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Beta Interferon-Mediated Activation of Signal Transducer and Activator of Transcription Protein 1 Interferes with *Rickettsia conorii* Replication in Human Endothelial Cells▽

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Infection of the endothelial cell lining of blood vessels with *Rickettsia conorii*, the causative agent of Mediterranean spotted fever, results in endothelial activation. We investigated the effects of *R. conorii* infection on the status of the Janus kinase (JAK)-signal transducer and activator of transcription protein (STAT) signaling pathway in human microvascular endothelial cells (HMECs), the most relevant host cell type, in light of rickettsial tropism for microvascular endothelium *in vivo*. *R. conorii* infection induced phosphorylation of STAT1 on tyrosine 701 and serine 727 at 24, 48, and 72 h postinfection in HMECs. Employing transcription profile analysis and neutralizing antibodies, we further determined that beta interferon (IFN-β) production and secretion are critical for STAT1 activation. Secreted IFN-β further amplified its own expression via a positive-feedback mechanism, while expression of transcription factors interferon regulatory factor 7 (IRF7) and IRF9, implicated in the IFN-β–STAT1 feedback loop, was also induced. Metabolic activity of rickettsiae was essential for the IFN-β-mediated response(s) because tetracycline treatment inhibited *R. conorii* replication, IFN-β expression, and STAT1 phosphorylation. Inclusion of IFN-β-neutralizing antibody during infection resulted in significantly enhanced *R. conorii* replication, whereas addition of exogenous IFN-β had the opposite inhibitory effect. Finally, small interfering RNA-mediated knockdown further confirmed a protective role for STAT1 against intracellular *R. conorii* replication. In concert, these findings implicate an important role for IFN-β-mediated STAT1 activation in innate immune responses of vascular endothelium to *R. conorii* infection.

Rickettsia conorii, the etiologic agent of Mediterranean spotted fever (MSF), is a Gram-negative, obligate intracellular bacterial pathogen transmitted to humans via arthropod vectors. As one of the prototypical species belonging to the spotted fever group (SFG) rickettsiae, *R. conorii* displays tropism for microvascular endothelium during human infections and in a well-established animal model of infection and robust actin-based directional motility for intracellular movements and intercellular spread. Compared to Rocky Mountain spotted fever, caused by *R. rickettsii*, MSF has traditionally been considered a relatively milder disease, with clinical symptoms of fever, headache, and maculopapular rash. Mainly because of delays in diagnosis and timely start of doxycycline therapy, however, MSF can result in significant morbidity and mortality. More recently, a severe form of MSF has also been reported, supported by evidence of vascular lesions in kidneys, gastrointestinal tract, liver, spleen, and skin and complications leading to meningoencephalitis, Guillain-Barré syndrome, hypoxemia, multiorgan failure, and death (25, 26, 28, 34, 35, 46). A clinical study of severe MSF cases in Algeria documented a fatality rate as high as 54.5% among the patients with multiorgan involvement of the disease, despite implementation of treatment with antibiotics (26). In spite of their global distribution and the resurgence of human rickettsial infections, the mechanisms underlying the virulence and pathogenesis of this group of diseases remain poorly characterized.

Infection with SFG rickettsiae activates otherwise quiescent vascular endothelium to acquire a procoagulant and proinflammatory phenotype by inducing the expression and/or secretion of a variety of cytokines and chemokines as well as surface adhesion molecules (17, 37, 42). As essential immunoregulators, interferons (IFNs) have also been demonstrated to play an important role in host innate and adaptive immune responses during bacterial infections. Upon production and secretion in response to microbial stimuli, type I interferons activate IFN-inducible genes, which in turn modulate the innate host defense to establish an antimicrobial state in infected cells. In comparison to the existing and rapidly expanding literature on the manipulation of IFN signaling by viruses, the knowledge of how IFN-inducible genes provide protection against bacterial pathogens is lagging far behind. Further, there is no published evidence on whether or not *Rickettsia*-infected endothelium is capable of secreting IFNs, and the potential roles of IFN-mediated cell signaling in host defense remain completely unknown.

To regulate host immunomodulatory genes, interferons trig-

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ger phosphorylation of latent transcription factors belonging to the signal transducer and activator of transcription (STAT) family of proteins via activation of Janus kinases (JAKs). The biological effects of type I IFNs are primarily mediated via formation of STAT1-STAT2-interferon regulatory factor 9 (IRF9) heterotrimeric complex named IFN-stimulated gene factor 3 (ISGF3) (8, 9, 20–22). Binding of the ISGF3 protein to IFN-stimulated response elements (ISREs) induces IFN-stimulated genes, thus activating cellular innate immune responses (3, 7). However, functions of JAK-STAT signaling in modulating critical host responses to *Rickettsia* infection remain as yet unidentified. With an aim to fill this critical knowledge gap in host-pathogen interactions and to better understand the role of JAK-STAT signaling in *Rickettsia*-infected endothelium, we investigated whether or not (i) interferons are produced and secreted by *R. conorii*-infected endothelial cells, (ii) the JAK-STAT1 pathway is activated by the infection, and (iii) IFN-mediated STAT1 activation is capable of inhibiting intracellular *R. conorii* replication in endothelium. Our data demonstrate that *R. conorii* infection triggers beta IFN (IFN-β)-mediated autocrine STAT1 activation in human endothelial cells, a response that requires host cell infection with viable intracellular rickettsiae. Moreover, IFN-β plays an important role in activating host innate immune responses to inhibit *R. conorii* replication in endothelial cells in a STAT1-dependent manner.

MATERIALS AND METHODS

Cell culture and *R. conorii* infection. HMEC-1, an immortalized line of human microvascular endothelial cells (HMECs), was grown under sterile culture conditions in MCDB 131 medium (Gibco) containing fetal bovine serum (FBS; 10% [vol/vol]; Aleken Biologicals), epidermal growth factor (10 ng/ml; Becton Dickinson), hydrocortisone (1 μg/ml; Sigma), and L-glutamine (10 mM; Gibco) as previously described (40). Human umbilical vain endothelial cells (HUVECs) were isolated from umbilical cords using an established procedure (32) and cultured in McCoy 5A medium (Gibco) supplemented with 20% FBS (Aleken Biologicals), endothelial cell (EC) growth supplement (Gibco), heparin (Sigma), and L-glutamine (Gibco). To ensure consistency, primary HUVECs were used after a second passage in culture for all experiments. *Rickettsia conorii* (Malish 7 strain) was propagated in Vero cells and purified by density gradient centrifugation, and plaque-purified stocks were kept frozen as aliquots of ≤0.5 ml (38). Both HMECs and HUVECs at an approximately 80 to 90% confluence level were infected with *R. conorii* according to our previously established protocols (30, 38), with the dose of infection ranging from 6×10^2 to 6×10^4 PFU per cm^2 of culture surface area, depending on the nature of endpoints for individual experiments. Because rickettsial invasion of host cells is almost instantaneous (43), extracellular bacteria in the culture medium were removed by gentle washing at 3 h postinfection and then replacing the initial infectious inoculum with fresh culture medium. To inhibit the metabolic activities of intracellular rickettsiae, tetracycline (Sigma) at a final concentration of 20 μg/ml was introduced into the culture medium at 3 h.

Preparation of conditioned medium from *R. conorii*-infected endothelial cells. Cell culture supernatants from *R. conorii*-infected or control uninfected cells were collected at different times postinfection and mixed with tetracycline (10 μg/ml) to inactivate any extracellular rickettsiae. The mixture was then passed through a 0.2-μm-pore-size nonpyrogenic filter (Whatman) to collect pathogen-free conditioned medium. Naïve endothelial cells were then incubated with the conditioned medium for 6 h for subsequent analysis of STAT1 activation.

Treatment with IFN-β and neutralization using antibodies. Cells were treated with 10 ng/ml of recombinant human IFN-β (PBL Interferon Source) after *R. conorii* infection. For IFN-β neutralization, culture medium was supplemented with 10 μg/ml of anti-human IFN-β antibody (R&D Systems). To neutralize the IFN-β in conditioned medium, 10 μg/ml of anti-human IFN-β antibody (R&D Systems) was added to the conditioned medium, which was then subjected to gentle mixing on a rocker platform for 90 min at 37°C.

***R. conorii* quantification by real-time PCR.** HMECs infected in individual wells of 6-well plates were scraped into the medium and pelleted by centrifugation at 10,000 × *g* for 30 min. Total DNA from the cellular pellets was extracted using a DNeasy blood and tissue kit (Qiagen). In the final preparatory step, DNA was eluted in 100 μl of Ultra-Pure distilled water (Gibco), followed by determination of its concentration in final preparations on a Nanodrop spectrophotometer (ND-1000; Thermo Scientific). The primer pair RR190.547F and RR190.701R, previously described for the quantification of SFG rickettsiae, was used to evaluate *R. conorii* replication in HMECs (5). Briefly, the PCR mixture (total volume, 20 μl) contained 5 ng of total DNA, primers at a final concentration of 10 μM, and iQ SYBR green Supermix (Bio-Rad). DNA template was initially denatured at 95°C for 3 min, followed by 95°C for 30 s, 57°C for 20 s, and 65°C for 1 min for 45 cycles and a final extension cycle at 72°C for 5 min. *R. conorii* copy number was calculated using a standard curve, generated by the pCR2.1-TOPO plasmid containing the RR190.547/RR190.701 PCR amplicon (5), and the *R. conorii* DNA copy number in each well was normalized to the amount of total DNA.

siRNA-mediated knockdown of STAT1. Validated small interfering RNA (siRNA) sequences for STAT1 and scrambled siRNA (control) were purchased from Qiagen (catalog nos. SI02662884 and SI03650318, respectively). HMECs at about 80% confluence were transfected with either STAT1-specific or scrambled siRNAs (both at 80 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 6 h, the medium containing transfection reagent and siRNAs was replaced with fresh culture medium and the cells were allowed to recover at 37°C for at least 12 h prior to infection with *R. conorii*.

Western blotting. Monolayers of uninfected and *R. conorii*-infected HMECs at different times postinfection were washed with prechilled phosphate-buffered saline (PBS) and disrupted by thorough scraping in a cell lysis solution (Tris buffer [pH 7.4], supplemented with protease and phosphatase inhibitor cocktails and 0.2% [wt/vol] SDS), followed by mild sonication. Total protein lysates thus obtained were separated on a polyacrylamide gel composed of a 5% stacking segment and a 10% resolution phase. The proteins were then transferred onto a nitrocellulose membrane using a wet blotting apparatus at 100 V for 90 min. The probing of blots was carried out employing anti-phospho-STAT1 (Tyr701), phospho-STAT1 (Ser727), total STAT1, and IRF7 (all from Cell Signaling Technology) and IRF9 (Santa Cruz) primary antibodies raised in rabbit and a compatible horseradish peroxidase (HRP)-linked secondary antibody for chemiluminescence-based detection. To normalize for variations in the loading of samples on different lanes, the blots from all experiments were stripped and probed with a mouse anti-human α-tubulin antibody (Accurate Chemical & Scientific Corporation) and a corresponding anti-mouse IgG-HRP (Santa Cruz). Protein-antibody complexes were detected using Western Lightning enhanced chemiluminescence reagent (Perkin Elmer).

Gene expression analysis by quantitative real-time PCR. Total RNA, isolated from *R. conorii*-infected and mock-infected endothelial cells using TRI-Reagent (Molecular Research Center, Inc.), was further purified using a quantitative PCR (qPCR)-grade RNA purification kit (SA Biosciences) and quantified by a Nanodrop spectrophotometer (ND-1000; Thermo Scientific). cDNA was then synthesized using an RT^2 first-strand kit (SA Biosciences) and pure total RNA as a template. Quantitative PCRs were performed in a MyiQ thermal cycler (Bio-Rad) using primers specific for the human IFN-β and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes with an RT^2 real-time SYBR green master mix (SA Biosciences) according to the manufacturer's instructions. Expression levels of target genes were normalized to that of the GAPDH gene, and relative expression was calculated by the $\Delta\Delta C_T$ (threshold cycle) method. For JAK-STAT pathway-focused PCR arrays, PCR was performed in a 96-well plate with 84 target genes using RT^2 real-time SYBR green master mix (SA Biosciences) in a MyiQ cycler (Bio-Rad), and data analysis was done using the SA Biosciences web-based PCR array data analysis tool.

Densitometry and statistical analysis. The autoradiograms for Western blots were scanned in grayscale mode at a resolution of 600 dpi, and band intensity was calculated with ImageJ software (version 1.42). Band intensities normalized to the levels of α-tubulin were assigned values relative to the corresponding untreated/uninfected controls, which were given a value of 1. All experiments were performed in triplicate, and statistical significance between the experimental and control groups was evaluated by Student's *t* test. Results were considered statistically significant when the *P* value was ≤0.05.

RESULTS

***R. conorii* infection induces STAT1 phosphorylation in endothelial cells.** The JAK-STAT pathway plays an important role in host defense against bacterial infections. STAT1 activation is regulated by phosphorylation of specific amino acid

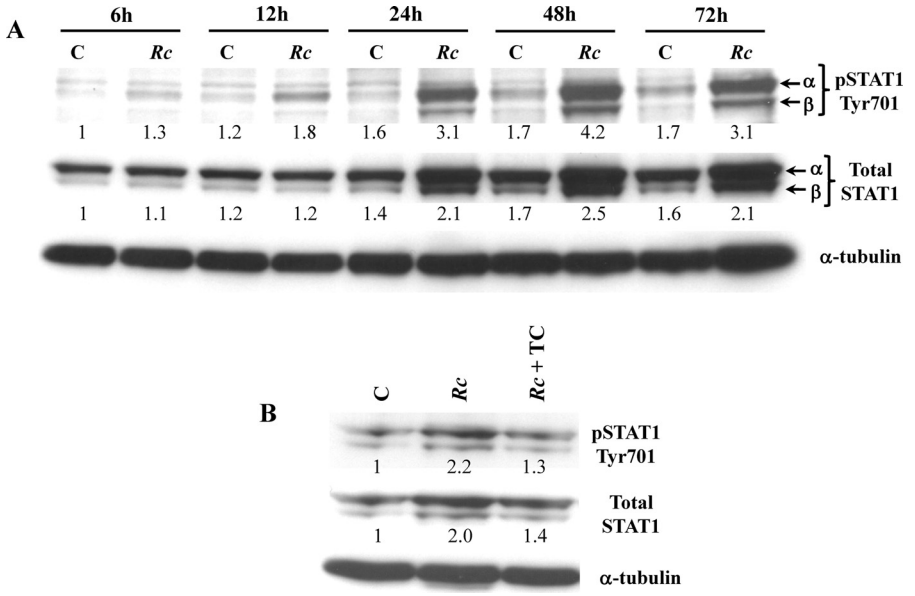

FIG. 1. Kinetics of STAT1 phosphorylation in *R. conorii*-infected HMECs. (A) STAT1 phosphorylation in *R. conorii*-infected HMECs at different times postinfection. The steady-state levels of STAT1 phosphorylated at Tyr701 and total STAT1 were determined by Western blot analysis, followed by probing for the levels of the housekeeping protein α-tubulin to normalize for any variations in the loading of samples on different lanes of the gel. For each time point, mock-infected HMECs were simultaneously processed as the corresponding controls. The relative positions of the α and β isoforms of STAT1 are indicated by arrows. For the ease of comparison between uninfected controls (C) and *R. conorii*-infected HMECs (*Rc*) at different times, the levels of phospho-STAT1 and total STAT1 in uninfected cells at 6 h (lane 1 from the left) were assigned a value of 1, and an increase of ≥2-fold relative to this baseline was considered to be a significant difference. The results from a representative blot ($n \geq 3$) are shown. (B) Effect of the presence of tetracycline (TC; 20 μg/ml) on the levels of phosphorylated and total STAT1 protein in HMECs infected for 48 h.

residues, which triggers dimerization, nuclear translocation, and binding to specific nucleotide sequences in the promoter regions of target genes. We initially infected HMECs with *R. conorii* at 6×10^3 PFU/cm^2 for various durations of time to evaluate the phosphorylation status of STAT1 by immunoblotting. In comparison to relatively low basal phosphorylation in mock-infected controls, *R. conorii* infection of HMECs resulted in significantly increased Tyr701 phosphorylation of both α and β isoforms of STAT1 at 24, 48, and 72 h postinfection (Fig. 1A). Moreover, *R. conorii* infection also increased the cellular abundance of total STAT1 protein, likely via increased protein synthesis or decreased and/or delayed degradation or possibly a combination of both. Infection of HMECs with various doses of *R. conorii* ranging from 6×10^4 PFU/cm^2 to 60 PFU/cm^2 yielded evidence for increased STAT1 phosphorylation even at lower doses of infection, indicating a high sensitivity of HMECs to trigger such a response (data not shown). We next investigated whether STAT1 activation was dependent on the intracellular replication/growth of viable rickettsiae. HMECs infected for 3 h with viable *R. conorii* were treated with tetracycline to inhibit the metabolic activity of intracellular rickettsiae. As shown in Fig. 1B, tetracycline treatment of *R. conorii*-infected HMECs led to the inhibition of STAT1 Tyr701 phosphorylation at 48 h postinfection. Because infection of HUVECs has traditionally been used as a model culture system to study *Rickettsia*-host cell interactions *in vitro*, we further investigated whether *R. conorii* triggers a similar pattern of STAT1 phosphorylation after infection of cultured HUVECs. As expected, the levels of phosphorylated STAT (pSTAT1) in mock-infected cells were very low, but *R. conorii*-infected HUVECs also displayed increased phosphorylation of STAT1 on Tyr701 starting at 24 h postinfection and markedly increased steady-state levels of pSTAT1 were present in HUVECs infected for 24, 48, and 72 h. Again, as was evident for infected HMECs, the amount of total cellular STAT1 was also increased during *R. conorii* infection of HUVECs (Fig. 2A). Supplementation of culture medium with tetracycline at 3 h postinfection inhibited STAT1 phosphorylation in infected HUVECs (Fig. 2B). Moreover, introduction of tetracycline into the culture medium at 24 h, a time point at which increased STAT1 phosphorylation is established, also resulted in at least partial inhibition of STAT1 phosphorylation at the later time of 48 h (Fig. 2C). Together, these results demonstrate that STAT1 is activated as a host response to intracellular infection with *R. conorii*. In addition, such a response not only requires but also is sustained by the metabolic activity of intracellular rickettsiae in both infected HMECs and HUVECs.

***R. conorii* infection of human endothelial cells induces interferon-regulated genes.** Next, we analyzed the expression profile of JAK-STAT-regulated genes by pathway-specific PCR arrays using RNA isolated from HUVECs infected with *R. conorii* for 48 h and corresponding uninfected controls. The qPCR-based microarray utilized in these experiments included interferon-inducible genes, STAT target genes, negative regulators of the JAK-STAT pathway, SH3/SH2 adaptor proteins, transcription factors, and genes related to cell growth, apoptosis, and innate immune responses. As shown in Table 1, *R. conorii* infection clearly induced interferon-regulated genes such as interferon-stimulated gene 15 (ISG15) and the genes

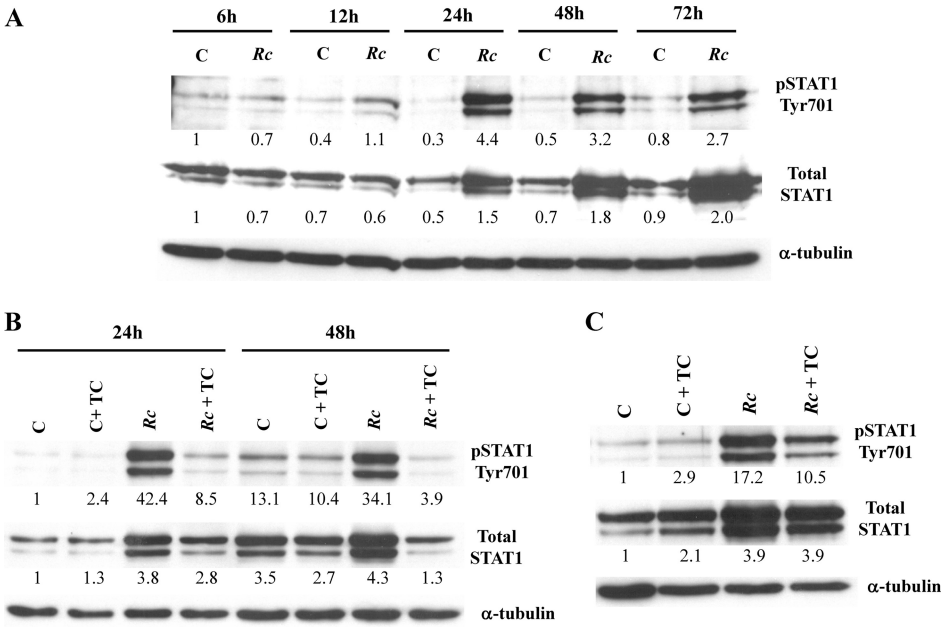

FIG. 2. Immunoblot-based measurement of STAT1 phosphorylation in *R. conorii*-infected HUVECs. (A) Time course analysis of Tyr701 phosphorylation of STAT1 and total STAT1 levels at different times in mock-infected (C) and *R. conorii*-infected (*Rc*) HUVECs. For comparison, the levels of phospho-STAT1 and total STAT1 in uninfected cells at 6 h (lane 1 from the left) were assigned a value of 1. (B) STAT1 activation requires infection with metabolically active rickettsiae. Tetracycline (TC; 20 μg/ml) was added to the culture medium at 3 h postinfection to inhibit rickettsial protein synthesis. Total protein lysates collected at 24 and 48 h postinfection in the presence (*Rc* + TC) and absence (*Rc*) of tetracycline were subjected to the determination of the levels of total and phospho-STAT1 in comparison with either uninfected cells alone (C) or uninfected cells treated with tetracycline (C + TC). (C) Tetracycline (20 μg/ml) was added to *R. conorii*-infected HUVECs (*Rc* + TC) and corresponding uninfected controls (C + TC) at 24 h, followed by the determination of the steady-state levels of phosphorylated and total STAT1 at 48 h.

TABLE 1. *R. conorii* infection induces expression of IFN-regulated genes in HUVECs[a]

Gene name	GenBank accession no.	Gene fold expression
ISG15	NM_005101	8.7
SOCS1	NM_003745	8.6
OAS1	XM_371292	6.4
IL2RA	NM_000417	3.2
GBP1	NM_002053	2.8
NFkB1	NM_003998	2
JAK3	NM_000215	1.9
SMAD3	NM_005902	1.9
IRF9	NM_006084	1.8
IRF1	NM_002198	1.8
CXCL9	NM_002416	1.8
STAT1	NM_007315	1.5
JUNB	NM_002229	1.4
SIT1	NM_014450	1.4
IFNGR1	NM_000416	1.3
STAT2	NM_005419	1.2
IFNAR1	NM_000629	1.1
JAK2	NM_004972	1
IFNG	NM_000619	−1
JAK1	NM_002227	−1
SOCS2	NM_003877	−1
PIAS2	NM_004671	−1
TYK2	NM_003331	−1.2
PIAS1	NM_016166	−1.4
STAT4	NM_003151	−1.7
SOCS3	NM_003955	−2

[a] HUVECs were infected with *R. conorii* for 48 h. The quantitative RT-PCR-based arrays were performed to screen a number of specific genes linked to the JAK-STAT pathway. The names of the different genes and their accession numbers are derived from GenBank. The fold induction values were calculated relative to the uninfected control, and a change of 2-fold was considered significant.

for oligoadenylate synthase 1 (OAS1) and guanylate-binding protein 1 (GBP1). Among these, ISG15 is mainly induced by type I interferons, whereas GBP1 and OAS1 are sensitive to both type I and type II IFNs. Suppressor of cytokine signaling 1 (SOCS1), a negative regulator of the JAK-STAT pathway, was also significantly induced, but the expression of SOCS2 remained relatively unaltered. Expression of protein inhibitors of activated STAT (PIAS) family proteins PIAS1 and PIAS2 was also not altered by the infection. On the other hand, SOCS3 gene expression was suppressed by about 2-fold. Expression of JAK1, JAK2, and TYK2 was not induced, whereas that of JAK3 exhibited an average increase of 1.9-fold. Transcriptional activation of interferon receptors IFNGR1 and IFNAR1 were also refractory to change as a consequence of *R. conorii* infection. Supporting our observation of increased levels of total STAT1 protein, its transcription was found to be induced approximately 1.5-fold. The expression levels of other STAT proteins, including STAT2, STAT3, STAT5A, or STAT5B, remained relatively unaltered, while that of STAT4 was downregulated by about 1.7-fold. These screening data thus clearly indicate that *R. conorii* infection leads to the upregulation of only a few, selective IFN-regulated genes in cultured human endothelial cells. Since pathogenic rickettsiae are known to exhibit a unique affinity for microvascular endothelium during human disease (46, 48), we employed HMECs for further detailed characterization of the STAT1 activation response during *R. conorii* infection.

Secreted IFN-β induces STAT1 phosphorylation. Because *Rickettsia* infection has previously been shown to activate en-

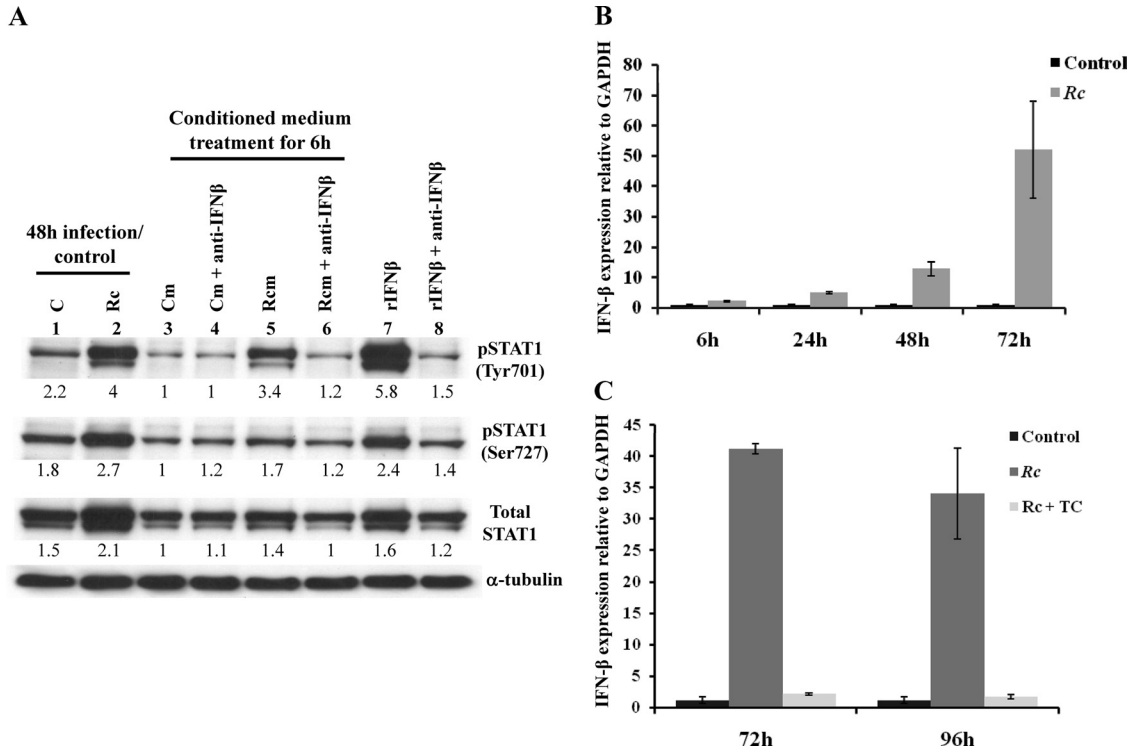

FIG. 3. Autocrine activation of STAT1 protein by IFN-β secreted from *R. conorii*-infected HMECs. (A) *R. conorii* infection induces STAT1 phosphorylation in HMECs infected with *R. conorii* for 48 h (*Rc*; lane 2) but not in mock-infected control cells (C; lane 1). Cell culture supernatants from HMECs, which were either left uninfected (Cm) or infected with *R. conorii* for 48 h (Rcm), were supplemented with tetracycline (10 μg/ml) and filter sterilized to remove inactivated rickettsiae from the medium. Fresh HMECs were then incubated with the conditioned medium for 6 h and analyzed for STAT1 phosphorylation (lanes 3 to 6). Also, conditioned media from uninfected HMECs (Cm) and *R. conorii*-infected HMECs (Rcm) were preincubated with an IFN-β-neutralizing antibody (10 μg/ml) for 90 min (Cm + anti-IFN-β and Rcm + anti-IFN-β in lanes 4 and 6, respectively). HMECs incubated with exogenous recombinant human IFN-β (10 ng/ml) for 6 h were used as a positive control (lane 7). To ensure the neutralizing ability of anti-IFN-β, recombinant IFN-β (rIFN-β) in the culture medium was incubated with the antibody for 90 min prior to addition to cultured HMECs for 6 h (lane 8). (B) Induction of IFN-β mRNA expression in *R. conorii*-infected HMECs. The levels of IFN-β gene expression were determined in cells infected with *R. conorii* for various durations of time by quantitative RT-PCR using the $\Delta\Delta C_T$ method and relative expression of GAPDH as a housekeeping gene. The data sets for each time point represent the mean ± standard error of the mean from three independent experiments performed in duplicate. Compared to the corresponding control, IFN-β expression in infected HMECs was higher by 2.1-fold, with relatively more robust induction at 24 h and later. (C) Analysis of IFN-β gene expression in HMECs infected with *R. conorii* in the presence and absence of tetracycline. Cells were infected with *R. conorii* and then treated with tetracycline as described in Materials and Methods. Total RNA from all experimental conditions (Control, uninfected cells; *Rc*, *R. conorii*-infected cells; Rc + TC, infection in the presence of tetracycline) was prepared in triplicate to measure IFN-β expression employing GAPDH as a housekeeping gene. The data are presented as the mean ± standard error from three independent experiments performed in duplicate.

dothelial cells to secrete proinflammatory cytokines and STAT1 phosphorylation in *R. conorii*-infected cells occurs relatively later than other well-characterized early signaling responses (16, 31), we hypothesized the possible involvement of a soluble mediator secreted by infected HMECs in triggering STAT1 activation. To investigate this possibility, we exposed naïve HMECs with conditioned culture medium collected from *R. conorii*-infected HMECs. As shown in Fig. 3A, incubation for 6 h with conditioned culture supernatant from infected cells clearly induced Tyr701 phosphorylation of STAT1 in fresh, naïve HMECs (lane 5). Interestingly, prior incubation of culture supernatant preparations with an antibody capable of neutralizing IFN-β resulted in marked inhibition of STAT1 activation (lane 6), whereas neutralization of IFN-γ had no effect (data not shown). Furthermore, STAT1 phosphorylation on Ser727 and total STAT1 expression were also found to be slightly induced by conditioned medium from *R. conorii*-infected cells (lane 5). As controls, both *R. conorii* infection and treatment of HMECs with exogenous IFN-β resulted in robust induction of Tyr701 and Ser727 phosphorylation of STAT1 and steady-state levels of total STAT1 protein (lanes 2 and 7, respectively). Also, the neutralizing antibody used in these experiments completely abolished IFN-β-mediated activation of STAT1, confirming its specificity and efficacy (lane 8). These data thus suggest that IFN-β secreted from *R. conorii*-infected endothelial cells functions in an autocrine manner to activate JAK-STAT signaling in the host cells. We further analyzed the transcriptional status of the IFN-β gene in *R. conorii*-infected host cells by quantitative reverse transcription-PCR (RT-PCR). As shown in Fig. 3B, steady-state cellular levels of IFN-β mRNA displayed average increases of about 2.1-, 6-, and 12-fold over basal expression in uninfected cells at 6, 24, and 48 h, respectively, reaching the peak at 72 h postinfection. Further, introduction of tetracycline into the culture medium at 3 h resulted in nearly complete inhibition of *R. conorii*-induced IFN-β gene expression at both 72 and 96 h postinfec-

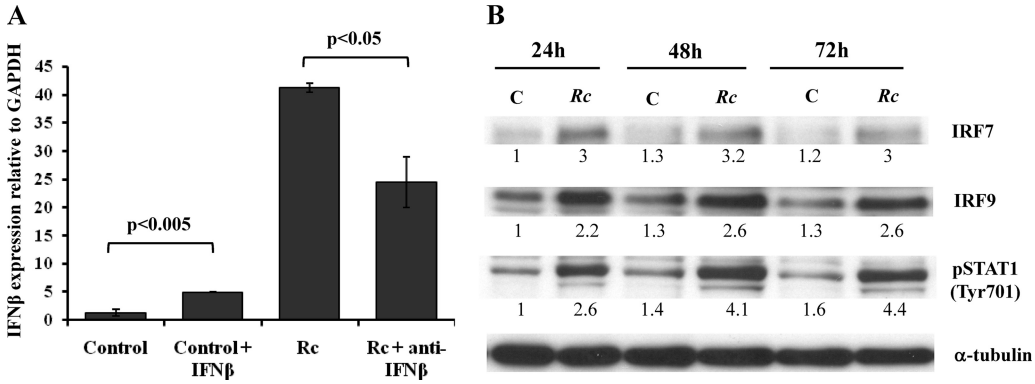

FIG. 4. Regulation of IFN-β gene expression in *R. conorii*-infected HMECs. (A) The effect of recombinant human IFN-β on the positive-feedback amplification of IFN-β gene expression in HMECs was analyzed by quantitative RT-PCR. Total RNA was extracted from HMECs that were left uninfected (control) or infected with *R. conorii* for 72 h (Rc) and treated with rIFN-β (10 ng/ml) for 6 h. Additionally, HMECs were also infected with *R. conorii* in the presence of an IFN-β-neutralizing antibody (10 μg/ml). Tests under all experimental conditions were carried out in triplicate in a minimum of two independent observations. The levels of gene expression were calculated relative to housekeeping gene GAPDH expression by the $\Delta\Delta C_T$ method. The data are presented as the mean ± standard error. Statistical significance was determined by comparing the data sets via paired Student's *t* test. (B) Western blot analysis of the expression of IRFs in *R. conorii*-infected HMECs. Total protein lysates from infected cells at 24, 48, and 72 h (*Rc*) and simultaneously processed uninfected controls (C) were subjected to the determination of the levels of IRF7 and IRF9. The levels of phospho-STAT1 were also evaluated to ensure the activation of STAT1. A representative blot from three independent experiments is shown. The band intensities for all experimental conditions were first normalized to the levels of α-tubulin and then compared to that of the earliest uninfected control (first lane from the left), which was assigned a value of 1.

tion (Fig. 3C), indicating a requisite for replication of viable intracellular rickettsiae to trigger the transcriptional activation of the IFN-β gene.

***R. conorii* infection induces expression of proteins involved in JAK-STAT signaling through IFN-β.** We next determined whether or not IFN-β is capable of self-induction via a positive-feedback loop. To this end, we measured its expression in HMECs treated with exogenous IFN-β (Fig. 4A). Our results suggest increased cellular mRNA expression of IFN-β following exposure to IFN-β as a stimulus. Interestingly, transcriptional activation of the IFN-β gene by exogenous IFN-β attained a significantly lower intensity than that observed in *R. conorii*-infected cells. Inclusion of IFN-β-neutralizing antibody in the medium only partially inhibited IFN-β gene expression in infected cells. Together, these observations point toward the existence of an endothelial surveillance system which is activated by intracellular rickettsiae to produce and secrete IFN-β from infected cells. IFN-β secreted in response to infection then further amplifies its own expression via a positive-feedback loop. Our data further demonstrate that IFN-β expression and secretion from *R. conorii*-infected HMECs also contribute to the activation of different components of the JAK-STAT signaling pathway (Fig. 4B). Clearly, STAT1 is activated by enhanced phosphorylation on Tyr701 at 24 to 72 h postinfection. An important consideration in this regard is that Tyr701 phosphorylation triggers STAT1 dimerization and nuclear translocation, whereas Ser727 phosphorylation increases its transcriptional activity. As shown in Fig. 4B, IRF9 and IRF7 were also induced by *R. conorii* infection. Activated by serine/threonine phosphorylation, IRF7 is a type 1 IFN-inducible gene which binds to ISREs to induce transcription of IFN-β. Similarly, IRF9 is another IFN-β-inducible transcription factor that binds with activated STAT1 and STAT2 proteins to form a heterotrimeric complex, known as ISGF3. Interestingly, the ISGF3 complex can induce the IRF7 gene, which contains ISREs, thereby initiating a positive-feedback loop for IFN-β expression via the JAK-STAT pathway.

IFN-β interferes with *R. conorii* replication in endothelial cells. IFN-β is a critical type I interferon known to initiate and govern innate immune responses against viral and bacterial infections. We therefore further investigated whether or not IFN-β-mediated host responses exert an effect on intracellular rickettsial replication in endothelial cells (Fig. 5A). Using quantitative RT-PCR, we first monitored the kinetics of *R. conorii* replication in HMECs to up to 96 h postinfection. An increase in rickettsial growth, as indexed by copy numbers of rickettsial outer membrane protein A (OMPA), was observed during the 96 h, with the maximum fold increase in rickettsial DNA seen during a 24-h period between 48 and 72 h postinfection (Fig. 5A and B). Again, the presence of neutralizing antibody against IFN-β (10 μg/ml) resulted in significant inhibition of STAT1 Tyr701 phosphorylation at 72 h and 96 h (Fig. 5C). Interestingly, neutralization of IFN-β significantly increased *R. conorii* replication at 72 h, whereas exogenous IFN-β treatment resulted in significant but not complete inhibition of *R. conorii* replication at both 72 h and 96 h (Fig. 5D). As expected, infection in the presence of tetracycline caused nearly complete inhibition of rickettsial replication (data not shown) (43, 48). Accordingly, detailed quantitative analysis revealed that neutralization of IFN-β activity caused an approximately 22% increase in the copy number of rickettsial DNA at 72 h postinfection, whereas addition of exogenous IFN-β led to partial inhibition of *R. conorii* replication by 35%. Thus, the net difference of *R. conorii* replication between IFN-β neutralization and exogenous IFN-β treatment was 57% in comparison to infection under control, untreated conditions, indicating that IFN-β-mediated host innate responses effectively interfere with intracellular rickettsial replication in endothelial cells. A similar protective role of IFN-β against *R. conorii* replication was also evident at 96 h postinfection, with

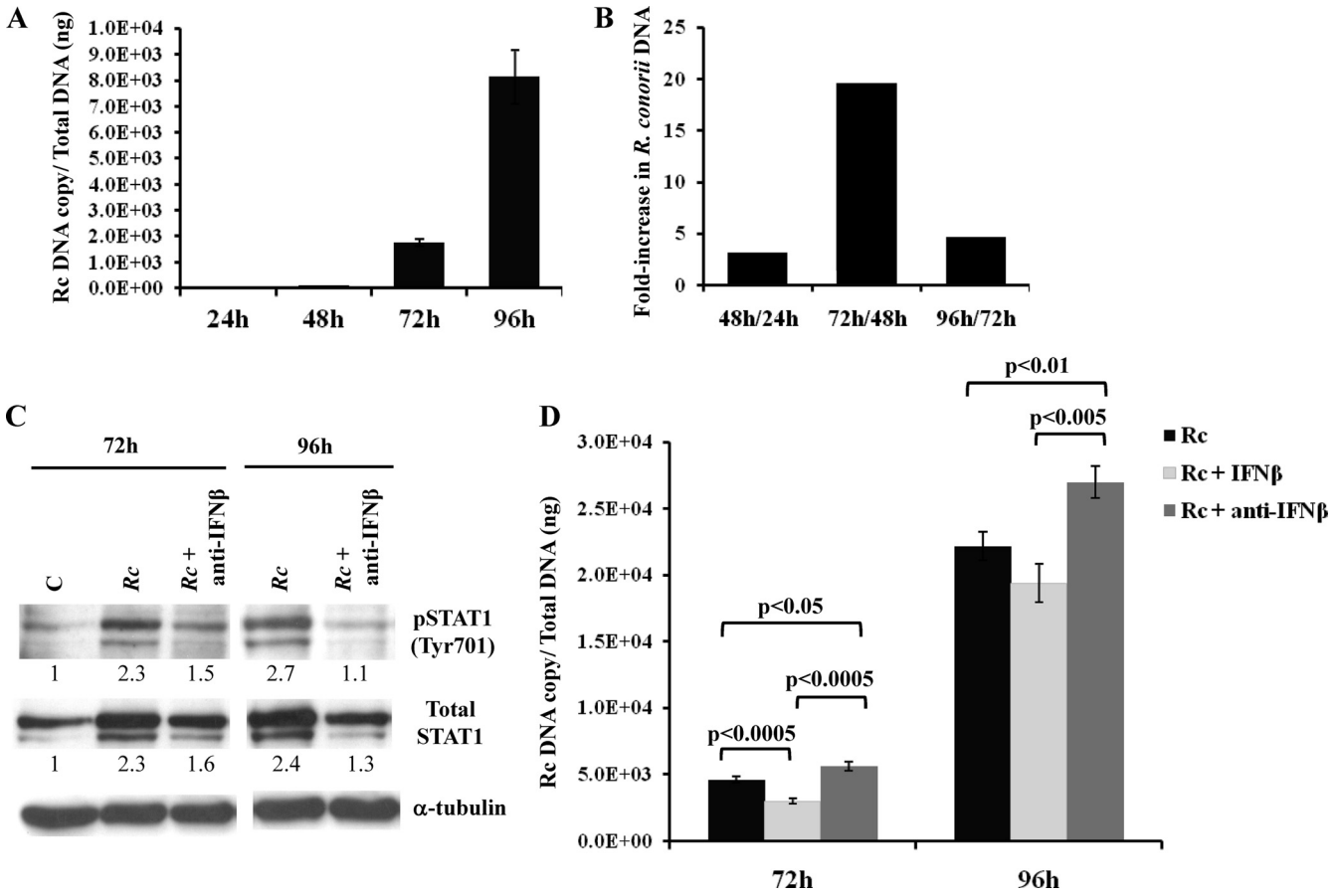

FIG. 5. IFN-β interferes with *R. conorii* replication in HMECs. (A) Quantification of *R. conorii* replication in HMECs by quantitative RT-PCR. Total DNA from infected cells was subjected to quantitative RT-PCR using a previously described primer pair specific for OMPA of spotted fever group rickettsiae (5). *R. conorii* DNA copy number was determined using a standard curve and normalized to the amount of total DNA. Data are presented as the mean ± standard error from a minimum of three separate observations. The average copy numbers of rickettsial OMPA (per ng of total DNA) in infected HMECs were 29 ± 2, 90 ± 5, 1,768 ± 133, and 8,120 ± 1,045 at 24, 48, 72, and 96 h postinfection, respectively. (B) Fold increase of *R. conorii* DNA within HMECs at 24-h intervals. (C) Neutralization of IFN-β during *R. conorii* infection of HMECs adversely affects STAT1 phosphorylation. Infection was carried out in the presence (*Rc* + anti-IFN-β) and absence (*Rc*) of an anti-IFN-β antibody (10 μg/ml; added at 3 h). Total protein lysates collected at 72 and 96 h were analyzed for STAT1 phosphorylation. For comparative analysis, the basal levels of phospho-STAT1 and total STAT1 in uninfected/untreated HMECs (lane C) were given a value of 1. (D) Effects of human IFN-β and neutralization of IFN-β on *R. conorii* replication in HMECs. HMECs infected with *R. conorii* were left alone (Rc) or treated with recombinant human IFN-β (10 ng/ml) or anti-IFN-β antibody (10 μg/ml) at 3 h postinfection. DNA from cell pellets collected at 72 and 96 h was then processed to quantify rickettsial DNA copy number by quantitative RT-PCR. The data are presented as the mean ± standard error of at least six independent replicates. Statistical significance was determined by subjecting the data sets from different experimental conditions to Student's *t* test.

an approximately 13% decrease by exogenous IFN-β treatment and an approximately 22% increase by IFN-β neutralization (Fig. 5D).

STAT1 is required for IFN-β-mediated effects on *R. conorii* replication. STAT1 plays an important role in signal transduction mechanisms induced by both type I and type II interferons, providing protective immunity against microbial infections. We hypothesized that STAT1 activation is likely critical for IFN-β-mediated interference with *R. conorii* replication in endothelial cells. To investigate this possibility, endothelial cells were transfected with sequence-specific siRNA to silence STAT1 expression (Fig. 6A). As shown, we achieved approximately 80% knockdown of STAT1 with STAT1-targeting siRNA, whereas a scrambled siRNA sequence (used as a negative control) had no effect, as expected. Infection-induced phosphorylation of STAT1 on Tyr701 was also inhibited by ~30% in STAT1 siRNA-transfected cells. We next examined the effects of STAT1 knockdown on *R. conorii* growth and replication (Fig. 6B). The data clearly suggest an average increase of 27% in rickettsial replication at 72 h in cells transfected with STAT-1-specific siRNA compared to the level of replication for the corresponding controls receiving a scrambled siRNA sequence. Moreover, the decrease in *R. conorii* replication due to exogenous IFN-β was also significantly affected by STAT1 knockdown (Fig. 6C). Exogenous IFN-β treatment resulted in approximately 44% inhibition of *R. conorii* replication in cells harboring control, noninterfering siRNA at 96 h, whereas those transfected with siRNA against STAT1 showed only 18% inhibition, suggesting an important role for STAT1 in mediating the effects of IFN-β on rickettsial replication.

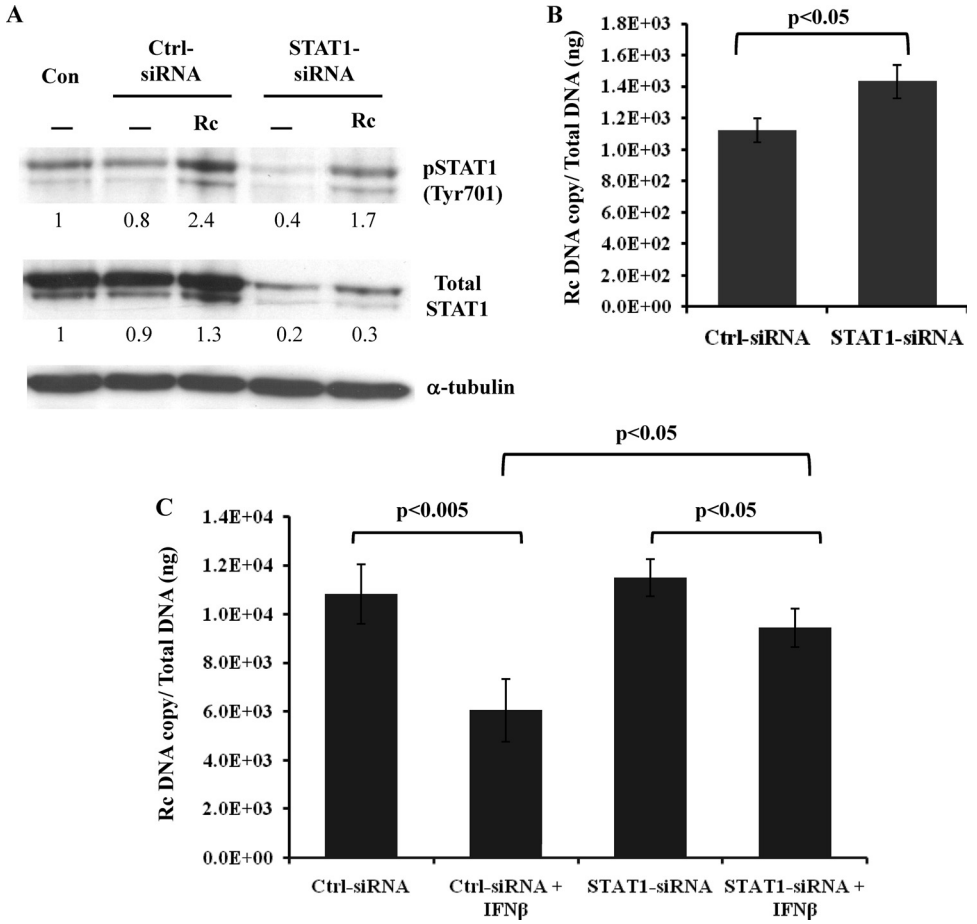

FIG. 6. siRNA-mediated STAT1 knockdown favors *R. conorii* replication but interferes with IFN-β's inhibitory effects on rickettsial replication in the endothelium. (A) HMECs were transfected with either a scrambled siRNA sequence (Ctrl-siRNA; 80 nM) or STAT1-specific siRNA (STAT1-siRNA; 80 nM) using Lipofectamine 2000. Cells were allowed to recover for 12 h. The status of STAT1 activation, as determined by the analysis of phospho-STAT1 via immunoblotting, was evaluated in HMECs that were transfected with control or STAT-1 siRNAs and that were subsequently either left uninfected or infected with *R. conorii* for 72 h. For the purpose of comparison among different experimental conditions, the baseline levels of phosphorylated and total STAT1 in untransfected/uninfected HMECs were assigned a value of 1. A typical result from three independent experiments is presented. (B) *R. conorii* replication in HMECs transfected with a scrambled control or STAT1-targeting siRNA. Data are presented as the mean ± standard error of three independent replicates. The level of statistical significance was determined by Student's *t* test. (C) Effect of STAT1 knockdown on IFN-β-mediated inhibition of *R. conorii* replication. HMECs transfected with either control siRNA or STAT1 siRNA were infected with *R. conorii* for 3 h, at which time human IFN-β (10 ng/ml) was added to the culture medium. The cell pellets collected at 96 h were processed for the isolation of DNA to estimate the copy numbers of rickettsial DNA by OMPA-based quantitative RT-PCR. The data are presented as the mean ± standard error from three independent experiments conducted in duplicate for each condition. The comparison of data between experimental conditions to determine the levels of statistical significance was performed by Student's *t* test.

DISCUSSION

Although activation of JAK-STAT signaling constitutes an important component of host defense mechanisms, the status of this pathway during *Rickettsia* infection of endothelial cells, the primary host cell niche preferably targeted by pathogenic rickettsiae during human spotted fever syndromes, has so far remained a neglected area of scientific enquiry. The findings of this study establish that the activation of STAT1 occurs during *R. conorii* infection of microvascular endothelial cells, implicate an IFN-β-mediated autocrine mechanism in sustaining this cellular response, and yield first evidence suggesting the involvement of both STAT1 and IFN-β in the control of intracellular replication of *R. conorii.* Widely accepted as an indicator of its activation, we observed phosphorylation of Tyr701 residue on both the full-length α isoform of STAT1 protein and the alternatively spliced, C-terminal truncated β isoform. Such a response was clearly evident in microvascular HMECs, included in our studies because of the tropism of pathogenic rickettsiae for the microvascular endothelium lining of small and medium-sized vessels during human disease and in experimental models of infection. In addition, infection of macrovascular HUVECs, a cell type used to establish *in vitro* models of infection and widely used thereafter to define host cell interactions with pathogenic rickettsiae (17, 36, 42), also displayed a similar pattern of STAT1 activation. Known to enhance the transcription of target genes (18), phosphorylation of serine 727 in the transactivation domain of the STAT1 α isoform was also induced in *R. conorii*-infected endothelial cells. Interestingly, *R. conorii* not only induced Tyr/Ser phosphorylation of STAT1 but also increased the abundance of the

STAT1 protein in endothelial cells. Increased levels of total STAT1 protein have previously been shown to occur in HeLa cells and mouse lung mononuclear cells infected with pathogenic *Chlamydia* species (19, 29). Recent studies have shown that unphosphorylated STAT1 is required to prolong the expression of IFN-induced immune regulatory genes (2). Although the specific biological functions and the mechanisms underlying STAT1 accumulation in *Rickettsia*-infected endothelial cells have yet to be defined in further detail, such an occurrence may be attributed to increased *de novo* synthesis, delay in the processing for degradation, or possibly a combination of both of these mechanisms.

In agreement with published evidence documenting the production of endogenous IFN-α/β by cultured mouse fibroblasts in response to infection with *R. prowazekii* (44), the present study unequivocally demonstrates that *R. conorii* infection stimulates the expression and secretion of IFN-β from endothelial cells. In this context, it is critically important to consider the intracellular behavior patterns of spotted fever group species (*R. conorii* and *R. rickettsii*) versus those of typhus group species (*R. prowazekii*), which display significantly higher intracellular accumulation of *R. prowazekii* due to the lack of actin-based motility seen in spotted fever rickettsiae. On the other hand, both of these major subgroups of pathogenic rickettsiae exhibit a tendency to preferentially infect vascular endothelium during human rickettsioses as well as in established animal models of infection. The outcomes of this study allow us to hypothesize that *R. conorii*-mediated IFN-β expression likely occurs via a two-component mechanism. As the first step, the presence of intracellular rickettsiae is sensed by an as yet unknown host surveillance system leading to the initiation of an IFN-β expression/secretion response. Although the recognition mechanism(s) of the host cell capable of detecting the proteins secreted into the cytoplasmic environment or the presence of intact intracellular rickettsiae remains to be identified, several intracellular as well as extracellular receptor signaling pathways are known to induce IFN-β expression during microbial infections. Toll-like receptors (TLRs) are capable of activating type I IFNs in response to microbial DNA and RNA. Specifically, TLR stimulation by specific microbial stimuli activates cellular transcription factors such as NF-κB, IRF3, and IRF7 via several adaptor proteins, culminating in the expression and release of proinflammatory mediators such as cytokines and type I IFNs. In addition, cytosolic receptors such as retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation antigen 5 (MDA5) also detect double-stranded RNA from RNA viruses to induce type I IFNs (49). Both IRF3 and IRF7 bind to the target response elements on the promoter regions of IFN-α and/or IFN-β to induce their transcription, and consequently, activation of IRF3, IRF7, or both is required for the IFN-α/β induction response (11–15). It has been shown that IFN-β induction in *Chlamydophila pneumoniae*-infected HUVECs is dependent on IRF3 and IRF7 (1). In a simultaneous follow-up step, secreted IFN-β induces its own expression through a positive-feedback loop. IFN-β is known to activate STAT1, STAT2, and IRF9 proteins to form the ISGF3 complex, which then binds to ISREs to induce IRF7 and IRF9. ISGF3-mediated IRF7 induction also contributes to a positive-feedback mechanism by upregulating IFN-α/β expression (23, 33). Our data are consistent with the potential involvement of such a mechanism in *R. conorii*-infected HMECs, which display increased expression of regulatory factors IRF7 and IRF9. Also, the presence of an IFN-β-neutralizing antibody inhibits IFN-β gene expression in *R. conorii*-infected endothelial cells, further suggesting the existence and contributions of an IFN-β-mediated positive-feedback loop. Such a positive feedback apparently functions in an autocrine/paracrine manner to augment IFN-β production from infected cells, thereby alerting uninfected neighbor cells to the presence of infection. Known to be activated by viral or bacterial infections, IRF3 is also capable of inducing IFN-β expression, yet there was no evidence for Ser396 phosphorylation as an indicator of IRF3 activation during *R. conorii* infection (data not shown). Although these data clearly implicate the presence of IRF3 in HMECs, the present study did not investigate its steady-state levels during the course of infection because unlike IRF7 and IRF9, IRF3 expression is not considered to be inducible by IFN-β (3, 13, 15).

IFN-β-mediated protective host responses against intracellular Gram-negative bacterial infections, for example, *Chlamydia trachomatis* infections, have previously been reported (19). However, IFN-β production in *Listeria*-infected lymphocytes functions to render the host more susceptible to infection (27). Thus, IFN-β is capable of triggering both protective and detrimental effects, depending on the nature of the pathogen. Our data yield the first evidence to support the proposal that endothelial IFN-β activation has protective functions against intracellular *R. conorii* replication. Whereas IFN-β neutralization results in increased *R. conorii* replication, treatment with recombinant IFN-β had the opposite inhibitory effect, as expected. Secreted IFN-β may act on uninfected cells to activate IFN-inducible genes that make cells more resilient to invading pathogenic rickettsiae. It has been shown that cross talk between IFN-α/β and IFN-γ can trigger strong cellular responses to IFN-γ and interleukin-6 (IL-6) (41). Interestingly, IFN-γ is capable of inhibiting rickettsial growth and has been documented to be critically important to clear the infection *in vivo* (6, 45, 47, 48). However, the significance of the potential cross talk between IFN-α/β and IFN-γ in rickettsiosis has not yet been evaluated either *in vitro* or *in vivo*.

STAT1 activation is required for both type I and type II IFN-mediated antimicrobial activity. STAT1-knockout mice are highly susceptible to bacterial infections, such as those caused by *Listeria monocytogenes* and *Mycobacterium tuberculosis* (24, 39). Furthermore, cells and tissues of STAT1-deficient mice are unresponsive to IFNs and show compromised innate immunity against viral infections (4). Intriguingly, interferons are also capable of inducing antiviral responses via STAT1-independent mechanisms (10), yet the molecular events underlying such pathways are not well elucidated. Thus, taking into consideration that antiviral responses are induced by both STAT1-dependent and STAT1-independent pathways by interferons, we investigated whether STAT1 protein is required for IFN-β-mediated antirickettsial activity by STAT1 gene knockdown in endothelium. Our data not only demonstrate that IFN-β-mediated inhibition of *R. conorii* replication requires STAT1 activation but also suggest that siRNA-mediated interference with STAT1 activity results in significantly enhanced replication of *R. conorii* in the host cells. We anticipate that STAT1 activation likely induces transcription of

antimicrobial compounds which resist intracellular bacterial growth and replication. Our quantitative RT-PCR-based array data show upregulation of ISG15, OAS1, and GBP1 proteins, all of which are known to possess antimicrobial activities. Although the antirickettsial mechanisms of these STAT1-inducible genes have not yet been characterized, their potential roles in *Rickettsia*-infected endothelium warrant further detailed investigations of this critically important aspect of innate immunity.

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