The Lyme Disease Spirochete *Borrelia burgdorferi* Utilizes Multiple Ligands, Including RNA, for Interferon Regulatory Factor 3-Dependent Induction of Type I Interferon-Responsive Genes[▽]

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We recently discovered a critical role for type I interferon (IFN) in the development of murine Lyme arthritis. *Borrelia burgdorferi*-mediated induction of IFN-responsive genes by bone marrow-derived macrophages (BMDMs) was dependent upon a functional type I IFN receptor but independent of Toll-like receptor 2 (TLR2), TLR4, TLR9, and the adapter molecule MyD88. We now demonstrate that induction of the IFN transcriptional profile in *B. burgdorferi*-stimulated BMDMs occurs independently of the adapter TRIF and of the cytoplasmic sensor NOD2. In contrast, *B. burgdorferi*-induced transcription of these genes was dependent upon a rapid STAT1 feedback amplification pathway. IFN profile gene transcription was IRF3 dependent but did not utilize *B. burgdorferi*-derived DNA or DNase-sensitive ligands. Instead, IFN-responsive gene expression could be induced by *B. burgdorferi*-derived RNA. Interferon regulatory factor 3 (IRF3)-dependent IFN profile gene transcription was also induced by sonicated bacteria, by the lipoprotein OspA, and by factors released into the BSKII medium during culture of *B. burgdorferi*. The IFN-stimulatory activity of *B. burgdorferi* culture supernatants was not destroyed by nuclease treatment. Nuclease digestion also had no effect on IFN profile induction mediated by sonicated *B. burgdorferi*. Thus, *B. burgdorferi*-derived RNA, OspA, and non-nucleic acid ligands present in both sonicated bacteria and *B. burgdorferi* culture medium contribute to type I IFN-responsive gene induction. These findings suggest that *B. burgdorferi* invasion of joint tissue and the resultant type I IFN induction associated with Lyme arthritis development may involve multiple triggering ligands.

Type I interferons (IFNs) have long been recognized as potent antiviral cytokines and have also been associated with the response to several intracellular bacterial and protozoan pathogens, including *Listeria monocytogenes*, *Francisella tularensis*, and *Trypanosoma cruzi* (15, 20, 23, 47). Recent reports have also linked type I IFN production with host defense to extracellular bacterial pathogens, such as group A and group B streptococci (11, 14). Type I IFN production has been implicated in the development of autoimmune diseases, including systemic lupus erythematosus (SLE) and juvenile idiopathic arthritis (JIA), and in arthritis associated with type I IFN treatment of hepatitis C and multiple sclerosis patients (13, 28, 40, 44, 52). In addition, we recently uncovered a novel role for type I IFN in the development of subacute murine arthritis caused by the Lyme disease spirochete *Borrelia burgdorferi* (30).

Production of type I IFN can occur as a direct downstream consequence of pattern recognition receptor (PRR) ligation of pathogen-associated molecular patterns (PAMPs). The best-characterized pathways that culminate in the transcription of type I IFNs involve Toll-like receptor (TLR) ligation of bacterium-derived lipopolysaccharide (LPS), CpG DNA motifs, or virus-derived nucleic acids. Signal transduction through most TLRs involved in type I IFN production proceeds through the adapter molecule MyD88. Exceptions include the double-stranded RNA (dsRNA)-sensing TLR3- and TLR4-mediated type I IFN transcription, both of which signal through TRIF. Other PRRs that sense RNA viruses and result in transcription of type I IFNs include the RIG-I-like receptor (RLR) family members RIG-I, MDA5, and LGP2, all of which utilize the adapter molecule MAVS (also known as VISA, CARDIF, or IPS-1) for further signal transduction events (21, 45). Muramyl dipeptide (MDP) derived from *Listeria monocytogenes* and *N*-glycolyl-MDP derived from *Mycobacterium tuberculosis* were recently demonstrated to induce type I IFN transcription as a consequence of signaling through the NOD-like receptor (NLR) NOD2 (16, 23, 34). Although the signal transduction pathways driving NOD2-dependent transcription of type I IFN have not yet been elucidated for *L. monocytogenes*-activated macrophages (16, 23), *M. tuberculosis*-derived *N*-glycolyl-MDP utilizes the adapter molecule RIP2 to trigger type I IFN production in a TBK1-, interferon regulatory factor 3 (IRF3)-, and IRF5-dependent manner (34).

Although TLRs and RLRs utilize different adapter molecules, both pathways culminate in the downstream production of type I IFNs through the activation of the IRF kinases TBK1 and/or IKKε1 and the subsequent phosphorylation of the transcription factors IRF3 and/or IRF7, depending on the cell type and pathogen involved. Type I IFNs can then amplify the production of more type I IFN and IFN-responsive genes through ligation of the type I IFN receptor and the resultant downstream activation of JAK-STAT-mediated signal transduction events (21, 45).

Previous reports from both our laboratory and those of

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colleagues have demonstrated that *B. burgdorferi* stimulation results in IFN-responsive gene induction in joint tissue of infected mice, in skin and blood from Lyme disease patients, and in cultured macrophages/monocytes derived from humans and mice (30, 38, 39, 49, 50). We recently reported that induction of IFN-responsive genes in *Borrelia burgdorferi*-stimulated murine bone marrow-derived macrophages (BMDMs) was mediated by type I IFN, as transcription of IFN-responsive genes was abolished in type I IFN receptor-deficient (IFNAR1$^{-/-}$) BMDMs. Intriguingly, although *B. burgdorferi* is an extracellular bacterial pathogen whose primary mode of PRR signaling involves lipoprotein interaction with TLR1/TLR2 heterodimers, transcription of IFN-responsive genes was TLR2, TLR4, TLR9, and MyD88 independent (17, 30, 50).

Although MyD88-independent induction of type I IFN-responsive genes has been reported for numerous intracellular viruses and bacteria (21, 45), two recent publications have described a MyD88-independent transcription of type I IFN by extracellular bacterial pathogens. Stimulation of macrophages by both group A streptococci (GAS) and group B streptococci (GBS) triggers a MyD88-independent, IRF3-dependent type I IFN response (11, 14). It was also reported that type I IFN induction by BMDMs could be mediated by the cytosolic delivery of GBS DNA (11). Intriguingly, a recent publication described the utilization of RIG-I and MDA5 macrophage receptors by the intracellular bacterium *Legionella pneumophila* for the MyD88-independent induction of a type I IFN response (31). In contrast, neither the *B. burgdorferi* ligand(s) that triggers IFN profile induction by macrophages nor the signal transduction pathways utilized by activated macrophages to transcribe type I IFN-responsive genes have been identified. This report provides insight into the nature of the *B. burgdorferi* ligand(s) and the signal transduction pathways utilized by macrophages in the induction of type I IFN-responsive gene transcripts.

MATERIALS AND METHODS

Bacteria and mice. C3H/HeN and C57BL/6 (B6) mice were purchased from Charles River Laboratories, and C3H/HeJ, B6 Nod2$^{-/-}$ (B6.129S1-*Nod2tm1Flv/J*), and B6 TRIF$^{-/-}$ (C57BL/6J-*Ticam1Lps2/J*) mice were obtained from the Jackson Laboratory. B6 MyD88$^{-/-}$ mice (1) were provided by S. Akira (Hyogo College of Medicine, Japan). C3H STAT1$^{-/-}$ (9) mice were a gift from C. Brown (University of Missouri, Columbia, MO), and IRF3$^{-/-}$ femurs (41) were provided by D. T. Golenbock and K. A. Fitzgerald (University of Massachusetts Medical School, Worchester, MA).

The clonal *B. burgdorferi* strain N40 (cN40), provided by S. Barthold (University of California, Davis, CA), was utilized in all studies (6). Spirochetes were cultured in BSKII medium (4) containing 6% rabbit serum (Sigma, St. Louis, MO) prepared under endotoxin-free conditions at 32°C.

Reagents. LPS from *Escherichia coli* J5 (Rc) was purchased from List Biological Laboratories (Campbell, CA) and repurified to eliminate endotoxin protein contaminants, as described previously (18). The phosphorothioate-modified class B CpG oligonucleotide 1668 (TCCATGACGTTCCTGATGCT) was synthesized by the University of Utah Oligonucleotide/Peptide Synthesis Core facility (Salt Lake City, UT). Poly(dI:dC) was obtained from GE Healthcare Life Sciences (Piscataway, NJ), and MDP was purchased from Sigma. The construction, expression, and purification of recombinant lipidated *B. burgdorferi* OspA were described elsewhere (51). Recombinant interleukin-6 (IL-6) and IL-12 proteins utilized as standards in sandwich enzyme-linked immunosorbent assays (ELISAs), as well as corresponding capture and biotinylated detection antibodies, were obtained from BD Pharmingen (San Diego, CA). Horseradish peroxidase (HRP)-avidin was purchased from Vector Laboratories (Burlingame, CA). DNase I was obtained from New England BioLabs (Ipswich, MA), and RNase A was purchased from Roche Applied Science (Indianapolis, IN).

BMDM cell culture. BMDMs were isolated from the femurs and tibias of mice (29) and cultured in RPMI 1640 (Invitrogen Life Science, Carlsbad, CA) containing 30% L929 culture supernatant and 20% horse serum (HyClone, Logan, UT) at 37°C with 5% CO_2. After reaching confluence, BMDMs were harvested and replated at a density of 7.5×10^5/ml in 12-well dishes in 1 ml of serum-free RPMI 1640 containing 1% Nutridoma (Roche Applied Science). Nutridoma was utilized in lieu of medium optimized to ensure the greatest viability of *B. burgdorferi* upon addition to BMDMs (22), because the IFN response was optimal in serum-free media. The MycoProbe *Mycoplasma* detection kit (R&D Systems, Minneapolis, MN) was utilized to confirm that the L929 culture supernatants and all other cell culture media were free of contaminating *Mycoplasma* species.

Following an overnight incubation, the medium was removed and cells were stimulated in 12-well plates for 6 or 24 h in 1 ml of medium containing 7.4×10^6/ml live (multiplicity of infection [MOI], 10) or 5 μg/ml sonicated *B. burgdorferi* organisms. Prior to use in BMDM experiments, live bacteria were washed twice with phosphate-buffered saline (PBS) to remove BSKII components (30). *B. burgdorferi* viability was at least 85 to 90%, as assessed by Petroff-Hauser counting of motile spirochetes via dark-field microscopy. Sonicated cN40 was prepared as described elsewhere (42). In some experiments, other stimuli, such as LPS or poly(dI:dC), were added to separate wells, as indicated in the appropriate figure legend(s). Upon assay completion, cellular supernatants were removed for IL-6 and IL-12 cytokine assessment by sandwich ELISA, as described elsewhere (10).

Nuclease digestion. Nucleic acids were prepared from log-phase cultures of *B. burgdorferi* using standard procedures for DNA (3) and Trizol for RNA (2). Two micrograms of *B. burgdorferi* genomic DNA, RNA, or live or sonicated bacteria was digested with DNase I or RNase A according to the manufacturer's specifications.

***B. burgdorferi* culture supernatant.** Supernatant from late-logarithmic-phase cultures of *B. burgdorferi* was collected via centrifugation and was then applied to 0.2-μm filters (Corning Life Sciences, Lowell, MA) to remove any remaining bacterial cellular debris. Volumes of recovered *B. burgdorferi* culture supernatant ranging from 50 μl to 150 μl were mixed with serum-free medium to total 1 ml and applied to BMDMs. Control wells received corresponding volumes of sterile complete BSKII medium that had been incubated at 32°C without the inclusion of *B. burgdorferi*.

RNA isolation and quantitative reverse transcription-PCR (RT-PCR). RNA was isolated from cultured BMDMs at 6 or 24 h poststimulation by homogenization in Trizol reagent (Invitrogen Life Technologies), as specified by the manufacturer. Five micrograms of RNA was reverse transcribed into cDNA, and transcripts for genes of interest were amplified with the LightCycler 480 (LC480) SYBR green I master mix and an LC480 LightCycler (Roche Applied Science), as previously described (32). The copy number of the gene of interest was calculated from the starting template sample and normalized to 1,000 copies of the mouse β-actin housekeeping gene, as detailed elsewhere (32). The following primer pairs span introns (except for *Ifit2*) and were utilized for PCR amplification in this study: β-actin gene, 5′-GTAACAATGCCATGTTCAAT-3′ (forward) and 5′-CTCCATCGTGGGCCGCTCTAG-3′ (reverse); *Cxcl9*, 5′-TTGG GCATCATCTTCCTGGAGCAG-3′ (forward) and 5′-GAGGTCTTTGAGGG ATTTGTAGTGG-3′ (reverse); *Cxcl10*, 5′-GAAATCATCCCTGCGAGCCTA TCC-3′ (forward) and 5′-GCAATTAGGACTAGCCATCCACTGGG-3′ (reverse); *Gbp2*, 5′-CTACCGCACAGGCAAATCCTAC-3′ (forward) and 5′-G TCATTCTGGTTGTCACCCTTCTC-3′ (reverse); *Ifit1*, 5′-ATGGGAGAGAA TGCTGATGGTG-3′ (forward) and 5′-TGTCAAGGAACTGGACCTGCTC-3′ (reverse); *Ifit2*, 5′-CAACGAGTAAGGAGTCACTGGAGAG-3′ (forward) and 5′-TTGCTGGATGAAGCCCTCAG-3′ (reverse); *Tyki*, 5′-AGGAGGTCCAGA AAGGGAAGTTC-3′ (forward) and 5′-TATGGCGTAGGTGGCTGTGCTAT G-3′ (reverse); and *Igtp*, 5′-TAGAGCAGACCCACAGAGTTCAGG-3′ (forward) and 5′-CAGCAGTCATAGATTTAGACCACGG-3′.

B. burgdorferi RNA content was assessed using the previously published 16S rRNA gene oligonucleotide primers (33).

RNA interference (RNAi) silencing of *Irf3*. The Amaxa nucleofector Y-001 program and mouse macrophage nucleofector kit (Lonza Walkersville Inc.) were utilized for the transfection of BMDMs with small interfering RNAs (siRNAs), as previously described (53). Briefly, 80 pmol of SMARTpool *Irf3* siRNAs or of control, SMARTpool scrambled siRNAs (Dharmacon, Chicago, IL) was added to 2×10^6 BMDMs resuspended in 100 μl of mouse macrophage nucleofector solution. Immediately following transfection, BMDMs were placed in complete RPMI medium (2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol [2-ME]) containing 20% fetal calf serum (FCS), transferred to bacteriological petri dishes, and incubated at 37°C in a 5% CO_2 incubator for 48 h. Transfected cells were then replated in 1% Nutridoma at a density of 3×10^5 cells/well in 24-well plates and incubated overnight. The
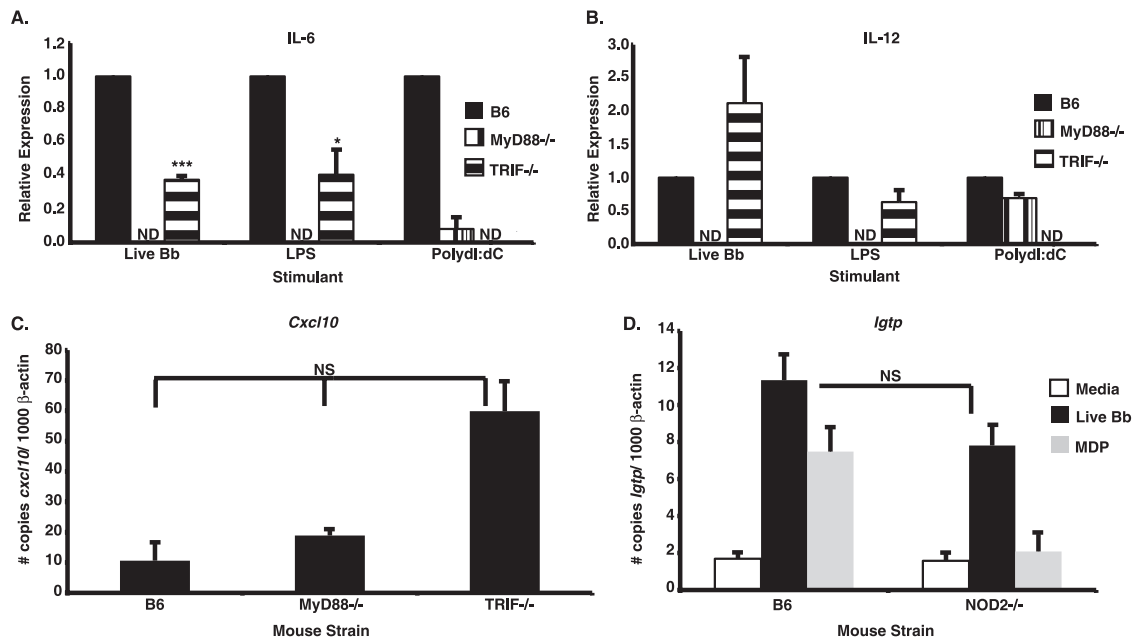
FIG. 1. *B. burgdorferi* induction of IFN-responsive genes is independent of TRIF and NOD2. (A and B) Wild-type B6, MyD88$^{-/-}$, or TRIF$^{-/-}$ BMDMs were stimulated with 7.4×10^6/ml live *B. burgdorferi* organisms (Bb), 100 ng/ml LPS, or 20 µg/ml poly(dI:dC) for 6 h. ND, not detected. The production of IL-6 (A) or IL-12 (B) was assessed by ELISA. Data indicate the means ± standard errors of the means (SEM) and are pooled from two independent experiments ($n = 4$). Statistical significance was assessed via a paired, two-tailed Student t test. ***, $P < 0.001$ for IL-6 levels obtained for TRIF$^{-/-}$ BMDMs stimulated with live *B. burgdorferi* versus comparably treated B6 BMDMs; *, $P < 0.05$ for LPS-treated TRIF$^{-/-}$ BMDMs versus similarly stimulated B6 BMDMs. In panel B, IL-12 levels for live *B. burgdorferi* ($P = 0.1996$)- or LPS ($P = 0.1010$)-stimulated TRIF$^{-/-}$ BMDMs were not significantly different from those for comparably treated B6 BMDMs. (C) Cellular RNA from B6, MyD88$^{-/-}$, or TRIF$^{-/-}$ BMDMs was analyzed by quantitative RT-PCR for IFN-responsive gene transcription at 6 h after *B. burgdorferi* stimulation. Data are depicted as the means ± SEM and are pooled from two independent experiments ($n = 4$). Statistical significance was assessed via an unpaired, two-tailed Student t test with the Welch correction. Transcript levels for *Cxcl10* are depicted as the fold change induction obtained for each normalized transcript following *B. burgdorferi* stimulation (7.4×10^6/ml) relative to values obtained for unstimulated cells. Although *B. burgdorferi*-induced IFN-responsive gene expression was elevated in TRIF$^{-/-}$ BMDMs, this increase was not statistically significant (NS) compared with values obtained for B6 ($P = 0.0519$) and MyD88$^{-/-}$ ($P = 0.0777$) BMDMs. (D) Cellular RNA from B6 or NOD2$^{-/-}$ BMDMs was analyzed by quantitative RT-PCR for IFN-responsive gene transcription at 24 h after *B. burgdorferi* stimulation. Data are depicted as the means ± SEM and are pooled from three independent experiments ($n = 6$). Statistical significance was assessed via the Mann-Whitney U test. *Igtp* transcript levels obtained from *B. burgdorferi* (7.4×10^6/ml)- or MDP (10 ng/ml)-stimulated B6 or NOD2$^{-/-}$ BMDMs are displayed as the number of copies normalized to β-actin. The *Igtp* expression levels obtained for B6 and NOD2$^{-/-}$ BMDMs following *B. burgdorferi* stimulation were not significantly different (NS) ($P = 0.1320$).

appropriate stimulus was added to each well, and cellular RNA was extracted at 6 h poststimulation. In some experiments, these protocols were used for the forced cytosolic delivery of *B. burgdorferi* sonicate or genomic DNA to BMDMs.

Statistics. A paired, two-tailed Student t test was utilized for two-group comparisons of parametric data exhibiting no differences in standard deviation. An unpaired, two-tailed Student t test was employed for data where pairing was ineffective, and for categorical data exhibiting differences in standard deviation, the Welch correction was applied to the t test. The Mann-Whitney U test was utilized for assessment of nonparametric data. A one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple-comparison test utilized as the posttest was employed for all multigroup comparisons. The threshold utilized to assess statistical significance for all tests was a P value of <0.05. GraphPad InStat3 version 3.0b for MacIntosh (GraphPad Software, San Diego, CA) was used for all statistical analyses.

RESULTS

Induction of IFN-responsive transcripts by BMDMs in response to *B. burgdorferi* does not require the adapter TRIF or the peptidoglycan sensor NOD2. We previously found that many components of the IFN-inducible gene profile found in joints of infected C3H mice were also induced following *B. burgdorferi* stimulation of BMDMs from wild-type and mutant mice. Importantly, BMDMs from both C3H and B6 mice dis-

play this response *in vitro*, although the magnitude of the response was greater in cells from C3H mice than in those from B6 mice (26, 30). The finding that IFN-responsive gene induction by BMDMs was MyD88 independent (30) suggested the possibility that the alternative TLR adapter, TRIF, could be involved in IFN-responsive gene induction by *B. burgdorferi*. Therefore, BMDMs obtained from wild-type B6 mice or from B6 mice with gene disruptions in MyD88 or TRIF were stimulated with live *B. burgdorferi* for 6 h. In agreement with previously published reports (7, 8, 12, 25), the cytokines IL-6 and IL-12 were detected in the supernatants of B6 BMDMs stimulated with *B. burgdorferi* but not in those obtained from MyD88$^{-/-}$ BMDMs. As anticipated, B6 BMDMs produced IL-6 and IL-12 in response to LPS and poly(dI:dC), whereas MyD88$^{-/-}$ BMDMs failed to secrete these cytokines following LPS administration. TRIF$^{-/-}$ BMDMs were unable to produce cytokines in response to poly(dI:dC) stimulation but responded to LPS, albeit at lower levels than the wild type (Fig. 1A and B). Intriguingly, TRIF$^{-/-}$ BMDMs produced significantly less IL-6 than wild-type B6 BMDMs in response to *B. burgdorferi* stimulation (Fig. 1A). Although we are not aware of

any previously published reports on IL-6 secretion by *B. burgdorferi*-infected TRIF$^{-/-}$ BMDMs, reduced BMDM IL-6 production in response to both *E. coli* and *Pseudomonas aeruginosa* has been previously documented (19, 37). IL-12 levels were also reduced following *B. burgdorferi* stimulation of TRIF$^{-/-}$ BMDMs, but this decrease did not achieve statistical significance (Fig. 1B). Both MyD88$^{-/-}$ and TRIF$^{-/-}$ BMDMs efficiently transcribed IFN-responsive genes following *B. burgdorferi* stimulation, as depicted in Fig. 1C for the representative IFN-inducible transcript *Cxcl10*. The "hyperinduction" of transcripts in TRIF$^{-/-}$ mice, although not statistically significant, was a consistent finding and possibly reflects streamlined activation of existing signaling pathways in the absence of competing pathways (unpublished observations with other mutant BMDMs). These data indicate that transcription of IFN-responsive genes by *B. burgdorferi*-stimulated BMDMs is not TRIF dependent and suggests that the extracellular spirochete may be utilizing a non-TLR receptor and signaling pathway previously associated with intracellular bacteria.

It was recently reported that *Listeria monocytogenes*- and *Mycobacterium tuberculosis*-mediated signaling through the NLR NOD2, which is known to be involved in inflammasome formation and signaling, results in increased TLR-independent induction of IFN-β (16, 23, 34). Since *B. burgdorferi* seemingly utilizes a TLR-independent receptor on BMDMs for the induction of IFN-responsive transcripts, we next asked whether NOD2 mediates transcription of these genes. Wild-type B6 and NOD2$^{-/-}$ BMDMs were stimulated for 24 h with live *B. burgdorferi* or with the NOD2 ligand MDP, cellular RNA was extracted, and IFN-responsive gene transcripts were quantified. MDP and *B. burgdorferi* stimulation of B6 BMDMs resulted in transcription of IFN-responsive genes. MDP stimulation of NOD2$^{-/-}$ BMDMs failed to induce transcription of IFN profile genes, as shown for the representative transcript *Igtp* (Fig. 1D). Induction of IFN profile genes was also observed in *B. burgdorferi*-treated NOD2$^{-/-}$ BMDMs (Fig. 1D), indicating that NOD2 is not required for *B. burgdorferi*-induced transcription of IFN-responsive genes.

STAT1 is required for *B. burgdorferi*-mediated induction of IFN-responsive gene transcription by murine BMDMs. Feedback amplification of type I IFNs and transcription of IFN-responsive genes are initiated via signaling through the type I IFN receptor and are dependent upon subsequent activation of JAK-STAT pathway members. STAT1 is the major transcriptional activator recruited for this pathway in macrophages (45). We previously demonstrated that IFN-responsive gene induction in *B. burgdorferi*-stimulated BMDMs was entirely dependent on a functional type I IFN receptor (30), and therefore we sought to assess whether STAT1 was critical for feedback amplification. IFN-responsive gene transcripts, as illustrated in Table 1 for the representative genes *Cxcl9*, *Cxcl10*, *Gbp2*, and *Ifit1*, were readily induced in wild-type C3H BMDMs stimulated with *B. burgdorferi*. In stark contrast, IFN-responsive gene expression was greatly reduced in BMDMs from C3H mice harboring the STAT1 gene ablation, as assayed at 24 h poststimulation (Table 1). Transcription of IFN profile genes was also observed in *B. burgdorferi*-activated TLR4-defective C3H/HeJ BMDMs (36, 48), indicating that IFN-responsive transcript induction was not due to potential endotoxin contamination of cultured bacteria (data not shown). Although

TABLE 1. IFN-responsive gene induction by *B. burgdorferi*-stimulated BMDMs is STAT1 dependent

Transcript	Level[a] (mean ± SEM) with BMDM genotype and stimulant:			
	C3H/HeN[b]		C3H STAT1$^{-/-c}$	
	Medium	Live *B. burgdorferi*	Medium	Live *B. burgdorferi*
Cxcl9	0.2 ± 0.1	0.6 ± 0.1**	0.0 ± 0.0	0.0 ± 0.0**
Cxcl10	2.6 ± 1.5	80.9 ± 10.8**	0.3 ± 0.2	0.3 ± 0.1**
Gpb2	15.0 ± 4.6	110.6 ± 9.8**	0.8 ± 0.2	1.6 ± 0.4**
Ifit1	13.9 ± 6.1	175.3 ± 35.6**	0.1 ± 0.0	0.4 ± 0.1**

[a] Number of copies normalized to 1,000 copies of the mouse β-actin housekeeping gene. Data are pooled from three independent experiments (*n* = 6). Statistical significance was assessed by the Mann-Whitney U test.
[b] **, *P* < 0.01 for live *B. burgdorferi* versus medium.
[c] **, *P* < 0.01 for STAT1$^{-/-}$/live *B. burgdorferi* versus C3H/HeN/live *B. burgdorferi*.

low-level IFN-responsive gene transcripts were detectable by 1 h in wild-type BMDMs, transcription of all genes examined was greatly reduced in STAT1$^{-/-}$ BMDMs, even at 1 h poststimulation (data not shown). Collectively, these data indicate that *B. burgdorferi*-mediated induction of type I IFN-responsive genes, including those reported to be involved in the earliest transcriptional response to *Listeria monocytogenes*, such as *Ifit1* (23), is entirely dependent upon an early feedback amplification loop that proceeds through STAT1.

IFN-responsive gene induction by *B. burgdorferi*-stimulated BMDMs is IRF3 dependent. IRF3-dependent signal transduction pathways are utilized by the majority of both extracellular and intracellular bacteria to trigger IFN-responsive gene transcription by macrophages (11, 14, 15, 23). For this reason, we assessed whether *B. burgdorferi*-mediated induction of IFN profile gene transcription by BMDMs is IRF3 dependent. Silencing of *Irf3* expression in C3H BMDMs by small interfering RNAs reduced, but did not abrogate, *Ifit1* and *Tyki* transcript levels in *B. burgdorferi*-stimulated BMDMs compared with values obtained from cells treated with a pool of control, scrambled siRNAs (Fig. 2A and B). To further address the involvement of IRF3, we next examined IFN-responsive gene expression in *B. burgdorferi*-stimulated BMDMs from IRF3$^{-/-}$ B6 mice. Transcription of the representative IFN profile genes *Cxcl10* and *Tyki* was nearly abrogated in IRF3$^{-/-}$ BMDMs, as these genes exhibited 97% and 86% decreases in expression levels, respectively, compared with values obtained for wild-type B6 BMDMs (Fig. 2C and D). Taken together, these data indicate that induction of IFN-responsive gene transcription by *B. burgdorferi*-stimulated BMDMs is IRF3 dependent.

IFN-responsive gene transcription can be induced by *B. burgdorferi* RNA. Previous studies utilizing group B streptococci indicated that IRF3-dependent induction of IFN-β by BMDMs was mediated by the cytosolic delivery of GBS DNA (11), whereas *L. pneumophila* utilizes the RNA-sensing receptors RIG-I and MDA5 for type I IFN gene transcription (31). These findings prompted us to examine whether *B. burgdorferi*-derived nucleic acid acts as the ligand triggering IRF3-dependent IFN-inducible gene expression by BMDMs. C3H BMDMs were stimulated with *B. burgdorferi* genomic DNA, RNA, live bacteria, or sonicated bacteria in both the presence and absence of nucleases, and the effects of these stimuli on
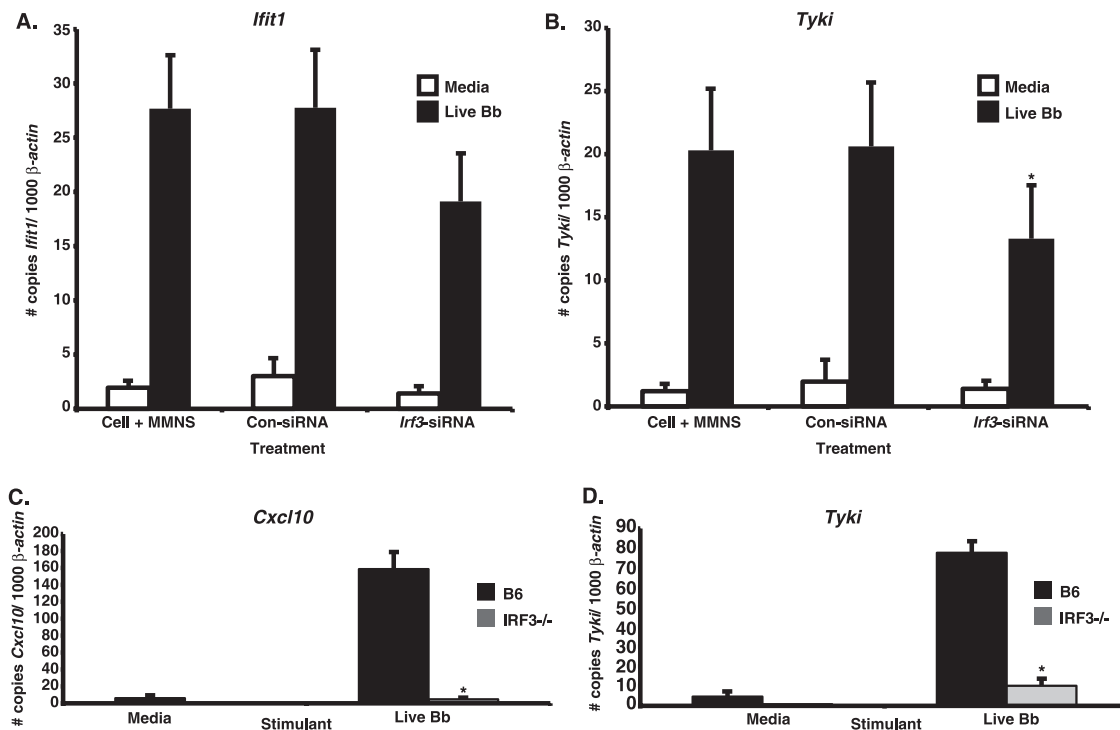
FIG. 2. *B. burgdorferi*-mediated induction of IFN-responsive genes is IRF3 dependent. (A and B) siRNA-mediated silencing of *Irf3*. Cultured C3H/HeN BMDMs were transfected with control siRNA (Con-siRNA), with *Irf3*-specific siRNA (*Irf3*-siRNA), or with transfection medium alone (MMNS) using the Amaxa nucleofector. At 72 h after transfection, cells were stimulated with 7.4×10^6/ml live *B. burgdorferi* organisms (Bb) for 6 h. Cellular RNA was assessed for IFN-responsive gene expression by RT-PCR, as shown for *Ifit1* (A) and *Tyki* (B). Data are shown as the means ± SEM and are pooled from four independent experiments ($n = 5$). For *Ifit1*, $P = 0.2491$ via a two-tailed, unpaired Student t test for live *B. burgdorferi*-stimulated *Irf3*-siRNA versus control siRNA. For *Tyki*, ∗, $P < 0.05$ via a two-tailed, paired Student t test for live *B. burgdorferi*-stimulated *Irf3*-siRNA versus control siRNA. (C and D) *B. burgdorferi* stimulation of IRF3$^{-/-}$ BMDMs. BMDMs obtained from B6 or IRF3$^{-/-}$ mice were stimulated with 7.4×10^6/ml live *B. burgdorferi* organisms for 6 h. RT-PCR results obtained for the representative IFN-responsive transcripts *Cxcl10* (C) and *Tyki* (D) are displayed as the number of copies of the gene of interest normalized to 1,000 copies of the mouse β-*actin* housekeeping gene. Data are displayed as the means ± SEM and are pooled from two independent experiments ($n = 4$). Differences between IRF3$^{-/-}$ and B6 live *B. burgdorferi*-stimulated BMDMs were determined via the Mann-Whitney U test ∗, $P < 0.05$.

IFN profile induction were assessed at 6 h poststimulation. As illustrated in Fig. 3A for the IFN-responsive transcripts *Tyki* and *Cxcl10*, the addition of *B. burgdorferi* RNA resulted in significant induction of IFN-responsive gene transcription. The stimulatory activity mediated by *B. burgdorferi* RNA was reduced upon incubation with RNase A (Fig. 3A). BMDM stimulation with *B. burgdorferi* genomic DNA did not result in IFN-inducible gene transcription, as shown in Fig. 3B for *Igtp*. In contrast, incubation with live or sonicated *B. burgdorferi* or with CpG DNA resulted in robust induction of IFN-responsive transcripts. As expected, DNase digestion of *B. burgdorferi* genomic DNA did not affect IFN-inducible gene expression, as the DNA does not act as a type I IFN-stimulatory ligand. Although DNase I and RNase A effectively digested *B. burgdorferi* genomic DNA and RNA, respectively (data not shown), neither DNase I nor RNase A digestion of live or sonicated *B. burgdorferi* caused a decrease in IFN-inducible transcript levels (Fig. 3B). In addition, forced cytosolic delivery of *B. burgdorferi* genomic DNA (0.7 ± 0.1 GBP/1,000 β-actin copies) via Amaxa nucleofector-mediated transfection did not result in the induction of IFN-responsive gene transcription by BMDMs, even though both sonicate (1.3 ± 0.8) and DNase I (2.1 ± 0.1)- or RNase A (2.2 ± 0.4)-digested sonicate preparations delivered

in this fashion triggered IFN profile transcription. These results indicate that *B. burgdorferi* RNA, but not genomic DNA, is utilized as a ligand for the induction of IFN-responsive gene transcription by BMDMs.

To determine why the IFN-stimulatory activity of live and sonicated *B. burgdorferi* was not destroyed by RNase A digestion, the 16S rRNA transcript levels of both live and sonicated *B. burgdorferi* and of RNase-digested samples were determined by RT-PCR. As expected, abundant 16S rRNA transcript was detected in live *B. burgdorferi*. Surprisingly, RNase A treatment failed to alter the 16S rRNA transcript levels obtained for live *B. burgdorferi* (Fig. 3C). In contrast to live *B. burgdorferi*, sonicated bacteria did not contain 16S rRNA, so RNase digestion had no effect (Fig. 3C). Collectively, our results indicate that *B. burgdorferi* RNA is one of at least two ligands capable of stimulating IFN profile transcription, since sonicate lacks 16S rRNA but still readily induces IFN gene expression.

B. burgdorferi culture supernatant contains components that trigger IRF3-dependent IFN-responsive gene transcription by BMDMs. Since *B. burgdorferi* sonicate, which is likely to include lipoproteins, contains a non-nucleic acid cell-associated component that mediates BMDM IFN profile induction, we next investigated whether OspA, a major lipoprotein induced
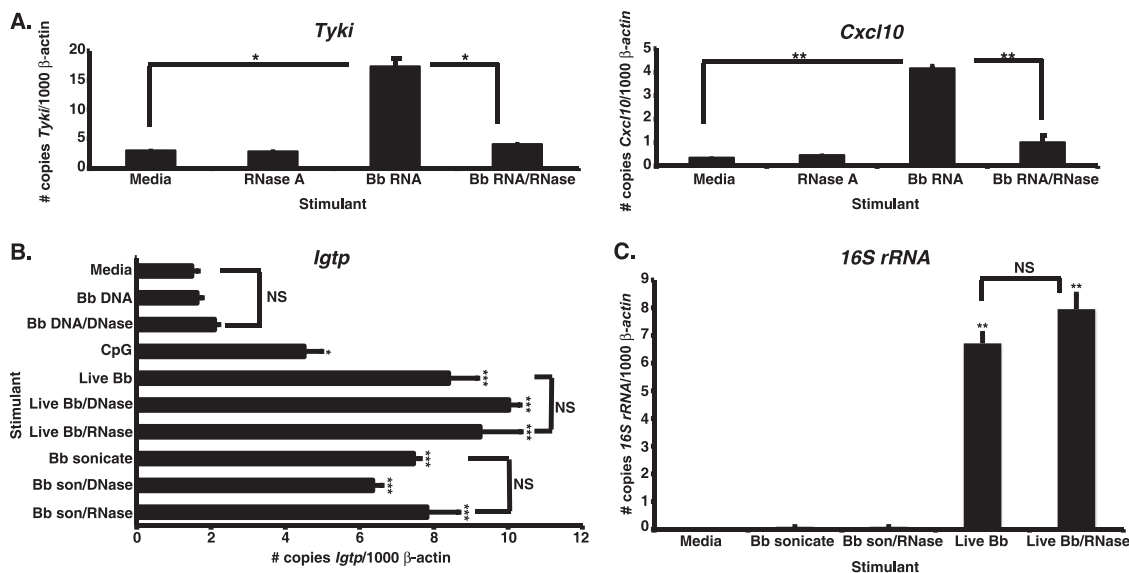
FIG. 3. *B. burgdorferi*-derived RNA induces IFN profile gene transcription by BMDMs. C3H/HeJ BMDMs were treated for 6 h with 2 μg *B. burgdorferi* (Bb) RNA, genomic DNA, RNase A-digested *B. burgdorferi* RNA, DNase I-digested *B. burgdorferi* DNA, 0.7 μM CpG oligonucleotide, 7.4×10^6/ml live *B. burgdorferi* organisms, live *B. burgdorferi* digested with DNase I or RNase A, 5 μg/ml sonicated *B. burgdorferi*, or sonicate digested with DNase I or RNase A. RT-PCR transcripts are displayed as the number of copies of the gene of interest normalized to 1,000 copies of the mouse β-actin housekeeping gene. (A) *B. burgdorferi* RNA stimulates IFN-responsive gene transcription by BMDMs. Transcript levels for *Tyki and Cxcl10* are shown. Data are depicted as the means ± SEM and are representative of two independent experiments ($n = 3$). Statistical significance was assessed via the two-tailed, unpaired Student t test with Welch correction. *, $P < 0.05$ for *B. burgdorferi* RNA versus medium and for *B. burgdorferi* RNA/RNase versus *B. burgdorferi* RNA. **, $P < 0.01$ for *B. burgdorferi* RNA versus medium and for *B. burgdorferi* RNA/RNase versus *B. burgdorferi* RNA. (B) *B. burgdorferi*-derived DNA is not a ligand for BMDM IFN profile gene induction. Transcript levels for *Igtp* are shown. Data are depicted as the means ± SEM and are representative of four independent experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA with the Tukey-Kramer multiple comparisons posttest. Significant differences for stimulated samples compared to medium alone: *, $P < 0.05$; ***, $P < 0.001$. Brackets indicate that there are no significant (NS) differences between live *B. burgdorferi*- or sonicate-stimulated samples versus the corresponding nuclease-digested treatment groups or for *B. burgdorferi* genomic DNA-stimulated versus *B. burgdorferi* DNA/DNase-digested samples. (C) Treatment with RNase A fails to destroy the 16S rRNA content in live *B. burgdorferi*. Transcript levels for *B. burgdorferi* 16S rRNA are shown. Data are shown as the means ± SEM and are representative of two independent experiments ($n = 3$). Statistical significance was assessed via the two-tailed, unpaired Student t test with the Welch correction. Differences between live *B. burgdorferi*- or live *B. burgdorferi*/RNase-stimulated BMDMs and medium: **, $P < 0.01$. Brackets indicate that there are no significant (NS) differences ($P = 0.1529$) between live *B. burgdorferi*- and live *B. burgdorferi*/RNase-digested samples.

during *in vitro* cultivation of *B. burgdorferi* (5), could elicit IFN-responsive gene transcription by BMDMs. As expected, addition of live or sonicated *B. burgdorferi* to TLR4-defective C3H/HeJ BMDMs resulted in increased IFN-responsive gene transcription, whereas LPS failed to activate the expression of these genes. Intriguingly, the addition of lipidated recombinant OspA (51) resulted in significant IFN profile gene transcription, as depicted in Fig. 4 for *Ifit1* (Fig. 4A) and *Ifit2* (Fig. 4B). We next assessed whether a *B. burgdorferi*-derived soluble component(s) could act as a ligand capable of eliciting IFN-responsive transcript induction from BMDMs. Volumes ranging from 50 to 150 μl of *B. burgdorferi* supernatant collected from late-logarithmic-phase cultures were mixed with serum-free cell culture medium and applied to cultured BMDMs obtained from C3H/HeJ mice. Equivalent volumes of bacterium-free BSKII medium served as controls for potential induction of IFN-responsive transcripts by BSKII components. Fifty microliters of culture supernatant resulted in increased IFN-responsive gene transcript levels, which was not seen in BMDMs treated with control BSKII medium. Addition of larger volumes of culture supernatant (shown in Fig. 4A and B for 150 μl) did not result in a further increase in IFN profile gene transcript levels, indicating that the stimulatory activity

was saturated with 50 μl of sample (Fig. 4A and B). Finally, we asked whether nucleic acids potentially released into the culture medium during *B. burgdorferi* growth were responsible for the IFN-inductive capacity of the culture supernatant. As demonstrated in Table 2 for *Cxcl10* and *Tyki*, neither DNase nor RNase digestion destroyed the IFN-stimulatory activity mediated by *B. burgdorferi* culture supernatant. These results suggest that there is a third ligand that is capable of inducing IFN-responsive gene transcription by BMDMs, as cultured *B. burgdorferi* releases a non-nucleic acid factor that stimulates IFN profile gene expression.

Since we have demonstrated in this report that IFN-responsive gene transcription by BMDMs stimulated with live *B. burgdorferi* is IRF3 dependent, we next asked whether IRF3 contributes to the inductive effect mediated by *B. burgdorferi* culture medium or sonicate. Application of *B. burgdorferi* culture supernatants to IRF3$^{-/-}$ BMDMs resulted in greatly diminished transcription of the IFN-responsive genes *Ifit1* (54.9 ± 12.8 copies/1,000 β-actin copies for B6 versus 7.4 ± 2.6 copies/1,000 β-actin copies for IRF3$^{-/-}$ BMDMs) and *Cxcl10* (79.8 ± 11.4 copies/1,000 β-actin copies for B6 versus 2.0 ± 0.2 copies/1,000 β-actin copies for IRF3$^{-/-}$ BMDMs) compared with the gene expression levels obtained from comparably
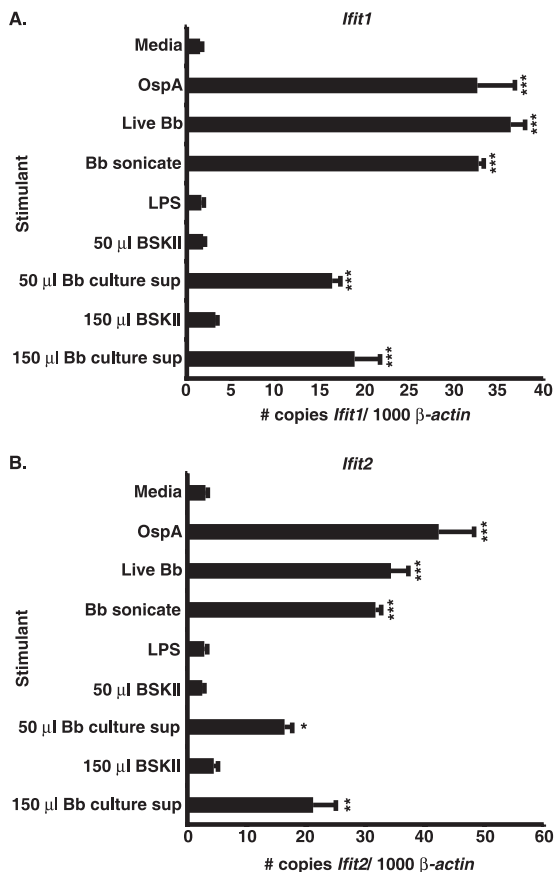
FIG. 4. Soluble components of *B. burgdorferi* spent medium govern IFN-responsive gene transcription by BMDMs. Cultured C3H/HeJ BMDMs were stimulated for 6 h with 5 µg/ml recombinant lipidated OspA, 7.4×10^6/ml live *B. burgdorferi* organisms (Bb), 5 µg/ml sonicated *B. burgdorferi*, 10 ng/ml LPS, 50 to 150 µl of *B. burgdorferi* culture supernatant, or 50 to 150 µl of clean BSKII medium. Data are depicted as the means ± SEM and are representative of two independent experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA with the Tukey-Kramer multiple comparisons posttest. Differences in *Ifit1* (A) and *Ifit2* (B) transcript levels between stimulated and unstimulated samples: *, $P < 0.05$; **, $P < 0.01$: ***, $P < 0.001$.

stimulated B6 BMDMs. Administration of sonicate to IRF3$^{-/-}$ BMDMs also resulted in reduced *Ifit1* (32.2 ± 1.8 copies/1,000 β-actin copies for B6 versus 13.7 ± 1.2 copies/ 1,000 β-actin copies for IRF3$^{-/-}$ BMDMs) and *Cxcl10* (43.5 ± 1.8 copies/1,000 β-actin copies for B6 versus 12.6 ± 2.1 copies/ 1,000 β-actin copies for IRF3$^{-/-}$ BMDMs) transcript levels. Taken together, these data indicate that a non-nucleic acid ligand(s) generated during *B. burgdorferi* growth contributes to the IRF3-dependent IFN-responsive gene transcription.

DISCUSSION

We recently uncovered a novel and previously unappreciated role for type I IFN in the development of severe Lyme arthritis in genetically susceptible C3H/HeN mice. In addition, we demonstrated that induction of IFN-responsive gene transcripts in BMDMs was entirely dependent upon a functional type I IFN receptor but independent of TLR2, -4, and -9 and of the adapter molecule MyD88 (30). The current report ex-

tends our previous observations by closely examining the nature of the ligand(s) and signal transduction pathways utilized by *B. burgdorferi* to elicit the downstream production of type I IFN-responsive genes by BMDMs. Here we present data indicating that *B. burgdorferi* utilizes multiple ligands for IRF3-dependent induction of type I IFN-responsive genes.

Although our previous work determined that IFN profile induction occurred independently of MyD88-dependent TLRs, we had not yet assessed whether this response was TRIF dependent. Similar to what has been reported for the extracellular group B streptococci (11) and for the intracellular bacteria *Listeria monocytogenes* (16, 23), *Francisella tularensis* (15), and *Mycobacterium tuberculosis* (34), we found that IFN-responsive gene transcripts are still highly induced in the absence of a functional TRIF adapter molecule (Fig. 1). These data indicated that initiation of a type I IFN response by *B. burgdorferi*-stimulated BMDMs does not involve TLR signaling. *L. monocytogenes*, *F. tularensis*, and *M. tuberculosis* each have the ability to escape from the phagosomal compartment of the macrophage and enter the cytosol of the cell. While in the cytosol, recognition of DNA derived from these pathogens, along with MDP-based signaling through NOD2, results in the downstream production of type I IFN by the infected macrophage (16, 23, 34). Our observation that *B. burgdorferi* does not utilize the cytoplasmic NLR NOD2 for the induction of IFN-responsive gene transcripts (Fig. 1) is not surprising, as it has been reported that this spirochete remains in the phagosome and does not escape to the cytosol (38). In the same vein, our finding that forced cytosolic delivery of *B. burgdorferi* genomic DNA or sonicate did not result in appreciable IFN-responsive gene transcription suggests that primary cytosolic signaling does not occur as a response to recognition of *B. burgdorferi* DNA. While we are unable to formally exclude the possibility of NLRP3 (cryopyrin) or other potential NLRs as potential receptors governing IFN profile induction, the failure of cytosolically administered *B. burgdorferi* to activate IFN-responsive gene transcription argues against this possibility. In addition, Liu and colleagues recently reported that the inflammasome is not utilized for *B. burgdorferi*-directed host defense (24). Collectively, our data indicate that *B. burgdorferi* likely utilizes an

TABLE 2. Nuclease treatment does not alter the IFN-stimulatory activity of *B. burgdorferi* culture supernatants

Stimulant	Transcript level (mean ± SEM)[a]	
	Cxcl10	*Tyki*
Medium	0.4 ± 0.0	3.0 ± 0.1
B. burgdorferi RNA[b]	4.2 ± 0.1***	17.3 ± 1.5*
B. burgdorferi RNA + RNase[c]	1.0 ± 0.3**	4.1 ± 0.2*
BSKII	0.5 ± 0.1	2.6 ± 0.1
B. burgdorferi culture supernatant[d]	3.4 ± 0.1**	9.0 ± 0.5**
B. burgdorferi supernatant + DNase	5.2 ± 0.7	10.6 ± 2.1
B. burgdorferi supernatant + RNase	3.0 ± 0.2	7.4 ± 1.5

[a] RT-PCR data are shown as the number of copies of the gene of interest per 1,000 copies of the β-actin gene and are representative of two independent experiments ($n = 3$). Statistical significance was assessed via a two-tailed, unpaired Student *t* test with the Welch correction.
[b] For *Cxcl10*: ***, $P < 0.001$ versus medium. For *Tyki*: *, $P < 0.05$ versus medium.
[c] For *Cxcl10*: **, $P < 0.01$ versus *B. burgdorferi* RNA. For *Tyki*: *, $P < 0.05$ versus *B. burgdorferi* RNA.
[d] For *Cxcl10*: **, $P < 0.01$ versus BSKII. For *Tyki*: **, $P < 0.01$ versus BSKII.

unidentified, potentially novel BMDM receptor to trigger transcription of IFN-responsive genes. Interestingly, *B. burgdorferi* also differs from other bacterial pathogens that elicit the production of IFN-responsive genes in that it does not have genes for a type III or IV secretion apparatus, nor does it possess phospholipases.

Type I IFN profile gene transcription by *B. burgdorferi*-stimulated BMDMs was entirely dependent upon an early STAT1-dependent feedback amplification loop, the effects of which were evident by 1 h poststimulation (Table 1 and data not shown). Interestingly, the induction of all analyzed IFN-responsive transcripts, including *Tyki*, *Ifit1*, and *Ifit2*, was suppressed in *B. burgdorferi*-stimulated STAT1$^{-/-}$ BMDMs (Table 1 and data not shown). These genes were reported to be among the first IFN-inducible genes transcribed by BMDMs without a requirement for secondary cytokine signaling in response to cytosolic *L. monocytogenes* (23). These results indicate that the temporal and compartment-specific transcription of these genes differs between *B. burgdorferi* and *L. monocytogenes*. The results obtained with *B. burgdorferi*-stimulated STAT1$^{-/-}$ BMDMs are intriguing but somewhat difficult to interpret, because antibody-mediated blockage of the type I IFN receptor prevents the development of severe Lyme arthritis (30), yet STAT1$^{-/-}$ mice still develop arthritis (9). However, joint tissue is very complex and multiple different types of cells are present, whereas cultured BMDMs are a homogenous single-cell population. Although absent in STAT1$^{-/-}$ BMDMs, compensatory pathways for type I IFN-responsive gene induction may be triggered by other cell types present within, or trafficking to, the joint microenvironment of STAT1$^{-/-}$ mice.

Similar to what has been reported for the majority of bacterial pathogens (11, 14, 15, 23), induction of IFN-responsive genes by *B. burgdorferi*-stimulated BMDMs was IRF3 dependent (Fig. 2). Previous studies conducted with *Listeria monocytogenes* (23, 43), *Legionella pneumophila* (43), and GBS (11) indicated that IRF3-dependent induction of type I IFN is driven by cytosolic recognition of pathogen-derived DNA. However, in stark contrast to the case for other bacterial pathogens, *B. burgdorferi*-mediated transcription of macrophage IFN-responsive genes was not governed by recognition, cytosolic or otherwise, of its DNA (Fig. 3). This finding was in agreement with our previous work utilizing *B. burgdorferi*-stimulated TLR9$^{-/-}$ BMDMs to demonstrate that this DNA sensor was not required for type I IFN profile gene transcription by *B. burgdorferi*-stimulated mouse macrophages (30). However, while this paper was in preparation, Petzke and colleagues reported that *B. burgdorferi* stimulated the TLR9-dependent production of type I IFN by human peripheral blood mononuclear cells (PBMCs) (35). Potential reasons for the discrepancies in the results obtained by these studies could include differences in experimental methodology and in both the species origin and cell types analyzed. We analyzed a single, pure cell population, the BMDM, from mice, whereas Petzke et al. utilized a mixed human cell population (PBMCs).

It was recently reported that *L. pneumophila* induces type I IFN production by BMDMs via ligation of the MyD88-independent RIG-I and MDA5 RNA recognition receptors (31). We now report that *B. burgdorferi*-derived RNA is one ligand that triggers IFN profile induction by BMDMs (Fig. 3A). Live

B. burgdorferi stimulates the MyD88-independent transcription of IFN-responsive genes (Fig. 1C) (30) and contains 16S rRNA (Fig. 3C), indicating that RNA likely contributes to the IFN-stimulatory capacity of live *B. burgdorferi*. Unfortunately, since live *B. burgdorferi* was resistant to RNase A digestion (Fig. 3C), it is unclear what proportion of the IFN-stimulatory activity can be attributed to RNA. However, in agreement with our results, Petzke and colleagues did find that inhibition of TLR7 on human PBMCs reduced type I IFN responses to live *B. burgdorferi* (35). IFN-responsive gene transcription was also governed by a second ligand: a non-nucleic acid, cell-associated molecule present in sonicated *B. burgdorferi* (Fig. 3B and C). The identity of this cell-associated molecule is currently unknown. Although studies with synthetic TLR2 ligands have failed to identify a signal transduction pathway that culminates in the downstream transcription of type I IFN, we demonstrated that BMDM IFN profile gene transcription could be induced by recombinant *B. burgdorferi* lipidated OspA (Fig. 4). These findings are in agreement with our previous report on the induction of IFN-β transcripts by purified OspA in macrophages from C3H/HeJ mice (27).

Significant induction of IFN-responsive gene transcripts was also mediated by a fourth ligand: a non-nucleic acid molecule(s) present in *B. burgdorferi* culture supernatant. Intriguingly, we also demonstrated that the inductive capacity mediated by components released during culture of *B. burgdorferi* was entirely dependent upon functional IRF3. Although LPS stimulation of TRIF can result in IRF3-dependent transcription of type I IFNs, *B. burgdorferi* lacks LPS (46). This allowed us to control for potential endotoxin contamination of medium components by utilizing the TLR4-defective C3H/HeJ BMDMs, where significant elevation of IFN-responsive gene transcription was stimulated by medium collected from cultured bacteria but not by unused medium (Fig. 4). To our knowledge, this is the first report to describe IRF3-dependent induction of type I IFN-responsive genes by soluble components present in bacterial culture supernatant. While the origin of these soluble factors is currently unknown, potential candidates include, but may not be limited to, proteins, small molecules, or lipids.

In conclusion, we have presented evidence that *B. burgdorferi* utilizes multiple ligands to trigger IFN-responsive gene transcription by BMDMs. Our data support the existence of at least four ligands: *B. burgdorferi*-derived RNA; a distinct non-nucleic acid, cell-associated molecule present in sonicated bacteria; the lipoprotein OspA; and a non-nucleic acid factor released by cultured *B. burgdorferi*. It is also possible that lipoprotein could be one constituent of the cell-associated or released factor. The novelty of these findings is of heightened significance since the production of type I IFN in response to *B. burgdorferi* assumes a critical role in the development of Lyme arthritis in mice but not in host defense (30). This important distinction may explain why the ligands/pathways utilized by *B. burgdorferi*-stimulated BMDMs for the transcription of type I IFN-responsive genes likely differ from those triggered as a host defense response to other bacterial pathogens. In addition, the recent findings that human monocytes also produce type I IFN in response to *B. burgdorferi* indicate that induction of this pathway may also have important clinical consequences in human Lyme disease patients (35, 38). Therefore, elucidation of the signal transduction pathways utilized

for type I IFN production by macrophages in response to *B. burgdorferi* infection could lead to the development of improved therapeutic agents for Lyme disease patients, particularly those afflicted with arthritis.

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