificed for harvest of bone marrow between 7 and 10 weeks of age. Bone marrow-derived macrophages (BMDM) were obtained by culture of bone marrow in L-cell-derived colony-stimulating factor 1 as described previously (45).

RNA preparation and Q-PCR. RNA isolation from macrophages was performed with a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. For RNA preparation from the colon, tissue pieces were homogenized in 700 μl RA1 buffer from the NucleoSpin II RNA isolation kit and processed according to the protocol. RNA quantities were determined using a NanoDrop-based protocol (ND1000; Peq lab). cDNA was prepared as described previously (46). Quantitative real-time PCR (Q-PCR) was performed on a Mastercycler EP Realplex S machine (Eppendorf, Vienna, Austria). Primers for individual mRNAs are given in Table S1 in the supplemental material. mRNA expression data were normalized to the housekeeping control gene (*Gapdh*). Data in Fig. 1 and 2 are displayed by setting the controls without inhibitor (Fig. 1) or short hairpin RNA (shRNA) (Fig. 2) to 100%. Data for samples from inhibitor-treated cells are thus displayed as percentages of expression relative to that of the uninhibited control. For Fig. 7, data are shown as relative expression compared to that of the Oaz1 housekeeping gene (47).

ChIP. Chromatin immunoprecipitation (ChIP) was performed as previously described (16), using DynaBeads protein G (Invitrogen) for precipitation. Antibodies used for ChIP are indicated in Table S2 in the supplemental material.

ChIP data were normalized to input and to the sample from untreated cells. Primers used for Q-PCR of the proximal Nos2 promoter were as follows (16): Nos2 prox fwd, 5′-GTCCCAGTTTTGAAGTGACTACG-3′; and Nos2 prox rev, 5′-GTTGTGACCCTGGCAGCAG-3′. The resulting PCR product spanned the proximal promoter with the NF-κB site and the transcription start. Exonic regions were amplified with the following primers: NOS2 exon12 for, 5′-CCACACAGCCTCAGAGTCCT-3′; NOS2 exon12 rev, 5′-CAACATCTCCTGGTGGAACA-3′; NOS2 exon22 for, 5′-CCTGGAGGTGCTTGAAGAGT-3′; and NOS2 exon22 rev, 5′-GAGTAGTAGCGGGGCTTCAA-3′. Primers for amplification of the interleukin-6 (IL-6) promoter were as follows: IL-6 fwd, 5′-ATCCAGTTGCCCTCTTGGGACTGA-3′; and IL-6 rev, 5′-ATCAGTTTCACAGCCTACCCACCT-3′.

Infection experiments. For *L. monocytogenes* infection, 7×10^5 bacteria/mouse were administered intraperitoneally (i.p.). Tumor necrosis factor (TNF) was injected i.p. at the indicated doses simultaneously with

L. monocytogenes. i.p. injection of JQ1 was started 24 h before infection and repeated every 24 h, as described previously (44). For survival experiments, mice were monitored for 10 days. For analyzing the bacterial loads in liver and spleen, mice were killed 48 h after i.p. infection. The organs were isolated, homogenized in phosphate-buffered saline (PBS), plated on BHI plates, and incubated at 37°C overnight. To assess resistance to influenza virus, C57BL/6 mice were infected intranasally under sedation with 500 PFU of influenza A virus strain WSN/33. JQ1 or vehicle controls were injected intraperitoneally once a day starting 1 day before infection and continuing throughout the duration of the experiment. Mice were monitored for health and weighed daily. The experiment was repeated twice ($n = 4$ for each group).

Retrovirally mediated RNA interference (RNAi). shRNAs (see Table S3 in the supplemental material) were cloned into an miR30-based shRNAmir backbone and expressed under the control of an optimized tetracycline (tet)-responsive element (TRE3G) coupled to Turbo-GFP, as previously described (48). Retroviral vectors were calcium phosphate transfected into Platinum-E packaging cells (Cellbiolabs) by using standard techniques. Virus-containing supernatant was harvested 4 times at 36 to 60 h posttransfection. Bone marrow-derived macrophages isolated from Rosa26-rtTA-M2 transgenic mice (49) were spin infected twice on day 3 after harvest in the presence of 4 μg/ml Polybrene (Sigma). shRNA expression was induced 2 days after infection by adding 1 μg/ml doxycycline (dox) to the medium, and shRNA-expressing (Turbo-GFP$^+$) cells were sorted by a fluorescence-activated cell sorter (FACS) after 5 days of dox treatment.

Determination of NO production. Measurement of splenic NO production was performed as described previously (50). Griess reagent was used to determine the amounts of NO in splenocyte supernatants.

DSS-induced colitis. For the colitis experiments, mice (6 to 8 weeks old) were transferred at least 1 week before treatment into individually ventilated cage isolators in an SPF facility. Colitis was induced by adding 2% DSS (molecular mass, 36 to 50 kDa; MP Biomedicals) to autoclaved drinking water, which was provided *ad libitum*, for 7 days. Daily weight measurement was performed during the course of the experiment. Upon sacrifice, the entire intestine was excised, flushed with PBS followed by 2% paraformaldehyde, prepared as a Swiss roll, fixed overnight at 4°C, and embedded in paraffin. Sections of the intestine were stained with hematoxylin and eosin (H&E) according to a standard protocol, and the level of inflammatory damage was scored blind.

Permeability assay. To assess intestinal permeability levels, mice were starved for 3 h and afterwards subjected to gavage with 0.4 mg fluorescein isothiocyanate (FITC)-dextran (3 to 5 kDa; Sigma) per g body weight. Three hours later, serum fluorescence levels were determined at 485/535 nm.

Statistical analysis. Differences between mean values for Q-PCR results of either mRNA expression or ChIP experiments were analyzed by paired *t* test analysis of at least three biological replicates. Differences in bacterial organ loads or splenic NO production were analyzed by the *t* test. Mouse survival data after infection with *L. monocytogenes* or influenza virus were analyzed by the log rank (Mantel-Cox) test. Statistical analysis of DSS-induced colitis data describing weight curves, colon lengths, pathology scores, and colon penetration by FITC-dextran was done using the *t* test.

RESULTS

BET inhibition reduces the expression of *Listeria monocytogenes*-induced genes. To assess the importance of Brd proteins for gene transcription in *L. monocytogenes*-infected cells, a subset of macrophages was treated with the BET inhibitor JQ1 prior to infection with *L. monocytogenes* (44). The inhibitor, but not its (−)-JQ1 enantiomer, reduced expression of Nos2 and of genes such as the IL1rn and IL-6 genes (Fig. 1A), which follow a similar pattern of coregulation by IFN-I and NF-κB pathways (16, 40). In line with previous reports, proinflammatory genes as well as ISGs were affected by JQ1 (Fig. 1B) (40–42). Inhibition of IFN-β mRNA synthesis during *L. monocytogenes* infection by use of JQ1 suggested that reduced IFN-β production and not a direct JQ1 effect might decrease Nos2 and ISG transcription. To test this assumption, the experiment was repeated by treating macrophages with a combination of heat-killed *L. monocytogenes* and exogenous IFN-β. In this experimental setup, heat-killed *L. monocytogenes* stimulates all *Listeria*-derived pathways except for the cytoplasmic pathway leading to IFN-I production; addition of exogenous IFN-β provides the signal for ISGF3 activation (16). This experimental protocol produced results nearly identical to those shown in Fig. 1A and B (Fig. 1C). Expression of Nos2 and other JQ1-sensitive genes was not rescued by the addition of exogenous IFN-β during infection, suggesting that the IFN-β, ISG, and Nos2 genes are direct Brd targets.

As a noteworthy difference to the results obtained after treatment of LPS-stimulated macrophages with the drug I-BET (40), expression of the TNF-α gene after *L. monocytogenes* infection was sensitive to BET inhibition. Furthermore, the IFN-inducible Gbp2 gene was unaffected by JQ1, unlike the ISGs Mxd1 and Ifitm1. This finding suggests heterogeneity in elongation control among ISGs.

Brd recruitment to the Nos2 promoter during *Listeria monocytogenes* infection. To investigate the role of BET proteins in the events leading to Nos2 expression, we analyzed the association of Brd2, -3, and -4 with promoter chromatin. Macrophages were treated with a combination of heat-killed *L. monocytogenes* and IFN-β and processed for ChIP. Figure 2A shows an approximately 12-fold enrichment of Brd4 at the Nos2 promoter as a consequence of treatment. In contrast, the BET proteins Brd2 and Brd3 increased between 2- and 3-fold.

While the data in Fig. 2A suggest that Brd4 is the predominant target of JQ1 at the Nos2 promoter, different affinities of the antibodies used for ChIP might influence the quantitative comparison of Brd2, -3, and -4 associations with Nos2 chromatin. To investigate this possibility, we first analyzed Brd binding to the IL-6 gene promoter. This gene shows a strong increase in both Brd2 and Brd3 binding upon LPS treatment (40), and reduced Brd2 expression causes a corresponding decrease of LPS-induced IL-6 production (41). In *Listeria*-infected macrophages, Brd2 and Brd3 associations with the IL-6 promoter were similar to that observed at the Nos2 promoter, but association with Brd4 was much weaker (Fig. 2B), in line with a larger relative importance of Brd2 and -3 for IL-6 production. For further examination of Brd function during *L. monocytogenes* infection, shRNA-mediated knockdown experiments were performed by retroviral transduction of primary bone marrow-derived macrophages. Two shRNAs were expressed for each Brd gene, i.e., the Brd2, -3, and -4 genes, and some (e.g., Brd3 301 and Brd4 552) showed some ability to cross-inhibit other family members. However, at least one shRNA (each) was absolutely specific for the targeted Brd (Brd2 1746, Brd3 448, and Brd4 1448) (Fig. 2C to E). The knockdown efficacy of the Brd2 shRNAs was lower than those of shRNAs targeting other family members. Examination of Nos2 expression after knockdown showed a slight inhibition by Brd2 and Brd3 shRNAs, which did not reach significance. In contrast, both Brd4 shRNAs caused a significant reduction of Nos2 expression (Fig. 2F). The data in Fig. 2C to F do not rule out a contribution of Brd2 and Brd3 to the transcriptional activation of the Nos2 gene. Importantly, a major role for Brd4 is suggested by these experiments.

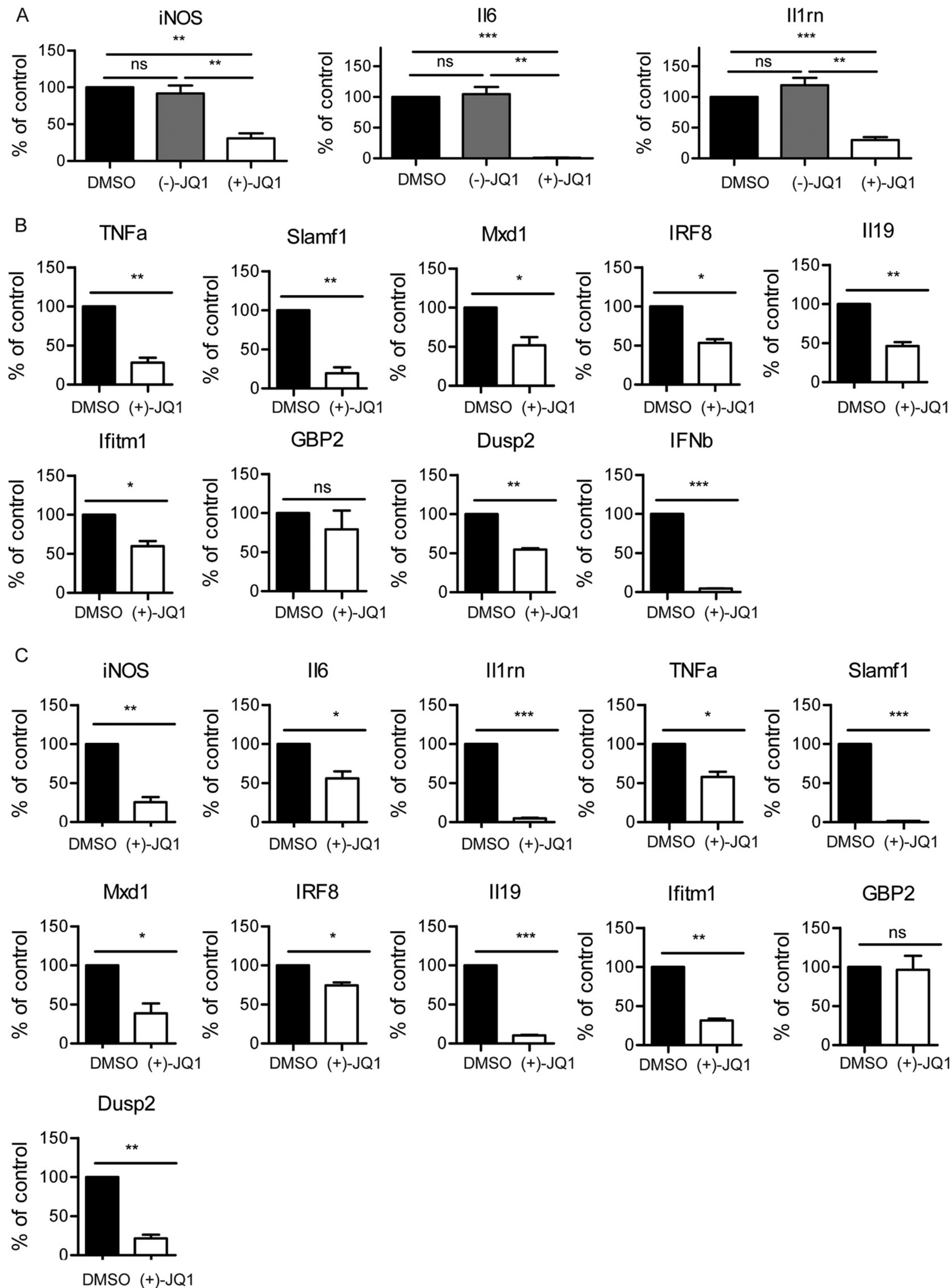

FIG 1 Sensitivity of *Listeria monocytogenes*-induced gene expression to BET protein inhibition with JQ1. Bone marrow-derived macrophages (BMDM) were infected with *L. monocytogenes* for 4 h (A and B) or treated with a combination of heat-killed *L. monocytogenes* and IFN-β (C). Where indicated, 250 nM JQ1 was added 1 h before infection and left in the culture medium during infection. Gene expression was determined by Q-PCR. Values represent means and standard errors for three independent biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Brd4 recruitment requires NF-κB signaling. We sought to determine whether the NF-κB or Stat pathway, or both, stimulates Brd4 binding to the Nos2 promoter. BI605906, a specific IKKβ inhibitor (51), inhibited Nos2 expression induced by *L. monocytogenes* infection (Fig. 3A). The level of inhibition was similar to that observed with JQ1 (Fig. 3B). Consistent with a role of NF-κB, treatment of macrophages with heat-killed *L. monocytogenes* alone stimulated Brd4 recruitment (Fig. 3C). Conversely, IFN-β did not stimulate Brd4 binding. Adding IFN-β together with heat-killed *L. monocytogenes* produced an increase in Brd4 binding which was

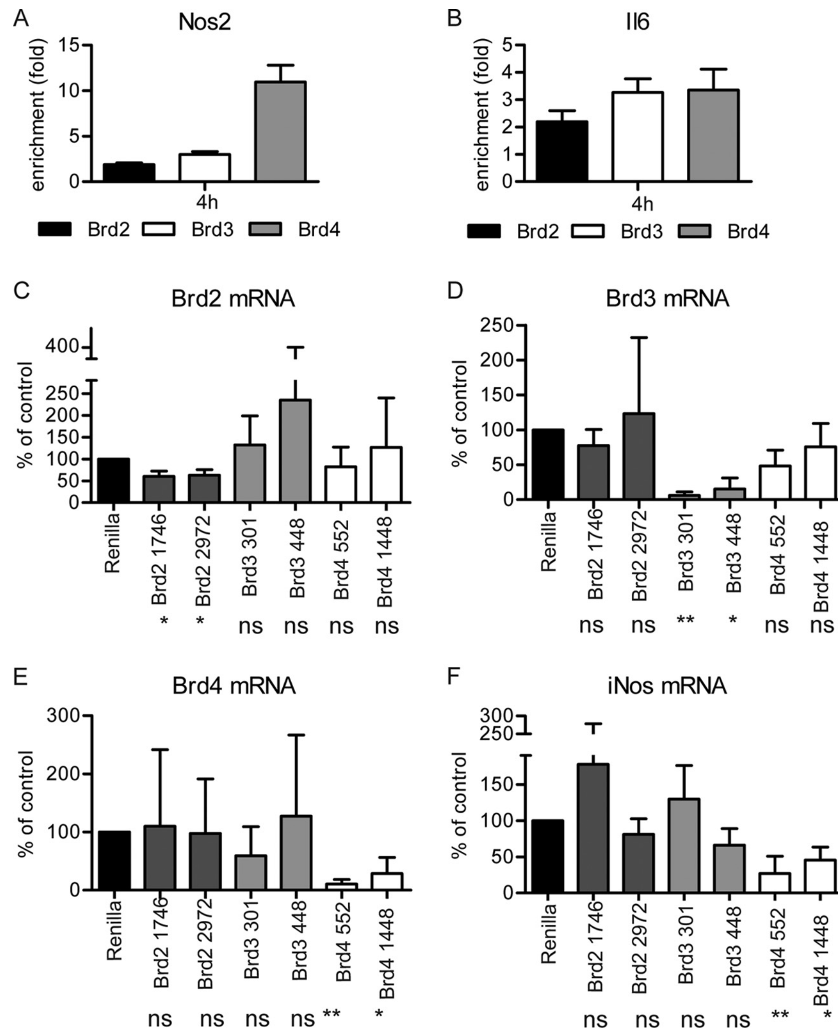

FIG 2 Recruitment of BET proteins to the Nos2 promoter and inhibition of Nos2 expression by Brd shRNAs. All experiments were carried out with BMDM. (A and B) The cells were treated with a combination of heat-killed *Listeria* and IFN-β, followed by ChIP with the indicated antibodies and amplification of the Nos2 promoter region (A) or IL-6 promoter region (B), including the TSS, by Q-PCR. $n = 5$ (A) or 3 (B). (C to E) BMDM isolated from Rosa26-rtTA-M2 transgenic mice (49) were spin infected as described in Materials and Methods with a retrovirus expressing tet-inducible Brd shRNA. shRNA expression was induced 2 days after infection by adding 1 μg/ml dox to the medium, and shRNA-expressing (Turbo-GFP$^+$) cells were FACS sorted after 5 days of dox treatment. The efficacy of the Brd knockdown in cells expressing shRNA was determined by Q-PCR ($n = 3$). (F) BMDM obtained as described for panels C to E were analyzed for shRNA-mediated inhibition of Nos2 expression by Q-PCR ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

not statistically significant. This demonstrates that NF-κB rather than ISGF3 is both necessary and sufficient for Brd4 recruitment. JQ1 did not inhibit NF-κB binding. Instead, increased p65 recruitment was observed after treatment of macrophages with heat-killed *L. monocytogenes* or both heat-killed *L. monocytogenes* and IFN-β (Fig. 3D and E). Nuclear localization and association of NF-κB with DNA are regulated by reversible acetylation (52), suggesting the possibility that JQ1 inhibits an acetylation-dependent molecular event involved in NF-κB recruitment. However, inhibition of histone deacetylases (HDAC1 to -3) with MS275 (53) or Ex-527 (Sirtuin 1) (54, 55) did not reproduce the JQ1 effect in *L. monocytogenes*-infected cells (Fig. 3F and G). At present, we cannot explain the increased association of p65 in the presence of JQ1. One possible explanation could be an active role of BET proteins in removing NF-κB from chromatin.

NF-κB–Brd4 interaction was shown to be regulated by p65 acetylation during infection with respiratory syncytial virus (56). In line with this, inhibition of histone deacetylases increased Brd4 association with the Nos2 promoter (Fig. 3H). This effect was particularly strong in the case of the Sirtuin 1 inhibitor Ex-527 (Fig. 3I).

BET protein inhibition decreases Pol II CTD phosphorylation at S5. Based on previous reports (27, 28, 31, 57), the most likely explanation for the JQ1 effect on Nos2 expression was the Brd4-mediated recruitment of CDK9. In line with this assumption, CDK9 binding to Nos2 chromatin increased during *L. monocytogenes* infection and was sensitive to the IKKβ inhibitor BI605906 (Fig. 4A). Surprisingly, however, CDK9 association remained unaffected by JQ1 (Fig. 4B). Therefore, the input of Brd4 to transcriptional activation of Nos2 differs from that observed for other genes.

To further investigate the input of BET proteins into Nos2 regulation, we examined Nos2 promoter binding of the TFIIH-associated Pol II S5 kinase CDK7 during infection with *L. mono-*

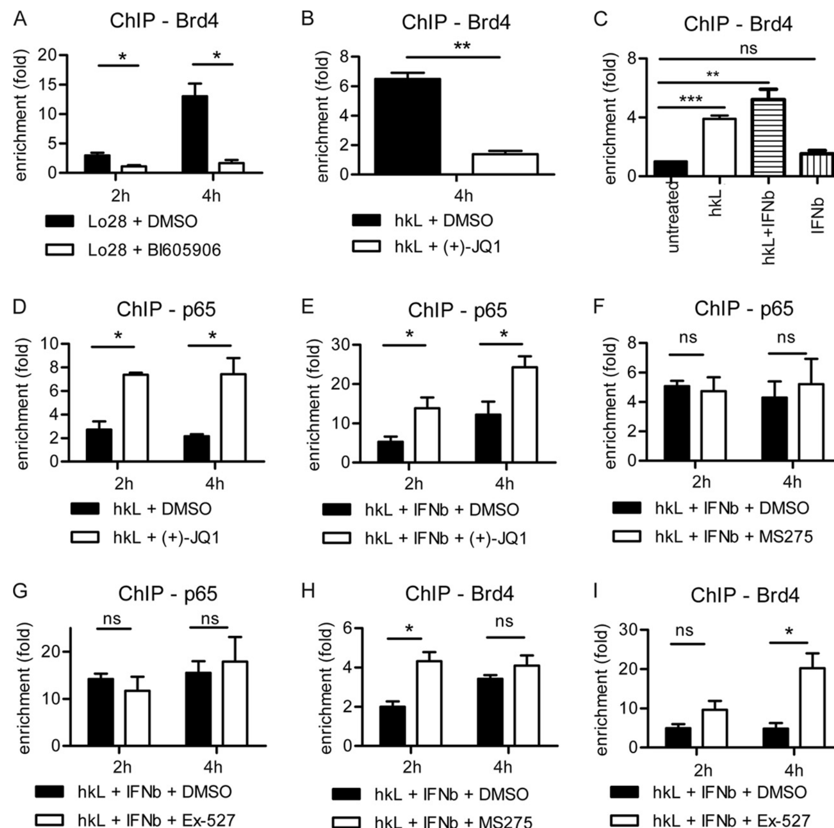

FIG 3 Impact of BET, IKKβ, or HDAC inhibition on the recruitment of Brd4 and NF-κB p65 to Nos2 chromatin. (A and B) BMDM were infected with *Listeria monocytogenes* strain Lo28 for the indicated time in the presence or absence of the IKKβ inhibitor BI605906 at 3 μM (A) or 250 nM JQ1 (B), followed by ChIP with antibodies to Brd4. (C) BMDM were treated with heat-killed *L. monocytogenes* (hkL), IFN-β, or a combination of both, and Brd4 binding to the Nos2 promoter was measured as described for panel A. (D and E) The cells were treated with either heat-killed *L. monocytogenes* (D) or a combination of heat-killed *Listeria* and IFN-β (E) in the presence or absence of 250 nM JQ1, followed by ChIP with antibodies to NF-κB p65 and amplification of the Nos2 promoter region, including the TSS, by Q-PCR. (F and G) The cells were treated with a combination of heat-killed *Listeria* and IFN-β in the presence or absence of the histone deacetylase inhibitor MS-275 at 2 μM (F) or Ex-527 at 10 μM (G), followed by ChIP with antibodies to NF-κB p65 and amplification of the Nos2 promoter region, including the TSS, by Q-PCR. (H and I) Treatment was the same as in panels F and G, but ChIP was done with antibodies to Brd4. The Nos2 promoter region, including the TSS, was amplified by Q-PCR. $n \geq 3$ for all experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

cytogenes. In contrast to CDK9, JQ1 reduced the stable association of CDK7 with the Nos2 promoter 4 and 6 h after *L. monocytogenes* infection (Fig. 4C). To confirm the role of JQ1-inhibitable Brd proteins in CDK7 recruitment, phosphorylation of the Pol II CTD was analyzed. Based on our data, BET inhibition should have a stronger impact on the phosphorylation of S5 in the Pol II CTD than on the phosphorylation of S2. To test this hypothesis, macrophages were treated with a combination of heat-killed *L. monocytogenes* and IFN-β. This treatment was chosen instead of infection because JQ1 reduces IFN-β synthesis during infection (Fig. 1). In contrast to the case for CDK7 and CDK9, recruitment of Pol II requires IFN-β signaling (16). Following treatment, the binding of Pol II to the Nos2 TSS and the phosphorylation of its CTD were determined by ChIP. The binding of Pol II was slightly inhibited by JQ1 4 h after treatment, but this reduction did not quite reach the lowest level of statistical significance ($P = 0.087$). At 6 h, the amount of inhibition was smaller (Fig. 4D). At present, we have no explanation for the function of BET proteins in Pol II recruitment.

Taking the inhibition of Pol II binding into account, JQ1 did not reduce CTD phosphorylation at S2 (Fig. 4E), i.e., the ratio of Pol II to pS2Pol II at the TSS or different regions of the Nos2 gene did not decrease (Fig. 4F). In contrast, CTD S5 phosphorylation was strongly inhibited, much more so than the binding of Pol II (Fig. 4G). The pS5Pol II/Pol II ratio increased as the enzyme proceeded to transcribe the Nos2 gene, most likely due to the decrease in S5 phosphorylation occurring during elongation, but it continued to show significant JQ1 inhibition (Fig. 4H). The data support the notion that at the Nos2 promoter, Brd4 and potentially other JQ1-sensitive Brds regulate the binding of TFIIH/CDK7 rather than the binding of pTEFb/CDK9.

Brd4 inhibition reduces NO synthesis and innate immunity to bacterial and viral pathogens. The impact of JQ1 on NO production by infected mice was tested using an experimental approach described by Serbina et al. (50). Splenocytes isolated after 1 day of *L. monocytogenes* infection were cultured for 36 h, and the amounts of NO in the culture supernatants were determined. This *ex vivo* study demonstrated a large impact of BET inhibition on NO synthesis (Fig. 5A), thus confirming the importance of Brds for Nos2 regulation in the context of an immune response. In accordance with previous papers (40–42), Fig. 1 shows inhibition of genes downstream of the NF-κB pathway (such as the TNF gene), the IFN-I pathway (such as the Mx1 gene), or both pathways (represented by Nos2). Hence, JQ1 inhibition can be expected to produce profound effects on innate responses to patho-

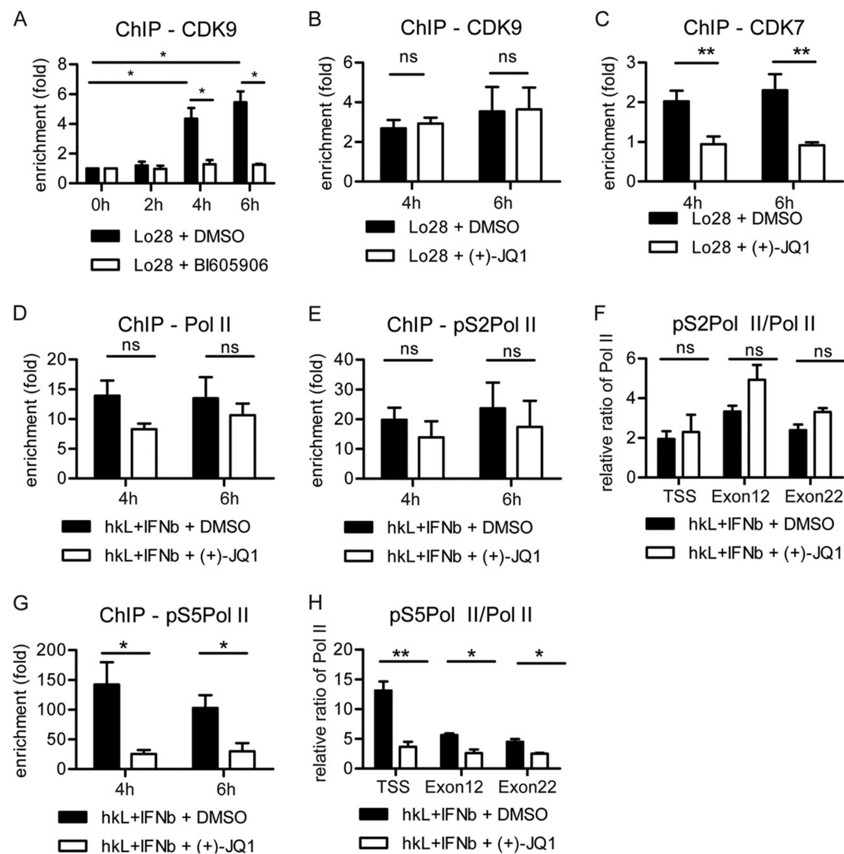

FIG 4 Impact of BET inhibition on CDK7, CDK9, and Pol II association with the Nos2 promoter and on phosphorylation of the Pol II CTD. (A) Recruitment of CDK9 to the Nos2 promoter of *L. monocytogenes* (Lo28)-infected BMDM as determined by ChIP and Q-PCR amplification of the proximal Nos2 promoter. White bars indicate CDK9 recruitment in the presence of the IKKβ inhibitor BI605906. (B and C) Impact of BET inhibition by JQ1 on the recruitment of CDK9 (B) and CDK7 (C). Untreated and *L. monocytogenes*-infected BMDM were subjected to ChIP with antibodies to CDK9 and CDK7. Where indicated, BET proteins were additionally inhibited by treatment with 250 nM JQ1. (D, E, and G) Impact of BET inhibition on recruitment of Pol II (D) and S2-phosphorylated (E) or S5-phosphorylated (G) Pol II to the Nos2 promoter or exonic regions. BMDM were left untreated or treated with a combination of heat-killed *L. monocytogenes* and IFN-β (black bars). Where indicated, BET proteins were additionally inhibited by treatment with 250 nM JQ1 (white bars). S2- or S5-phosphorylated Pol II association was determined by ChIP. (F) Ratio of S2-phosphorylated Pol II and total Pol II at different regions of the Nos2 gene. (H) Ratio of S5-phosphorylated Pol II and total Pol II at different regions of the Nos2 gene. Values represent means and standard errors for biological replicates. $n = 3$ (B, F, and H) or 4 (A, C, D, E, and G). *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

gens or inflammatory disease. To further examine the extent to which Brd proteins regulate innate immunity, macrophages were treated with JQ1 and infected with *L. monocytogenes*, and numbers of intracellular bacteria were determined by CFU assay. JQ1 treatment had no impact on the uptake or phagocytosis-associated killing of *L. monocytogenes* within 1 h of infection. In contrast, the inhibitor strongly reduced the ability of macrophages to inhibit bacterial replication in an 8-h period (Fig. 5B). To extend these findings to an organismic immune response, mice were treated with JQ1 according to a recently established regimen (44). Cohorts of JQ1-treated and control animals were infected with *L. monocytogenes*, followed by determination of liver and splenic bacterial loads after 48 h as well as survival over a 10-day observation period. JQ1 treatment strongly increased both the numbers of bacteria in internal organs (Fig. 5C and D) and the number of animals that succumbed to infection (Fig. 5E). In addition, it strongly reduced the time of survival. TNF-α provides protection to *L. monocytogenes*-infected mice, and the Tnfa gene was suggested to require Brd4-mediated pTEFb recruitment (31, 58). To test whether TNF inhibition by JQ1 (Fig. 1) was responsible for the reduced survival of mice, the infection experiment was repeated with mice that had received TNF in addition to JQ1. The administered doses of 0.5 and 1 μg i.p. were chosen based on publications showing that 100 ng TNF will strongly protect from herpes simplex virus infection and that 6 μg given intravenously (i.v.) suffices to kill a vast majority of treated C57BL/6 mice, the strain used in our experiments (59, 60). A slight prolongation of the survival period was observed in TNF-treated animals, but the cytokine did not rescue any of the infected animals (Fig. 5F and G). This shows that although TNF inhibition may be a contributing factor, Brd-dependent genes other than the TNF gene are important in innate resistance to *L. monocytogenes*.

Survival of influenza virus-infected mice is increased by the antiviral response but decreased by inflammation and impaired tissue repair (61). NO production by Nos2 contributes to inflammatory lung pathology (62). Since both antiviral and inflammatory responses are potentially suppressed by BET inhibition, we sought to determine the outcome for mice given JQ1 treatment prior to influenza virus infection. This experiment clearly established a protective role for Brd-dependent genes, as a larger frac-

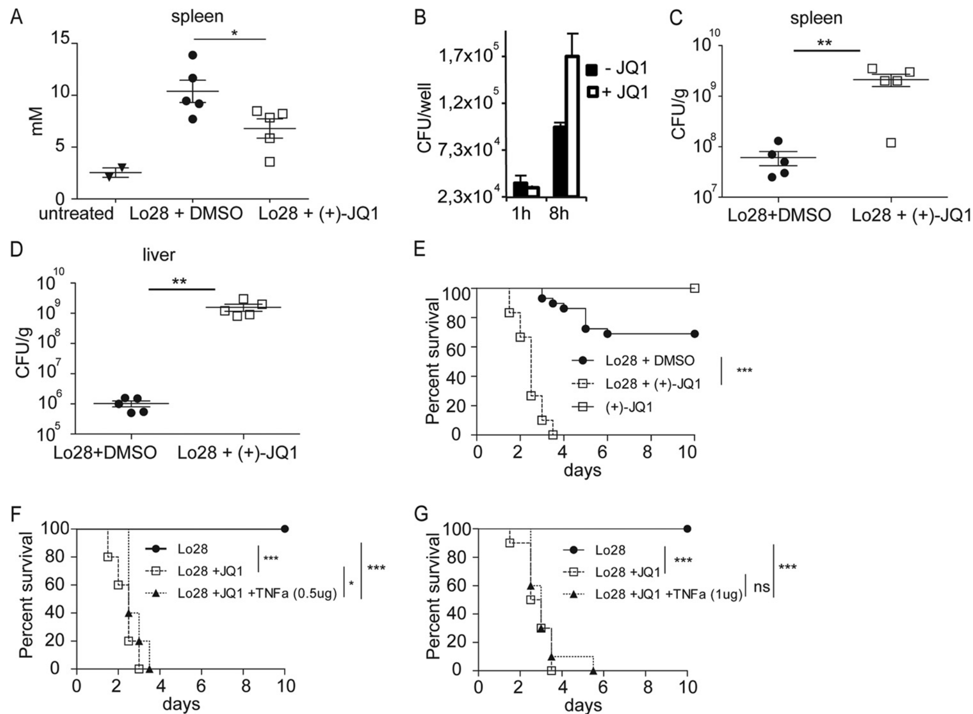

FIG 5 Impact of Brd4 inhibition on NO production and innate immunity to *Listeria monocytogenes*. (A) Untreated or JQ1-treated mice (daily injections of 50 mg/kg i.p.) were infected intraperitoneally with *L. monocytogenes* (Lo28). Twenty-four hours after infection, the spleen was removed. Splenic leukocytes were cultured for 36 h, and supernatants were collected for the determination of NO with Griess reagent ($n = 5$ per group). (B) BMDM were left untreated or treated with 250 nM JQ1. The cells were infected with *L. monocytogenes* for the indicated times, followed by an assessment of intracellular *L. monocytogenes* by CFU assay. The experiment is representative of more than three independent biological replicates. (C to G) Untreated mice ($n = 5$) or mice treated with JQ1 as in panel A ($n = 5$) were infected intraperitoneally with *L. monocytogenes*. Infected mice were analyzed after 48 h for the bacterial burdens in the spleen and liver (C and D) or for survival over a 10-day observation period (E to G) ($n = 10$ per group; data from three independent experiments were combined). Panels F and G show data for animals additionally treated intraperitoneally with 0.5 or 1 μg ($n = 10$ per group), respectively, of TNF-α before infection with *L. monocytogenes* to test the cytokine's ability to rescue the JQ1 effect. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

tion of the mice treated with JQ1 succumbed to infection (Fig. 6). This experiment suggests a predominance of JQ1-mediated suppression of the innate and/or adaptive antiviral response.

JQ1 treatment increases DSS-induced colitis. A recent report demonstrated that concomitant inhibition of Brd2, -3, and -4 by the synthetic acetylhistone mimetic I-BET reduces adverse effects of systemic inflammation caused by bacteria or their products (40). In the case of colitis, the same potentially inflammatory

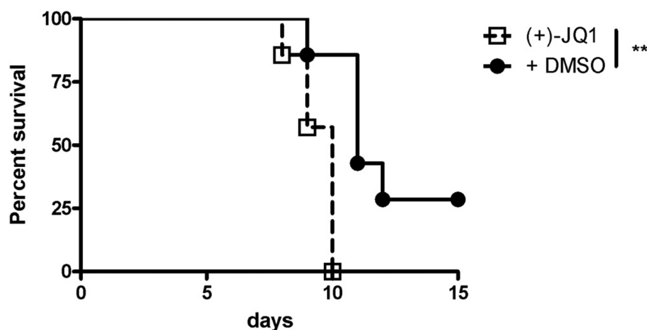

FIG 6 Effect of BET inhibition on resistance to influenza virus. Untreated or JQ1-treated mice (daily injections at 50 mg/kg) were infected with 500 PFU of a mouse-adapted influenza A virus (H1N1 subtype; strain WSN/33), and survival was monitored over 15 days ($n = 8$; data from two independent experiments with $n = 4$ were combined). **, $P < 0.01$.

pathways can protect from colitis or contribute to the damage inflicted by the inflammatory response (63–65). This prompted us to examine whether colitis was prevented or exacerbated by JQ1. Mice were treated with DSS to induce colitis, and one group of animals was treated with JQ1. Treatment of wt animals with 2% DSS caused a 20% weight loss within 10 days (Fig. 7A). The effect of 2% DSS, with or without JQ1, was determined by weight loss (Fig. 7B), shortening of the colon (Fig. 7C), and pathology scores (Fig. 7D). All criteria for intestinal inflammation were profoundly exacerbated by JQ1; in fact, the experiment had to be terminated already after 7 days of treatment because the JQ1-DSS-treated animals had reached 80% of their original weight, after which Austrian law requires their euthanasia. In keeping with a recent report (44), JQ1 treatment alone did not cause mice to lose weight or to develop apparent tissue pathology (Fig. 7B and data not shown). Histological examination at day 7 after DSS treatment revealed increased epithelial damage and mucosal infiltration in the presence of JQ1 (Fig. 7E and F). JQ1 treatment *per se* did not affect the tightness of the epithelial layer, as suggested by a similar appearance of FITC-labeled dextran in the blood after application of the chemical by gavage (Fig. 7G). In keeping with our observations with *L. monocytogenes* infection, expression of Nos2 in colon tissue was decreased by JQ1 in both the steady state and the DSS-induced state, although the reduction reached significance only in the former situation (Fig. 7H). This was similarly true for the gene

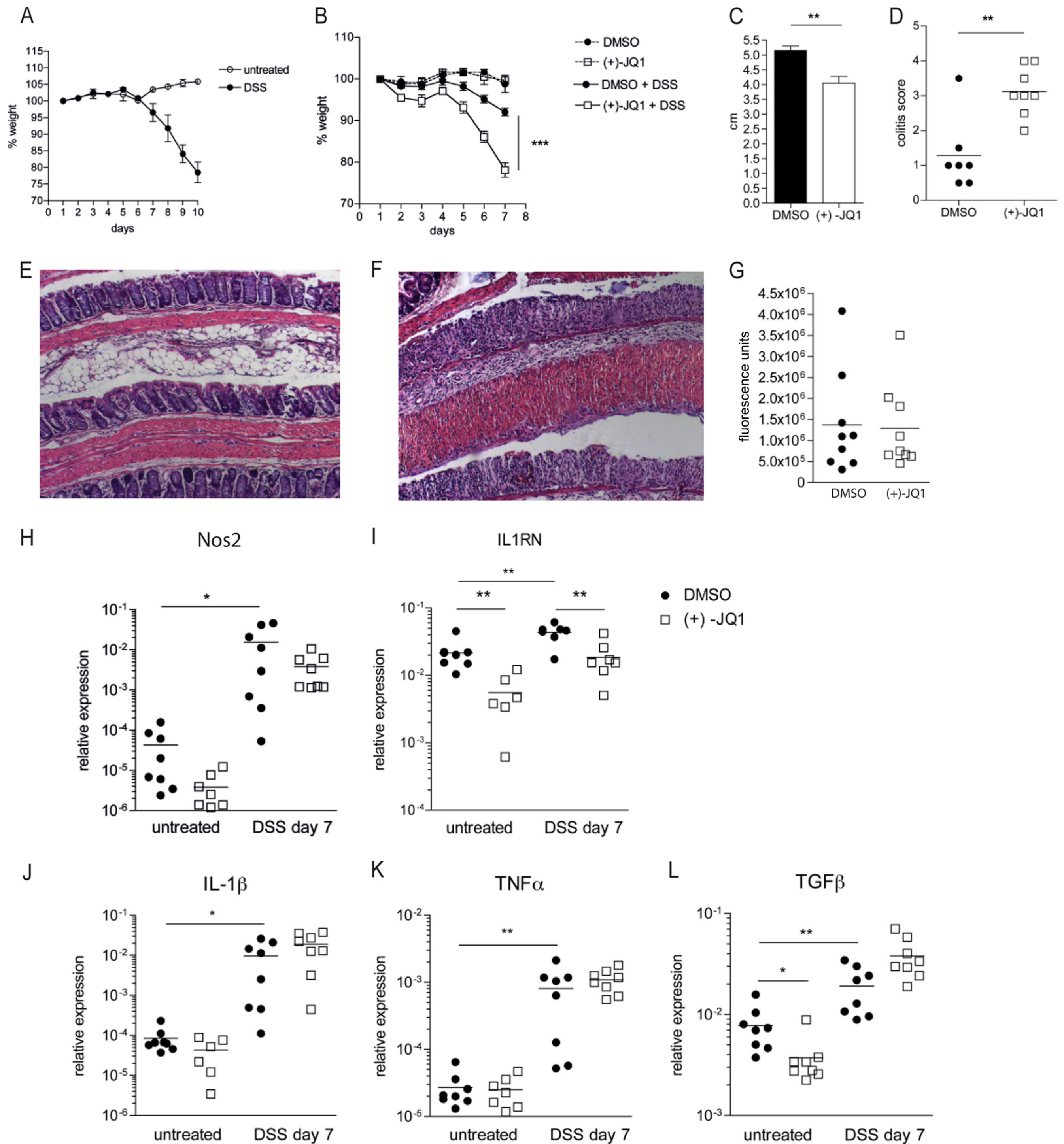

FIG 7 Effect of BET inhibition on DSS-induced colitis. (A to D) Untreated or JQ1-treated mice (daily injections of 50 mg/kg i.p.) were given 2% DSS in their drinking water or kept on regular drinking water over a 7-day period. Colitis was assessed by weight loss over 10 days (A) or 7 days (B) (see the text for further information), shortening of the colon (C), and pathology score (D) ($n = 8$; data from two independent experiments with $n = 4$ were combined). (E and F) Histological examination of the colon mucosa on day 7 of the DSS treatment protocol in the absence (E) or presence (F) of JQ1. Micrographs represent thin sections of paraffin-embedded tissue stained with hematoxylin and eosin. (G) FITC-labeled dextran (molecular mass of 3,000 to 5,000 Da) was given to mice via gavage. The appearance of fluorescent material in the blood was measured 3 h later. (H to L) Expression of the indicated genes was measured by Q-PCR following mRNA extraction from the colon mucosa. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

encoding IL-1 receptor antagonist (IL-1RN), whose regulation follows that of Nos2 during *L. monocytogenes* infection (16) (Fig. 7H and I). The proinflammatory IL-1β and TNF-α cytokines remained unaffected by JQ1 treatment (Fig. 7J and K). Similarly, expression of the chemokines CXCL1, CCL2, and CCL7 was the same in the colons of DSS-treated mice irrespective of the additional presence of JQ1 (data not shown). The gene for the anti-inflammatory cytokine transforming growth factor beta (TGFβ) was decreased by JQ1 in the steady state but not after DSS treatment (Fig. 7L). The IL-10 gene was unaffected by JQ1 treatment before DSS or at day 7 after treatment (data not shown). The data show that unlike systemic LPS-induced inflammation, JQ1 increases the susceptibility to DSS-induced colitis.

DISCUSSION

The primary aim of our study was to elucidate steps involved in the initiation and elongation of Nos2 transcription. Given the importance of BET proteins in the regulation of many genes involved in the establishment of innate immunity and the availability of a specific inhibitor, our second aim was to shed light on the importance of Brd-dependent gene regulation for antimicrobial and inflammatory responses of cells and organisms. Brd4 received particular attention in our studies due to the strong increase of this BET family member at the Nos2 promoter in *L. monocytogenes*-infected macrophages and to the strong inhibition of Nos2 expression by Brd4 shRNA. However, our knockdown experiments suggest that JQ1 inhibition of Brd2 and Brd3 may additionally contribute to decreased Nos2 expression.

Nos2 expression as well as that of the ISG Mx or Ifitm1 during *L. monocytogenes* infection was sensitive to Brd4 inhibition. A common denominator of the associated genes is their regulation by the ISGF3 complex. Whereas ISGF3 may be responsible for Brd4 recruitment in the case of ISGs (42), binding of the BET protein to the Nos2 promoter requires NF-κB and can be caused by stimulation of the NF-κB pathway alone. This is suggested by the sensitivity of Brd4 binding to IKKβ inhibition and by data showing Brd4 binding in response to treatment with heat-killed *L. monocytogenes*, i.e., in the absence of IFN-I production (16). Therefore, Nos2 gene-like genes and ISGs employ ISGF3 in different steps of transcriptional initiation/elongation; most likely, some of the ISGF3 activities at ISG promoters are taken over by NF-κB at Nos2 gene-like genes. Surprisingly, some ISGs, represented in our study by the Gbp2 gene, appear to be insensitive to JQ1 action. This finding points to heterogeneity in the molecular mechanisms driving the transcriptional response to IFN-I.

BET proteins play an important role in the regulation of the Tnfa gene, encoding a critical cytokine of inflammation and immunity. Hargreaves et al. (31) deduced an involvement of Brd4 in pTEFb recruitment and LPS-induced TNF-α expression in macrophages from binding kinetics and small interfering RNA (siRNA)-mediated knockdown. In line with this, Nicodeme et al. (40) found a Brd4 requirement based on siRNA experiments. Surprisingly, though, inhibition with I-BET had no effect on TNF expression. Based on this result, the authors proposed that a histone acetylation-independent mechanism tethers Brd4 to the Tnfa promoter after LPS stimulation. In our studies, TNF-α expression in response to *L. monocytogenes* infection was inhibited by JQ1 but was insensitive to the drug when induced by DSS treatment in mice. Therefore, both histone acetylation-dependent and -independent molecular events appear to associate BET proteins with the Tnfa promoter in a stimulus- and/or cell type-specific fashion. The prevalence of one or the other may be determined by preexisting histone modification or a differential ability of proinflammatory stimuli to modify promoter chromatin.

According to the model of Hargreaves et al., NF-κB is employed for histone acetyltransferase (HAT) recruitment leading to H4 acetylation as a prerequisite for Brd4 binding and pTEFb recruitment. Alternatively, or additionally, direct association with acetylated NF-κB p65 may tether Brd4 to Nos2 chromatin, as recently described for virus-infected cells (56). Our data at present do not allow us to clearly distinguish which of these mechanisms is represented at the Nos2 promoter; however, we favor a role for direct association with NF-κB, because we noted an increase in physical interaction between NF-κB and Brd4 during infection (data not shown). In addition, inhibition of histone deacetylases increased Brd4 recruitment. Our data disagree with the mode of pTEFb recruitment proposed for immediate early genes of inflammation, because CDK9 binding was insensitive to inhibition with JQ1. Molecular complexes, including Brd4 and the recently described Brd4-independent superelongation complex, provide alternative platforms for pTEFb recruitment (66). Moreover, Brd4-independent tethering of pTEFb to promoters via direct interaction with transcriptional activators (22, 57) or through the multisubunit Mediator complex, particularly its CDK8 or Med26 subunit, has been reported (67–70).

Whereas BET proteins were dispensable for bringing pTEFb/CDK9 to the Nos2 promoter, they did play a role in the binding of TFIIH/CDK7. This is consistent with a recent biochemical study reporting an interaction between Brd4 and CDK7 (71). The measured increase in CDK7 binding was not more than 2- to 3-fold, most likely due to antibody affinity and/or instability of TFIIH association with the Nos2 promoter. In spite of this, a strong impact of BET inhibition on CDK7 recruitment is suggested by the strong and selective reduction of S5 phosphorylation at the Pol II CTD. S2 phosphorylation of the Pol II CTD was inhibited much less by comparison, confirming an important role of BET proteins in CDK7 but not CDK9 recruitment.

During infection with *L. monocytogenes*, NO is produced by various cell types, including infected macrophages and inflammatory dendritic cells such as Tip-DC (15, 50). It is unclear whether all NO-producing cell types regulate Nos2 in an identical manner. JQ1 treatment strongly reduced NO production of splenocytes isolated from infected mice, suggesting that a Brd-dependent mechanism of transcriptional regulation is widely employed by cells participating in the innate response to *L. monocytogenes*. Treatment of mice with I-BET demonstrated that many genes involved in inflammation are regulated by BET proteins; in fact, both I-BET and JQ1 rescued the survival of mice in animal models of bacterial sepsis (40, 41). JQ1 inactivation of Brd proteins is likely to reduce the expression of many genes orchestrating the inflammatory response. In the case of *L. monocytogenes*, the immediate production of inflammatory mediators is protective, as judged by the increased mortality of mice lacking TNF, IL-1, or IL-6 genes (58, 72, 73). Consistent with this, JQ1 treatment increased bacterial replication in infected cells and mice, and it strongly decreased the ability of mice to survive the infectious disease caused by *L. monocytogenes*. TNF-α treatment did not rescue the survival of JQ1-treated animals, suggesting that this cytokine alone cannot compensate the immune defects inflicted by JQ1 treatment. In the case of influenza virus infection, the benefit

of inhibiting tissue-destructive proinflammatory genes appears to be overcompensated by the simultaneous inhibition of critical IFN-responsive antiviral genes. Examining the impact of JQ1 on DSS-induced colitis was particularly interesting because the same cellular pathways can be protective or detrimental, depending on the cell type that employs them. This has been shown convincingly for MyD88 and NF-κB signaling (63–65, 74, 75). Unlike I-BET or JQ1 treatment in the case of bacterial sepsis, JQ1 treatment dramatically worsened the condition of animals suffering from DSS-induced intestinal inflammation. The data suggest that intrinsic differences in the pathomechanisms of bacterium-induced sepsis and DSS-induced colitis are revealed by BET inhibition. The ability of Brd4 to coactivate most inflammatory genes but corepress others may be relevant in this context (40). Surprisingly, the protective effects of the JQ1-sensitive pathways strongly overcome their role in inflammatory pathology. Importantly, JQ1 treatment *per se* does not induce colitis or affect epithelial integrity. This notion is derived from the maintenance of normal body weight of mice treated with JQ1 only and from the identical abilities of FITC-dextran to penetrate the epithelial barrier with and without JQ1 treatment. In spite of this, both steady-state and DSS-induced expression of some genes was notably altered, consistent with an exacerbated inflammatory response.

JQ1 holds considerable promise for clinical application against tumors or as a reversible inhibitor of spermatogenesis (76–79). The data presented in our study suggest that the benefit of JQ1 treatment must be weighed carefully against a potential impairment of protective immunity.

ACKNOWLEDGMENTS

We thank Christian Seiser and Anna Sawicka for critical discussions.

Funding was provided by the Austrian Science Fund (FWF) through grant SFB-28 to M. Müller and T. Decker and grant P25235-B13 to A. M. Jamieson. S. Wienerroither was supported by the FWF through the doctoral program Molecular Mechanisms of Cell Signaling.

S. Wienerroither, F. Rosebrock, J. Bradner, A. M. Jamieson, I. Rauch, J. Zuber, M. Müller, and T. Decker conceived the study, designed the experiments, and analyzed data. S. Wienerroither carried out most of the experiments, with important contributions by F. Rosebrock, I. Rauch, M. Muhar, and A. M. Jamieson. J. Bradner produced and contributed critical reagents. T. Decker coordinated the project. The manuscript was written by T. Decker, with contributions from S. Wienerroither, I. Rauch, A. M. Jamieson, and M. Müller.

J. Bradner issues the following statement: the Dana-Farber Cancer Institute has licensed drug-like derivatives of the JQ1 BET bromodomain inhibitor, created in the Bradner laboratory, to Tensha Therapeutics. All other authors declare no financial interests.

REFERENCES

1. **Kawai T, Akira S.** 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int. Immunol. **21:**317–337. http://dx.doi.org/10.1093/intimm/dxp017.
2. **Iwasaki A, Medzhitov R.** 2010. Regulation of adaptive immunity by the innate immune system. Science **327:**291–295. http://dx.doi.org/10.1126/science.1183021.
3. **Portnoy DA, Auerbuch V, Glomski IJ.** 2002. The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity. J. Cell Biol. **158:**409–414. http://dx.doi.org/10.1083/jcb.200205009.
4. **Hamon M, Bierne H, Cossart P.** 2006. Listeria monocytogenes: a multifaceted model. Nat. Rev. Microbiol. **4:**423–434. http://dx.doi.org/10.1038/nrmicro1413.
5. **Seki E, Tsutsui H, Tsuji NM, Hayashi N, Adachi K, Nakano H, Futatsugi-Yumikura S, Takeuchi O, Hoshino K, Akira S, Fujimoto J, Nakanishi K.** 2002. Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of Listeria monocytogenes in mice. J. Immunol. **169:**3863–3868.
6. **Mancuso G, Gambuzza M, Midiri A, Biondo C, Papasergi S, Akira S, Teti G, Beninati C.** 2009. Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat. Immunol. **10:**587–594. http://dx.doi.org/10.1038/ni.1733.
7. **Woodward JJ, Iavarone AT, Portnoy DA.** 2010. c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. Science **328:**1703–1705. http://dx.doi.org/10.1126/science.1189801.
8. **Sauer J-D, Sotelo-Troha K, von Moltke, J, Monroe KM, Rae CS, Brubaker SW, Hyodo M, Hayakawa Y, Woodward JJ, Portnoy DA, Vance RE.** 2011. The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to Listeria monocytogenes and cyclic dinucleotides. Infect. Immun. **79:**688–694. http://dx.doi.org/10.1128/IAI.00999-10.
9. **O'Riordan M, Yi CH, Gonzales R, Lee KD, Portnoy DA.** 2002. Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. Proc. Natl. Acad. Sci. U. S. A. **99:**13861–13866. http://dx.doi.org/10.1073/pnas.202476699.
10. **Stockinger S, Materna T, Stoiber D, Bayr L, Steinborn R, Kolbe T, Unger H, Chakraborty T, Levy DE, Muller M, Decker T.** 2002. Production of type I IFN sensitizes macrophages to cell death induced by Listeria monocytogenes. J. Immunol. **169:**6522–6529.
11. **Levy DE, Darnell JE, Jr.** 2002. Stats: transcriptional control and biological impact. Nat. Rev. Mol. Cell Biol. **3:**651–662. http://dx.doi.org/10.1038/nrm909.
12. **Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S, Williams BR, Major J, Hamilton TA, Fenton MJ, Vogel SN.** 2002. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat. Immunol. **3:**392–398. http://dx.doi.org/10.1038/ni774.
13. **McCaffrey RL, Fawcett P, O'Riordan M, Lee KD, Havell EA, Brown PO, Portnoy DA.** 2004. A specific gene expression program triggered by Gram-positive bacteria in the cytosol. Proc. Natl. Acad. Sci. U. S. A. **101:**11386–11391. http://dx.doi.org/10.1073/pnas.0403215101.
14. **Gao JJ, Filla MB, Fultz MJ, Vogel SN, Russell SW, Murphy WJ.** 1998. Autocrine/paracrine IFN-alphabeta mediates the lipopolysaccharide-induced activation of transcription factor Stat1alpha in mouse macrophages: pivotal role of Stat1alpha in induction of the inducible nitric oxide synthase gene. J. Immunol. **161:**4803–4810.
15. **Bogdan C.** 2001. Nitric oxide and the immune response. Nat. Immunol. **2:**907–916. http://dx.doi.org/10.1038/ni1001-907.
16. **Farlik M, Reutterer B, Schindler C, Greten F, Vogl C, Muller M, Decker T.** 2010. Nonconventional initiation complex assembly by STAT and NF-kappaB transcription factors regulates nitric oxide synthase expression. Immunity **33:**25–34. http://dx.doi.org/10.1016/j.immuni.2010.07.001.
17. **MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson N, Chen H, Mudget JS.** 1995. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell **81:**641–650. (Erratum, **81:**following 1170.)
18. **Fehr T, Schoedon G, Odermatt B, Holtschke T, Schneemann M, Bachmann MF, Mak TW, Horak I, Zinkernagel RM.** 1997. Crucial role of interferon consensus sequence binding protein, but neither of interferon regulatory factor 1 nor of nitric oxide synthesis for protection against murine listeriosis. J. Exp. Med. **185:**921–931. http://dx.doi.org/10.1084/jem.185.5.921.
19. **Stockinger S, Decker T.** 2008. Novel functions of type I interferons revealed by infection studies with Listeria monocytogenes. Immunobiology **213:**889–897. http://dx.doi.org/10.1016/j.imbio.2008.07.020.
20. **Cole C, Thomas S, Filak H, Henson PM, Lenz LL.** 2012. Nitric oxide increases susceptibility of Toll-like receptor-activated macrophages to spreading Listeria monocytogenes. Immunity **36:**807–820. http://dx.doi.org/10.1016/j.immuni.2012.03.011.
21. **Smale ST.** 2012. Transcriptional regulation in the innate immune system. Curr. Opin. Immunol. **24:**51–57. http://dx.doi.org/10.1016/j.coi.2011.12.008.
22. **Adelman K, Lis JT.** 2012. Promoter-proximal pausing of RNA polymerase II: emerging roles in metazoans. Nat. Rev. Genet. **13:**720–731. http://dx.doi.org/10.1038/nrg3293.
23. **Nechaev S, Adelman K.** 2011. Pol II waiting in the starting gates: regulating the transition from transcription initiation into productive elonga-

tion. Biochim. Biophys. Acta **1809:**34–45. http://dx.doi.org/10.1016/j.bbagrm.2010.11.001.
24. **Medzhitov R, Horng T.** 2009. Transcriptional control of the inflammatory response. Nat. Rev. Immunol. **9:**692–703. http://dx.doi.org/10.1038/nri2634.
25. **Egloff S, Murphy S.** 2008. Cracking the RNA polymerase II CTD code. Trends Genet. **24:**280–288. http://dx.doi.org/10.1016/j.tig.2008.03.008.
26. **Dey A, Chitsaz F, Abbasi A, Misteli T, Ozato K.** 2003. The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. Proc. Natl. Acad. Sci. U. S. A. **100:**8758–8763. http://dx.doi.org/10.1073/pnas.1433065100.
27. **Yang Z, Yik JHN, Chen R, He N, Jang MK, Ozato K, Zhou Q.** 2005. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. Mol. Cell **19:**535–545. http://dx.doi.org/10.1016/j.molcel.2005.06.029.
28. **Jang MK, Mochizuki K, Zhou M, Jeong H-S, Brady JN, Ozato K.** 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. http://dx.doi.org/10.1016/j.molcel.2005.06.027.
29. **Wu S-Y, Chiang C-M.** 2007. The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation. J. Biol. Chem. **282:**13141–13145. http://dx.doi.org/10.1074/jbc.R700001200.
30. **Bisgrove DA, Mahmoudi T, Henklein P, Verdin E.** 2007. Conserved P-TEFb-interacting domain of BRD4 inhibits HIV transcription. Proc. Natl. Acad. Sci. U. S. A. **104:**13690–13695. http://dx.doi.org/10.1073/pnas.0705053104.
31. **Hargreaves DC, Horng T, Medzhitov R.** 2009. Control of inducible gene expression by signal-dependent transcriptional elongation. Cell **138:**129–145. http://dx.doi.org/10.1016/j.cell.2009.05.047.
32. **Kanno T, Kanno Y, Siegel RM, Jang MK, Lenardo MJ, Ozato K.** 2004. Selective recognition of acetylated histones by bromodomain proteins visualized in living cells. Mol. Cell **13:**33–43. http://dx.doi.org/10.1016/S1097-2765(03)00482-9.
33. **LeRoy G, Rickards B, Flint SJ.** 2008. The double bromodomain proteins Brd2 and Brd3 couple histone acetylation to transcription. Mol. Cell **30:**51–60. http://dx.doi.org/10.1016/j.molcel.2008.01.018.
34. **Denis GV, Vaziri C, Guo N, Faller DV.** 2000. RING3 kinase transactivates promoters of cell cycle regulatory genes through E2F. Cell Growth Differ. **11:**417–424.
35. **Wu F, Dassopoulos T, Cope L, Maitra A, Brant SR, Harris ML, Bayless TM, Parmigiani G, Chakravarti S.** 2007. Genome-wide gene expression differences in Crohn's disease and ulcerative colitis from endoscopic pinch biopsies: insights into distinctive pathogenesis. Inflamm. Bowel Dis. **13:**807–821. http://dx.doi.org/10.1002/ibd.20110.
36. **Huang B, Yang X-D, Zhou M-M, Ozato K, Chen L-F.** 2009. Brd4 coactivates transcriptional activation of NF-kappaB via specific binding to acetylated RelA. Mol. Cell. Biol. **29:**1375–1387. http://dx.doi.org/10.1128/MCB.01365-08.
37. **Ramirez-Carrozzi VR, Braas D, Bhatt DM, Cheng CS, Hong C, Doty KR, Black JC, Hoffmann A, Carey M, Smale ST.** 2009. A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell **138:**114–128. http://dx.doi.org/10.1016/j.cell.2009.04.020.
38. **Paulson M, Press C, Smith E, Tanese N, Levy DE.** 2002. IFN-stimulated transcription through a TBP-free acetyltransferase complex escapes viral shutoff. Nat. Cell Biol. **4:**140–147. http://dx.doi.org/10.1038/ncb747.
39. **Jamieson AM, Farlik M, Decker T.** 2012. How Stats interact with the molecular machinery of transcriptional activation, p 65–89. *In* Decker T, M̈uller M (ed), Jak-Stat signaling: from basics to disease. Springer, Vienna, Austria.
40. **Nicodeme E, Jeffrey KL, Schaefer U, Beinke S, Dewell S, Chung C-W, Chandwani R, Marazzi I, Wilson P, Coste H, White J, Kirilovsky J, Rice CM, Lora JM, Prinjha RK, Lee K, Tarakhovsky A.** 2010. Suppression of inflammation by a synthetic histone mimic. Nature **468:**1119–1123. http://dx.doi.org/10.1038/nature09589.
41. **Belkina AC, Nikolajczyk BS, Denis GV.** 2013. BET protein function is required for inflammation: Brd2 genetic disruption and BET inhibitor JQ1 impair mouse macrophage inflammatory responses. J. Immunol. **190:**3670–3678. http://dx.doi.org/10.4049/jimmunol.1202838.
42. **Patel MC, Debrosse M, Smith M, Dey A, Huynh W, Sarai N, Heightman TD, Tamura T, Ozato K.** 2013. BRD4 coordinates recruitment of pause-release factor P-TEFb and the pausing complex NELF/DSIF to regulate transcription elongation of interferon stimulated genes. Mol. Cell. Biol. **33:**2497–2507. http://dx.doi.org/10.1128/MCB.01180-12.
43. **Tian B, Zhao Y, Kalita M, Edeh CB, Paessler S, Casola A, Teng MN, Garofalo RP, Brasier AR.** 2013. CDK9-dependent transcriptional elongation in the innate ISG response to RSV infection in airway epithelial cells. J. Virol. **87:**7075–7092. http://dx.doi.org/10.1128/JVI.03399-12.
44. **Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, Morse EM, Keates T, Hickman TT, Felletar I, Philpott M, Munro S, McKeown MR, Wang Y, Christie AL, West N, Cameron MJ, Schwartz B, Heightman TD, La Thangue N, French CA, Wiest O, Kung AL, Knapp S, Bradner JE.** 2010. Selective inhibition of BET bromodomains. Nature **468:**1067–1073. http://dx.doi.org/10.1038/nature09504.
45. **Baccarini M, Bistoni F, Lohmann Matthes ML.** 1985. In vitro natural cell-mediated cytotoxicity against Candida albicans: macrophage precursors as effector cells. J. Immunol. **134:**2658–2665.
46. **Stockinger S, Reutterer B, Schaljo B, Schellack C, Brunner S, Materna T, Yamamoto M, Akira S, Taniguchi T, Murray PJ, Muller M, Decker T.** 2004. IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism. J. Immunol. **173:**7416–7425.
47. **Meyer FRL, Grausgruber H, Binter C, Mair GE, Guelly C, Vogl C, Steinborn R.** 2013. Cross-platform microarray meta-analysis for the mouse jejunum selects novel reference genes with highly uniform levels of expression. PLoS One **8:**e63125. http://dx.doi.org/10.1371/journal.pone.0063125.
48. **Zuber J, McJunkin K, Fellmann C, Dow LE, Taylor MJ, Hannon GJ, Lowe SW.** 2011. Toolkit for evaluating genes required for proliferation and survival using tetracycline-regulated RNAi. Nat. Biotechnol. **29:**79–83. http://dx.doi.org/10.1038/nbt.1720.
49. **Hochedlinger K, Yamada Y, Beard C, Jaenisch R.** 2005. Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Cell **121:**465–477. http://dx.doi.org/10.1016/j.cell.2005.02.018.
50. **Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG.** 2003. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity **19:**59–70. http://dx.doi.org/10.1016/S1074-7613(03)00171-7.
51. **Clark K, Peggie M, Plater L, Sorcek RJ, Young ERR, Madwed JB, Hough J, McIver EG, Cohen P.** 2011. Novel cross-talk within the IKK family controls innate immunity. Biochem. J. **434:**93–104. http://dx.doi.org/10.1042/BJ20101701.
52. **Chen LF, Greene WC.** 2004. Shaping the nuclear action of NF-kappaB. Nat. Rev. Mol. Cell Biol. **5:**392–401. http://dx.doi.org/10.1038/nrm1368.
53. **Dai Y, Rahmani M, Dent Grant PS.** 2005. Blockade of histone deacetylase inhibitor-induced RelA/p65 acetylation and NF-kappaB activation potentiates apoptosis in leukemia cells through a process mediated by oxidative damage, XIAP downregulation, and c-Jun N-terminal kinase 1 activation. Mol. Cell. Biol. **25:**5429–5444. http://dx.doi.org/10.1128/MCB.25.13.5429-5444.2005.
54. **Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW.** 2004. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J. **23:**2369–2380. http://dx.doi.org/10.1038/sj.emboj.7600244.
55. **Matkar S, Thiel A, Hua X.** 2013. Menin: a scaffold protein that controls gene expression and cell signaling. Trends Biochem. Sci. **38:**394–402. http://dx.doi.org/10.1016/j.tibs.2013.05.005.
56. **Brasier AR, Tian B, Jamaluddin M, Kalita MK, Garofalo RP, Lu M.** 2011. RelA Ser276 phosphorylation-coupled Lys310 acetylation controls transcriptional elongation of inflammatory cytokines in respiratory syncytial virus infection. J. Virol. **85:**11752–11769. http://dx.doi.org/10.1128/JVI.05360-11.
57. **Peterlin BMB, Price DHD.** 2006. Controlling the elongation phase of transcription with P-TEFb. Mol. Cell **23:**297–305. http://dx.doi.org/10.1016/j.molcel.2006.06.014.
58. **Pfeffer K, Matsuyama T, Kundig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Kronke M, Mak TW.** 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell **73:**457–467.
59. **Rossol-Voth R, Rossol S, Schütt KH, Corridori S, de Cian W, Falke D.** 1991. In vivo protective effect of tumour necrosis factor alpha against experimental infection with herpes simplex virus type 1. J. Gen. Virol. **72:**143–147. http://dx.doi.org/10.1099/0022-1317-72-1-143.

60. **Van Hauwermeiren F, Armaka M, Karagianni N, Kranidioti K, Vandenbroucke RE, Loges S, Van Roy M, Staelens J, Puimège L, Palagani A, Berghe WV, Victoratos P, Carmeliet P, Libert C, Kollias G.** 2013. Safe TNF-based antitumor therapy following p55TNFR reduction in intestinal epithelium. J. Clin. Invest. **123:**2590–2603. http://dx.doi.org/10.1172/JCI65624.
61. **Jamieson AM, Pasman L, Yu S, Gamradt P, Homer RJ, Decker T, Medzhitov R.** 2013. Role of tissue protection in lethal respiratory viral-bacterial coinfection. Science **340:**1230–1234. http://dx.doi.org/10.1126/science.1233632.
62. **Jayasekera JP, Vinuesa CG, Karupiah G, King NJC.** 2006. Enhanced antiviral antibody secretion and attenuated immunopathology during influenza virus infection in nitric oxide synthase-2-deficient mice. J. Gen. Virol. **87:**3361–3371. http://dx.doi.org/10.1099/vir.0.82131-0.
63. **Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F.** 2004. Recognition of commensal microflora by Toll-like receptors is required for intestinal homeostasis. Cell **118:**229–241. http://dx.doi.org/10.1016/j.cell.2004.07.002.
64. **Kirkland D, Benson A, Mirpuri J, Pifer R, Hou B, DeFranco AL, Yarovinsky F.** 2012. B cell-intrinsic MyD88 signaling prevents the lethal dissemination of commensal bacteria during colonic damage. Immunity **36:**228–238. http://dx.doi.org/10.1016/j.immuni.2011.11.019.
65. **Hoshi N, Schenten D, Nish SA, Walther Z, Gagliani N, Flavell RA, Reizis B, Shen Z, Fox JG, Iwasaki A, Medzhitov R.** 2012. MyD88 signalling in colonic mononuclear phagocytes drives colitis in IL-10-deficient mice. Nat. Commun. **3:**1120. http://dx.doi.org/10.1038/ncomms2113.
66. **Smith E, Lin C, Shilatifard A.** 2011. The super elongation complex (SEC) and MLL in development and disease. Genes Dev. **25:**661–672. http://dx.doi.org/10.1101/gad.2015411.
67. **Malik S, Roeder RG.** 2010. The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nat. Rev. Genet. **11:**761–772. http://dx.doi.org/10.1038/nrg2901.
68. **Conaway RC, Conaway JW.** 2013. The Mediator complex and transcription elongation. Biochim. Biophys. Acta **1829:**69–75. http://dx.doi.org/10.1016/j.bbagrm.2012.08.017.
69. **Donner AJ, Ebmeier CC, Taatjes DJ, Espinosa JM.** 2010. CDK8 is a positive regulator of transcriptional elongation within the serum response network. Nat. Struct. Mol. Biol. **17:**194–201. http://dx.doi.org/10.1038/nsmb.1752.
70. **Takahashi H, Parmely TJ, Sato S, Tomomori-Sato C, Banks CAS, Kong SE, Szutorisz H, Swanson SK, Martin-Brown S, Washburn MP, Florens L, Seidel CW, Lin C, Smith ER, Shilatifard A, Conaway RC, Conaway JW.** 2011. Human mediator subunit MED26 functions as a docking site for transcription elongation factors. Cell **146:**92–104. http://dx.doi.org/10.1016/j.cell.2011.06.005.
71. **Devaiah BN, Singer DS.** 2012. Cross-talk among RNA polymerase II kinases modulates C-terminal domain phosphorylation. J. Biol. Chem. **287:**38755–38766. http://dx.doi.org/10.1074/jbc.M112.412015.
72. **Dalrymple SA, Lucian LA, Slattery R, McNeil T, Aud DM, Fuchino S, Lee F, Murray R.** 1995. Interleukin-6-deficient mice are highly susceptible to Listeria monocytogenes infection: correlation with inefficient neutrophilia. Infect. Immun. **63:**2262–2268.
73. **Labow M, Shuster D, Zetterstrom M, Nunes P, Terry R, Cullinan EB, Bartfai T, Solorzano C, Moldawer LL, Chizzonite R, McIntyre KW.** 1997. Absence of IL-1 signaling and reduced inflammatory response in IL-1 type I receptor-deficient mice. J. Immunol. **159:**2452–2461.
74. **Eckmann L, Nebelsiek T, Fingerle AA, Dann SM, Mages J, Lang R, Robine S, Kagnoff MF, Schmid RM, Karin M, Arkan MC, Greten FR.** 2008. Opposing functions of IKKbeta during acute and chronic intestinal inflammation. Proc. Natl. Acad. Sci. U. S. A. **105:**15058–15063. http://dx.doi.org/10.1073/pnas.0808216105.
75. **Liu SF, Malik AB.** 2006. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. Am. J. Physiol. Lung Cell. Mol. Physiol. **290:**L622–L645. http://dx.doi.org/10.1152/ajplung.00477.2005.
76. **Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, Kastritis E, Gilpatrick T, Paranal RM, Qi J, Chesi M, Schinzel AC, McKeown MR, Heffernan TP, Vakoc CR, Bergsagel PL, Ghobrial IM, Richardson PG, Young RA, Hahn WC, Anderson KC, Kung AL, Bradner JE, Mitsiades CS.** 2011. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell **146:**904–917. http://dx.doi.org/10.1016/j.cell.2011.08.017.
77. **Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA, Magoon D, Qi J, Blatt K, Wunderlich M, Taylor MJ, Johns C, Chicas A, Mulloy JC, Kogan SC, Brown P, Valent P, Bradner JE, Lowe SW, Vakoc CR.** 2011. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. Nature **478:**524–528. http://dx.doi.org/10.1038/nature10334.
78. **Matzuk MM, McKeown MR, Filippakopoulos P, Li Q, Ma L, Agno JE, Lemieux ME, Picaud S, Yu RN, Qi J, Knapp S, Bradner JE.** 2012. Small-molecule inhibition of BRDT for male contraception. Cell **150:**673–684. http://dx.doi.org/10.1016/j.cell.2012.06.045.
79. **Ott CJ, Kopp N, Bird L, Paranal RM, Qi J, Bowman T, Rodig SJ, Kung AL, Bradner JE, Weinstock DM.** 2012. BET bromodomain inhibition targets both c-Myc and IL7R in high-risk acute lymphoblastic leukemia. Blood **120:**2843–2852. http://dx.doi.org/10.1182/blood-2012-02-413021.