Suppressor of cytokine signalling protein SOCS1 and UBP43 regulate the expression of type I interferon-stimulated genes in human microvascular endothelial cells infected with *Rickettsia conorii*

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Rickettsia conorii, the causative agent of Mediterranean spotted fever, preferentially infects human microvascular endothelium and activates pro-inflammatory innate immune responses as evidenced by enhanced expression and secretion of cytokines and chemokines. Our recent studies reveal that human microvascular endothelial cells (HMECs) infected with *R. conorii* also launch 'antiviral' host defence mechanisms typically governed by type I interferons. To summarize, infected HMECs secrete IFN-β to activate STAT1 in an autocrine/paracrine manner and display increased expression of IFN-stimulated genes, for example *ISG15*, which in turn activate innate responses to interfere with intracellular replication of rickettsiae. We now present evidence that UBP43 and SOCS1, known negative regulators of JAK/STAT signalling, are also induced in *R. conorii*-infected HMECs, of which UBP43 but not SOCS1 functions to negatively regulate STAT1 activation. Interestingly, UBP43 induction is almost completely abolished in the presence of IFN-β-neutralizing antibody, implicating an important role for UBP43 as a feedback inhibitor for IFN-β-mediated STAT1 activation. In contrast, SOCS1 expression is only partially affected by IFN-β neutralization, implicating potential involvement of as-yet-unidentified IFN-independent mechanism(s) in *SOCS1* induction during *R. conorii* infection. A number of IFN-stimulated genes, including *ISG15*, *OAS1*, *MX1*, *IRF1*, *IRF9* and *TAP1* are also induced in an IFN-β-dependent manner, whereas *GBP1* remains unaffected by IFN-β neutralization. Increased STAT1 phosphorylation in HMECs subjected to *UBP43* knockdown led to transcriptional activation of *OAS1*, *MX1* and *GBP1*, confirming the negative regulatory role of UBP43. Although *IRF1*, *IRF9* and *TAP1* were induced by IFN-β, siRNA-mediated silencing of *UBP43* or *SOCS1* did not significantly affect their transcriptional activation. Expression of *ISG15* was, however, increased in HMECs transfected with siRNA for *UBP43* and *SOCS1*. Thus, unique regulatory patterns of induced expression of *UBP43*, *SOCS1* and IFN-stimulated genes represent pathogen-specific responses underlying IFN-β-mediated host endothelial signalling during the pathogenesis of spotted fever group rickettsiosis.

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INTRODUCTION

Rickettsia conorii, a Gram-negative, obligate intracellular α-proteobacterium known to cause Mediterranean spotted fever (MSF) in humans, represents one of the major spotted fever group (SFG) *Rickettsia* species. Typically transmitted to humans by infected ticks and characterized by visible skin lesions termed 'tache-noire' at the bite site, the disease symptoms include high fever, headache and body rash (Raoult *et al.*, 1986; Sousa *et al.*, 2003). Although MSF is traditionally considered a benign disease, significant morbidity and mortality are evident among people exposed to strains with higher levels of virulence and in cases with delayed diagnosis due to non-specific, initial flu-like

Abbreviation: HEMC, human microvascular endothelial cell.

symptoms and late intervention with doxycycline therapy. As a prototypic member of SFG rickettsiae, *R. conorii* preferentially infects the vascular endothelial monolayer lining small and medium-sized blood vessels, causing 'endothelial activation' as well as injury (George *et al.*, 1993; Mansueto *et al.*, 2012). Vascular endothelial cells infected with *R. conorii* acquire a pro-adhesive and pro-inflammatory phenotype characterized by increased expression of surface adhesion molecules and secretion of cytokines and chemokines such as interleukin (IL)-1α, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1 and fractalkine (Kaplanski *et al.*, 1995; Valbuena *et al.*, 2003). In addition, endothelial cells stimulated with interferon (IFN)-γ and tumour necrosis factor-α are capable of killing intracellular *R. conorii* via nitric oxide-dependent mechanism(s) (Walker *et al.*, 1997) and mice lacking IFN-γ exhibit more than 100-fold greater susceptibility to infection with *Rickettsia australis* (Walker *et al.*, 2001).

Since their discovery, IFNs have generally been considered as cytokines secreted by virus-infected cells to induce an 'antiviral state' in neighbouring host cells through autocrine/paracrine signalling mechanism(s). Among these, a single IFN-β along with a number of IFN-α proteins are classified as type I IFNs, which interfere with viral replication by inducing host gene expression. Although some of these target genes also display anti-bacterial activity (Monroe *et al.*, 2010), type I IFNs have traditionally been assigned a relatively minor role and consequently received much less attention in anti-bacterial host defence mechanisms. Recently, we have reported on the expression and secretion of IFN-β from cultured microvascular endothelial cells and demonstrated an important role for this type I IFN in modulating innate immune responses to inhibit intracellular growth of *R. conorii*. Intriguingly, as a component of the Janus kinase–signal transducer and activator of transcription (JAK/STAT) signalling pathway, phosphorylation/activation of transcription factor STAT1 primarily involves autocrine/paracrine effects of IFN-β and is indispensable for IFN-β's anti-rickettsial activity in cultured human endothelium (Sahni *et al.*, 2009; Colonne *et al.*, 2011a). Our findings further suggest that *R. conorii* infection also induces the expression of an IFN-stimulated gene of 15 kDa (*ISG15*) via IFN-β-dependent JAK/STAT signalling in human endothelial cells. Moreover, intracellular levels of free ISG15 as well as ISG15 conjugated to other as yet unknown host cellular proteins are also increased during infection and participate in protective innate immune response by suppressing the intracellular rickettsial growth in infected host cells (Colonne *et al.*, 2011b).

Activation of JAK/STAT signalling by IFNs is tightly regulated by specific negative regulators at multiple levels. Three families of proteins known as phospho-tyrosine phosphatases (PTPs), suppressors of cytokine signalling (SOCS), and protein inhibitors of activated STAT (PIAS) have been implicated in negative regulation of the JAK/STAT signalling pathway. Specifically, members of the PTP family proteins, namely SHP1 and SHP2, negatively regulate IFN signalling by dephosphorylating activated JAK1 and JAK2 proteins (Klingmüller *et al.*, 1995; You *et al.*, 1999). SH2 domains of SOCS proteins, on the other hand, inhibit IFN signalling by competing with STATs for the receptor binding sites, inhibiting JAKs by direct binding, or by targeting bound proteins for proteasomal degradation (Kamizono *et al.*, 2001; Kile *et al.*, 2002). SOCS1, a prototype member of the SOCS family, can interact with different cellular proteins such as JAKs, IRAK (interleukin-1-receptor-associated kinase) and NF-κB subunits p50/p65 to regulate a wide range of cellular functions, including proliferation, differentiation, apoptosis and immune responses in a cell-specific manner (Fujimoto & Naka, 2010). Negative regulators belonging to the PIAS family inhibit STAT-mediated gene activation. PIAS1 and PIAS3 bind to STAT1 and STAT3, respectively, to inhibit DNA-binding activity of phosphorylated STAT proteins thereby inhibiting transcriptional activation of IFN-stimulated genes (Chung *et al.*, 1997; Liao *et al.*, 2000). UBP43, a ubiquitin-specific protease, has also been identified as a negative regulator of type I IFN signalling. It associates with IFN-α receptor 2 subunit (IFNAR2), preventing its binding to JAK and thereby inhibiting downstream IFN-β signalling cascades (Malakhova *et al.*, 2006).

Subversion of JAK/STAT signalling through negative regulatory proteins has emerged as an important survival strategy for intracellular microbes. As an example, influenza A and herpes simplex viruses interfere with IFN signalling by activating SOCS3 expression during host invasion (Pauli *et al.*, 2008; Yokota *et al.*, 2004). Although *R. conorii* infection augments IFN-β response during endothelial cell infection, the status of negative regulators of the JAK/STAT pathway remains completely unknown. To address this critical regulatory aspect of IFN signalling, we have investigated whether or not *R. conorii* infection alters the expression of SOCS1 and UBP43 and further determined the effects of such changes on IFN-β-dependent STAT1 activation and stimulation of responsive downstream genes in human endothelial cells. The presented results reveal that, although *R. conorii* infection induces the expression of both SOCS1 and UBP43 in endothelium, IFN-β-dependent STAT1 activation is selectively regulated by UBP43 but not SOCS1 protein. Moreover, we have also identified a specific subset of IFN-stimulated genes induced by *R. conorii* infection and evaluated the inhibitory effects of UBP43 and SOCS1 on these IFN-stimulated genes in *R. conorii*-infected endothelium.

METHODS

Cell culture and infection. *Rickettsia conorii* (Malish 7 strain) was propagated in Vero cells and stocks prepared by density-gradient centrifugation followed by plaque formation assay to estimate the infectivity titres were kept frozen as aliquots. An immortalized line of human dermal microvascular endothelial cells (HMEC-1) was grown under sterile culture conditions in MCDB 131 medium (Gibco), supplemented with FBS (10 % v/v; Aleken Biologicals), epidermal growth factor (10 ng ml^{-1}; Becton Dickinson), hydrocortisone (1 μg ml^{-1}; Sigma) and L-glutamine (10 mM; Gibco). At approximately

80 % confluence, the monolayers of HMECs were infected with 6×10^4 p.f.u. of *R. conorii* per cm^2 of culture surface area according to our established protocols (Sporn *et al.*, 1997; Colonne *et al.*, 2011a). At 3 h post-infection, extracellular bacteria in the culture medium were removed by aspiration and gentle washing and infected cells were placed in fresh culture medium alone for the remaining duration of incubation. This protocol consistently results in the infection of 80 to 90 % of cells with a mean of 3 to 4 rickettsiae per cell at 6 h post-infection (Sporn *et al.*, 1997; Rydkina *et al.*, 2007; Colonne *et al.*, 2011a). In each experiment, the viability of both mock- and *R. conorii*-infected host cells at different time points was ascertained microscopically.

Cell treatment. To neutralize secreted IFN-β, culture medium was supplemented with anti-human IFN-β antibody (10 μg ml^{-1}; R&D Systems) immediately after 3 h infection. To inhibit the metabolic activity of intracellular *R. conorii*, tetracycline (20 μg ml^{-1}; Sigma) was added to the culture medium at 3 h post-infection. For treatment, recombinant human IFN-β (10 ng ml^{-1}; PBL Interferon Source) was added to the cell culture medium.

Small interfering RNAs for *SOCS1* and *UBP43*. Specific ON-TARGETplus smart pools of siRNA for *SOCS1* and *UBP43* along with a control (scrambled) sequence were obtained from Thermo Scientific. HMECs at 80 % confluence were transfected with *SOCS1*-specific, *UBP43*-specific, or scrambled siRNAs (final concentration of 100 nM) using Lipofectamine 2000 (Invitrogen) according to our published protocols (Colonne *et al.*, 2011a, b). After 6 h, transfection medium was replaced with fresh culture medium and the cells were allowed to recover for at least 12 h prior to infection with *R. conorii*.

Gene expression analysis by quantitative real-time PCR. Total RNA isolated from mock- and *R. conorii*-infected endothelial cells using TRI-Reagent (Molecular Research Center) was further purified using an RNA purification kit (Qiagen) and quantified on a Nanodrop spectrophotometer (ND-1000, Thermo Scientific). Complementary DNA (cDNA) was then synthesized using the RT^2 First Strand kit (Qiagen). Validated primers quantifying the expression of *SOCS1*, *UBP43*, *ISG15*, *OAS1*, *TAP1*, *MX1*, *GBP1*, *IRF1*, *IRF9* and *GAPDH* were purchased from Qiagen. Quantitative PCRs were performed in a MyiQ thermal cycler (Bio-Rad) with RT^2 Real-time SYBR Green Master mix (Qiagen) according to the manufacturer's instructions. The levels of expression of target genes were normalized to *GAPDH* and relative expression was calculated by the $\Delta\Delta C_t$ method.

Western blot analysis. Monolayers of uninfected and *R. conorii*-infected HMECs were washed with PBS and disrupted by scraping and suspension in a cell lysis solution [Tris buffer (100 mM, pH 7.4), supplemented with a mixture of protease and phosphatase inhibitors and 0.2 % w/v SDS] followed by mild sonication. Total protein lysates thus prepared were separated on a 10 % w/v polyacrylamide gel. The proteins were then transferred onto a nitrocellulose membrane by wet blotting at 100 V for 90 min. The blots were probed with primary antibodies against pSTAT1 (Tyr701) and UBP43 (Cell Signaling Technology) and a compatible 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), followed by detection with an anti-mouse IgG-HRP (Santa Cruz). Protein–antibody complexes were revealed using the Western Lightning enhanced chemiluminescence detection system (PerkinElmer) and exposure to X-ray film.

Densitometric and statistical analysis. Blots were scanned in grayscale mode at a resolution of 600 d.p.i. Band intensities were calculated using ImageJ software (version 1.42), normalized to the housekeeping gene α-tubulin, and assigned values relative to the corresponding uninfected control, which was given a value of 1 for ease of comparison. All experiments were performed at least in triplicate and statistical significance between control and experimental conditions was evaluated by Student's *t*-test. Results were considered to be statistically significant at a threshold *P*-value of $\leqslant 0.05$.

RESULTS

R. conorii infection induces SOCS1 and UBP43 expression in human endothelial cells

Human microvascular endothelial cells respond to *R. conorii* infection *in vitro* by secreting IFN-β, which is responsible for activating autocrine and/or paracrine innate immune responses via transcriptional activation of *STAT1* to inhibit intracellular rickettsial replication (Colonne *et al.*, 2011a). Because negative regulators of the JAK/STAT pathway represent an important arm of the signalling network involved in the regulation of expression of host IFN-responsive genes, we first determined which specific players are induced in *R. conorii*-infected HMECs. We found that expression of SOCS1 and UBP43, known inhibitors of IFN signalling, was induced during rickettsial infection (Fig. 1). UBP43 expression was significantly higher during *R. conorii* infection in comparison with the corresponding uninfected controls at 24 and 48 h, which was followed by the peak level of response at 72 h and then sustained through 96 h post-infection. SOCS1 expression, on the other hand, displayed only minimal changes early during the infection followed by significant increase of about 3.5-fold at 72 h post-infection and a subsequent decline to a mean of 2-fold induction at 96 h. These results demonstrate induced expression of SOCS1 and UBP43 and reveal clearly noticeable differences in the intensity and kinetics of such responses during *R. conorii* infection of host endothelial cells (Fig. 1a). Further, upregulation of UBP43 expression was attributable to IFN-β produced and secreted by endothelial cells since infection in the presence of an antibody capable of neutralizing IFN-β completely abolished this host cell response. This finding also implies the dependence of cellular *UBP43* induction during infection predominantly on autocrine/paracrine effects of IFN-β and rules this response out as a consequence of pathogen invasion and/or intracellular replication (Fig. 1b). Interestingly, *SOCS1* expression was only partially inhibited at 72 h and completely attenuated by neutralization of IFN-β at 96 h (Fig. 1c), implicating potential contributions from IFN-β-independent transcriptional activation mechanism(s), likely triggered by *R. conorii* invasion and intracellular multiplication. To this end, we further quantified the levels of SOCS1 mRNA expression in cells treated with recombinant IFN-β alone in comparison with those infected with *R. conorii* in the presence and absence of tetracycline and an IFN-β neutralizing antibody. As shown in Fig. 1d, SOCS1 expression during *R. conorii*

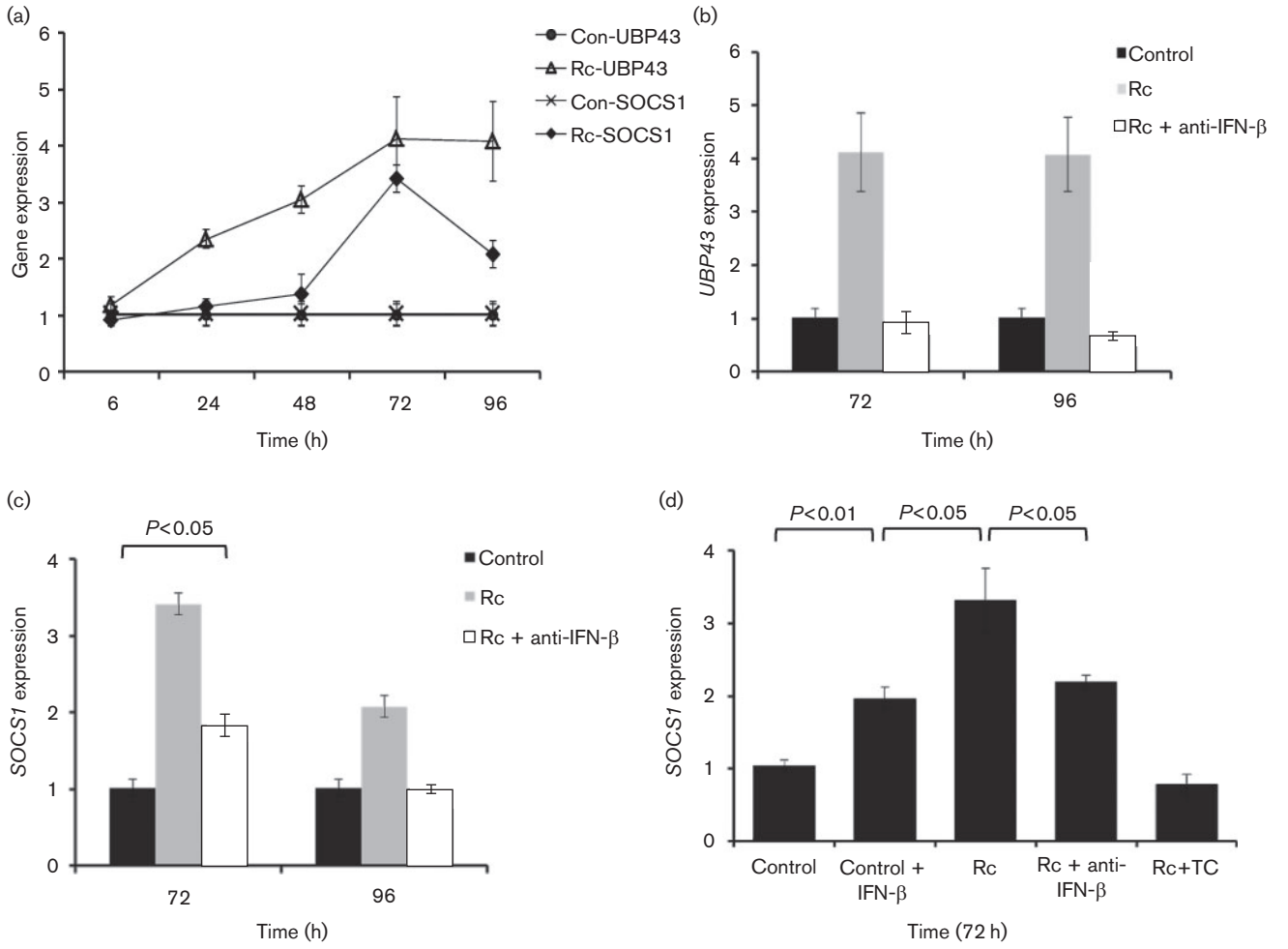

Fig. 1. *R. conorii* infection induces *SOCS1* and *UBP43* expression in human microvascular endothelial cells. (a) Time-course analysis of *SOCS1* and *UBP43* expression during *R. conorii* infection. The levels of *SOCS1* and *UBP43* expression were determined relative to the housekeeping gene *GAPDH* by quantitative RT-PCR. (b) Inclusion of an IFN-β-neutralizing antibody during *R. conorii* infection inhibits *UBP43* expression in HMECs. Infection was carried out in the absence (Rc) or presence of an antibody capable of neutralizing soluble IFN-β (Rc + anti-IFN-β) in comparison with the corresponding uninfected cells (Con). (c) Partial dependence of SOCS1 expression on *R. conorii*-induced IFN-β at 72 and 96 h post-infection. SOCS1 expression was determined in RNA preparations from uninfected (Con) and *R. conorii*-infected (Rc) HMECs and those infected in the presence of an IFN-β-neutralizing antibody (Rc + anti-IFN-β) as mentioned above. (d) Further analysis of IFN-β-independent expression of SOCS1. The expression of SOCS1 transcript was quantified in HMECs subjected to the following experimental conditions: uninfected/untreated (Control), recombinant human IFN-β-treated (Control + IFN-β), *R. conorii*-infected (Rc), infection in the presence of an anti-IFN-β antibody (Rc + anti-IFN-β) and infection in the presence of tetracycline as described in Methods (Rc + TC). The datasets represent the mean ± SE of the mean from a minimum of three independent experiments performed in duplicate.

infection was significantly higher than in HMECs subjected to IFN-β treatment alone and neutralization of IFN-β yielded only partial inhibition of SOCS1 expression in infected cells. Also, inhibition of rickettsial metabolic activity by tetracycline treatment completely attenuated SOCS1 expression, indicating that infection with viable intracellular rickettsiae is essential for SOCS1 induction. Taken together, these data suggest that *R. conorii* infection induces the expression of SOCS1 and UBP43, known negative regulators of IFN signalling, in host HMECs.

Inhibitory effect of UBP43, but not SOCS1, on *R. conorii*-induced STAT1 activation

Since both UBP43 and SOCS1 have been implicated in the negative regulation of the STAT1 activation response, we next investigated whether IFN-β-dependent STAT1 phosphorylation during *R. conorii* infection of HMECs is regulated by SOCS1 or UBP43. To investigate this, we utilized the RNA interference approach to transiently knockdown the expression of *UBP43* or *SOCS1* in HMECs

prior to infection with *R. conorii*. As expected, infection stimulated the expression of both *SOCS1* and *UBP43* by a mean of 3- and 5-fold, respectively, in HMECs transfected with a scrambled siRNA sequence used as a negative control. Also, transcriptional activation of both of these genes following infection was significantly inhibited in HMECs subjected to introduction of specific ON-target siRNA for *SOCS1* (Fig. 2a) and *UBP43* (Fig. 2b), respectively. Interestingly, siRNA-mediated knockdown of *SOCS1* did not have a significant impact on the transcriptional activation of *UBP43*, but HMECs transfected with *UBP43*-specific siRNA and subsequently infected with *R. conorii* had significantly higher levels of SOCS1 expression (Fig. 2a, b). Although analysis of protein expression further ascertained that *SOCS1* knockdown did not adversely affect the expression of both full-length protein as well as a truncated isoform (splice variant) of UBP43 in infected endothelial cells (Fig. 2c), we were not able to determine the levels of SOCS1 protein by Western blotting owing to relatively very low abundance in HMECs.

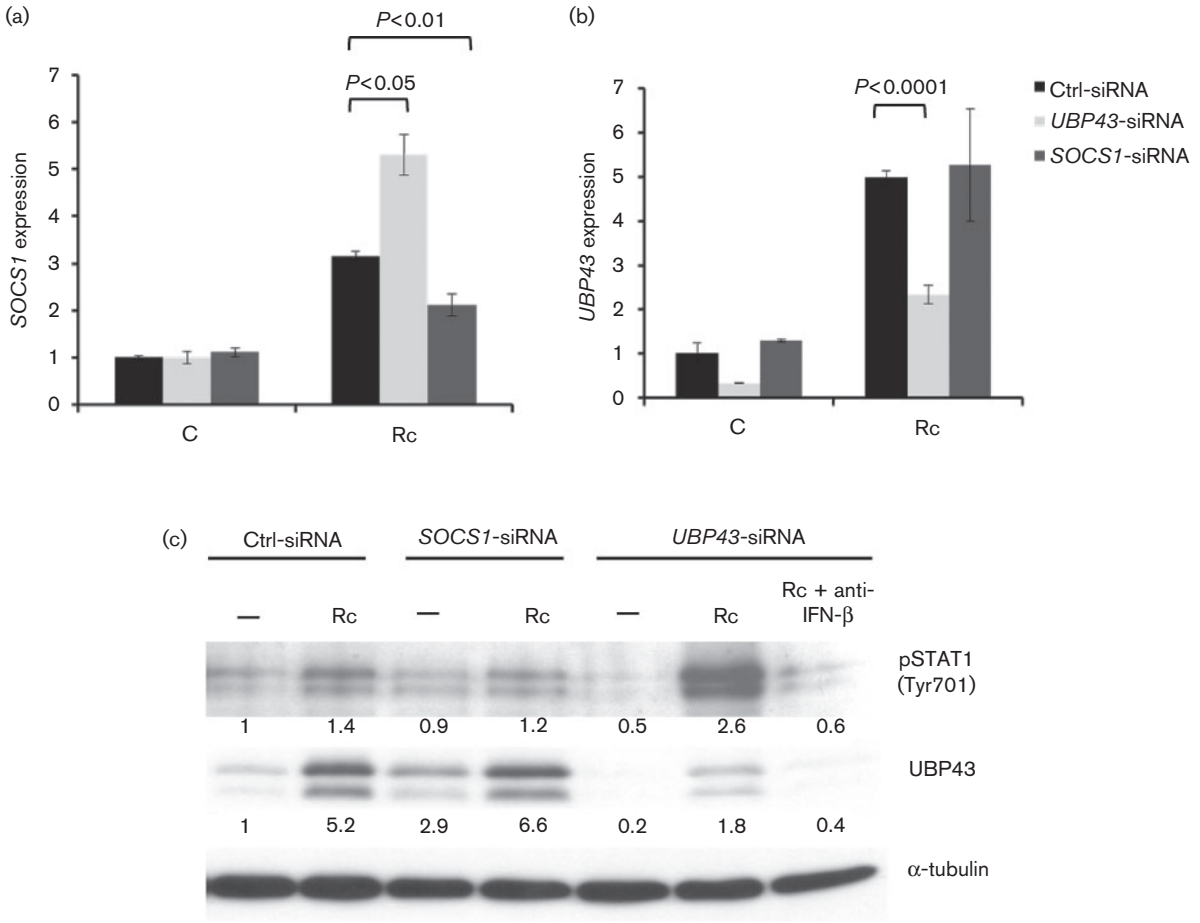

Fig. 2. Effect of *UBP43* and *SOCS1* silencing with small interfering RNA on the status of STAT1 activation in *R. conorii*-infected HMECs. Human microvascular endothelial cells were transfected with siRNA sequences against *SOCS1* (*SOCS1*-siRNA) or *UBP43* (*UBP43*-siRNA) and then infected with *R. conorii* for 72 h. The levels of expression were determined relative to the housekeeping gene *GAPDH*. (a) Effect of *UBP43* knockdown on *SOCS1* expression. *UBP43*-siRNA transfected cells were either left uninfected (C) or infected with *R. conorii* (Rc) and *SOCS1* expression was analysed by quantitative RT-PCR. *UBP43* expression was also measured to confirm gene knockdown. Cells transfected with non-specific, scrambled sequences were used as controls (Ctrl-siRNA). (b) Effect of *SOCS1* knockdown on *UBP43* expression in HMECs. Cells were transfected with *SOCS1*-specific siRNA and infected with *R. conorii* followed by quantitative RT-PCR measurements to measure *UBP43* expression. The data are presented as the mean ± SE of three independent experiments. (c) Effects of *SOCS1* and *UBP43* siRNA on STAT1 phosphorylation induced by *R. conorii* infection. Total proteins were extracted at 72 h post-infection and processed for Western detection using antibodies directed against pSTAT1 (Tyr701) and UBP43. Primary antibody used in this experiment is capable of binding with both the full-length and the truncated isoform of UBP43 protein. The band intensities for pSTAT1 and UBP43 were determined by quantitative densitometry, for which the levels of α-tubulin were used to account for any variations in the loading of samples.

R. conorii infection resulted in the stimulation of STAT1 Tyr701 phosphorylation in HMECs transfected with both scrambled sequence (control) and *SOCS1*-specific siRNA, the intensity of which was not significantly different (Fig. 2c). Thus, interference with SOCS1 expression did not affect the status of STAT1 phosphorylation, indicating that IFN-β-dependent STAT1 activation due to *R. conorii* infection is not regulated by SOCS1. In contrast, depletion of UBP43 led to the potentiation of STAT1 phosphorylation and this response was completely blocked by the presence of IFN-β-neutralizing antibody in the culture medium. Together, these data show that UBP43, but not SOCS1, functions as a feedback inhibitor to regulate IFN-β-dependent STAT1 activation in infected endothelium.

Transcriptional activation of IFN-stimulated genes in *R. conorii*-infected endothelium

Previous studies from our laboratory have shown that secreted IFN-β from *R. conorii*-infected HMECs induces an IFN-stimulated gene *ISG15* to activate innate host defence to inhibit intracellular growth of the pathogen (Colonne et.al, 2011b). Using quantitative PCR, we have further identified a series of IFN-stimulated genes whose mRNA expression is significantly upregulated in endothelial cells infected with *R. conorii*. As shown, transcriptional activation of IFN regulatory factor-1 (*IRF1*), *IRF9*, transporter of antigen peptides-1 (*TAP1*), myxovirus resistance protein (*MX1*) and oligoadenylate synthetase-1 (OAS1) was clearly evident and occurred in an IFN-β-dependent manner, because presence of an antibody capable of neutralizing the activity of IFN-β effectively curtailed the expression of these genes (Fig. 3a–e). IFN-β neutralization, however, did not have an effect on the induction of guanylate binding protein-1 (GBP1) (Fig. 3f).

Role of SOCS1 and UBP43 in regulating IFN-stimulated genes in *R. conorii*-infected HMECs

Because expression of IFN-stimulated downstream genes during inflammatory and microbial insults is subject to tight regulatory control in order to prevent severe immunopathology and damage to the host cells and our findings suggest increased expression of UBP43 and SOCS1 during *R. conorii* infection of HMECs, we reasoned that UBP43 and SOCS1 may have an important role in the regulation of transcriptional activation of IFN-stimulated genes. Therefore, HMECs transfected with either *UBP43* or *SOCS-1*-specific siRNA along with a control scrambled sequence were infected with *R. conorii* prior to the analysis of expression of IFN-stimulated genes. As expected, *R. conorii* infection induced the expression of GBP1, OAS1, MX1, ISG15, IRF1, IRF9 and TAP1 in cells transfected with control siRNA (Figs 4 and 5). Further, siRNA-mediated knockdown of *UBP43* led to significant increase in the expression of GBP1, OAS1 and MX1, whereas *SOCS1* knockdown had no significant effect on these genes (Fig. 4a–c). ISG15 expression during infection was, however, induced further in host cells subjected to *UBP43* as well as *SOCS1* siRNA, although the effect was much more pronounced with the knockdown of *UBP43* as compared with *SOCS1* (Fig. 4d). On the other hand, the expression of IRF1, IRF9 and TAP1 was not significantly affected by silencing the expression of either SOCS1 or UBP43 (Fig. 5). These results thus suggest that UBP43 and SOCS1 not only are induced in response to *R. conorii* infection, but also selectively regulate the expression of specific IFN-stimulated genes in microvascular endothelial cells.

DISCUSSION

We have recently demonstrated the activation of host cell JAK/STAT signalling and an essential role for IFN-β, a type I IFN, in STAT1-mediated interference with intracellular replication of *R. conorii* in vascular endothelial cells *in vitro* (Sahni *et al.*, 2009; Colonne *et al.*, 2011a). Signalling mechanisms downstream of JAKs and STATs are controlled at many steps through a variety of distinct mechanisms, including key negative regulators such as the suppressor of cytokine signalling (SOCS) proteins. Microbial pathogens are known to subvert and subterfuge IFN-governed innate immune responses of the host via modulation of negative regulators of the JAK/STAT pathway, yet the potential importance of such mediators in determining IFN-β-mediated responses during host cell interactions with pathogenic *Rickettsia* species is not at all understood. In the present study, we have identified increased expression of UBP43 and SOCS1, proteins implicated in the feedback inhibitory loop of the JAK/STAT signalling pathway, during *R. conorii* infection of HMECs and report on their selective involvement in the regulation of downstream IFN-β-responsive genes.

UBP43, a member of the family of ubiquitin isopeptidases, specifically cleaves a ubiquitin-like molecule ISG15, which is induced by stress signals such as type I IFNs and viral infections and associates with other cellular proteins to form ISGylated complexes. Documented evidence suggests that UBP43 negatively regulates IFN signalling by blocking interactions between JAK kinase and the IFNAR2 receptor subunit, thereby interfering with STAT1 activation (Malakhova *et al.*, 2006). Our findings are in agreement with such a role for UBP43, because neutralization of IFN-β during *R. conorii* infection results in complete attenuation of *UBP43* induction, indicating that UBP43 likely functions as a feedback inhibitory mechanism to regulate STAT1 activation. Silencing of UBP43 expression also results in even stronger STAT1 phosphorylation in infected cells, yielding further support to the notion that activation of STAT1 is negatively regulated by UBP43. In addition to its role as an inhibitor for IFN signalling, UBP43 catalyses the deISGylation reaction by cleaving ISG15–protein complexes and in doing so regulates the extent of cytosolic protein ISGylation (Malakhov *et al.*, 2002). Thus, UBP43 maintains the delicate cellular equilibrium between free cytosolic ISG15 and ISG15–protein conjugates, which are

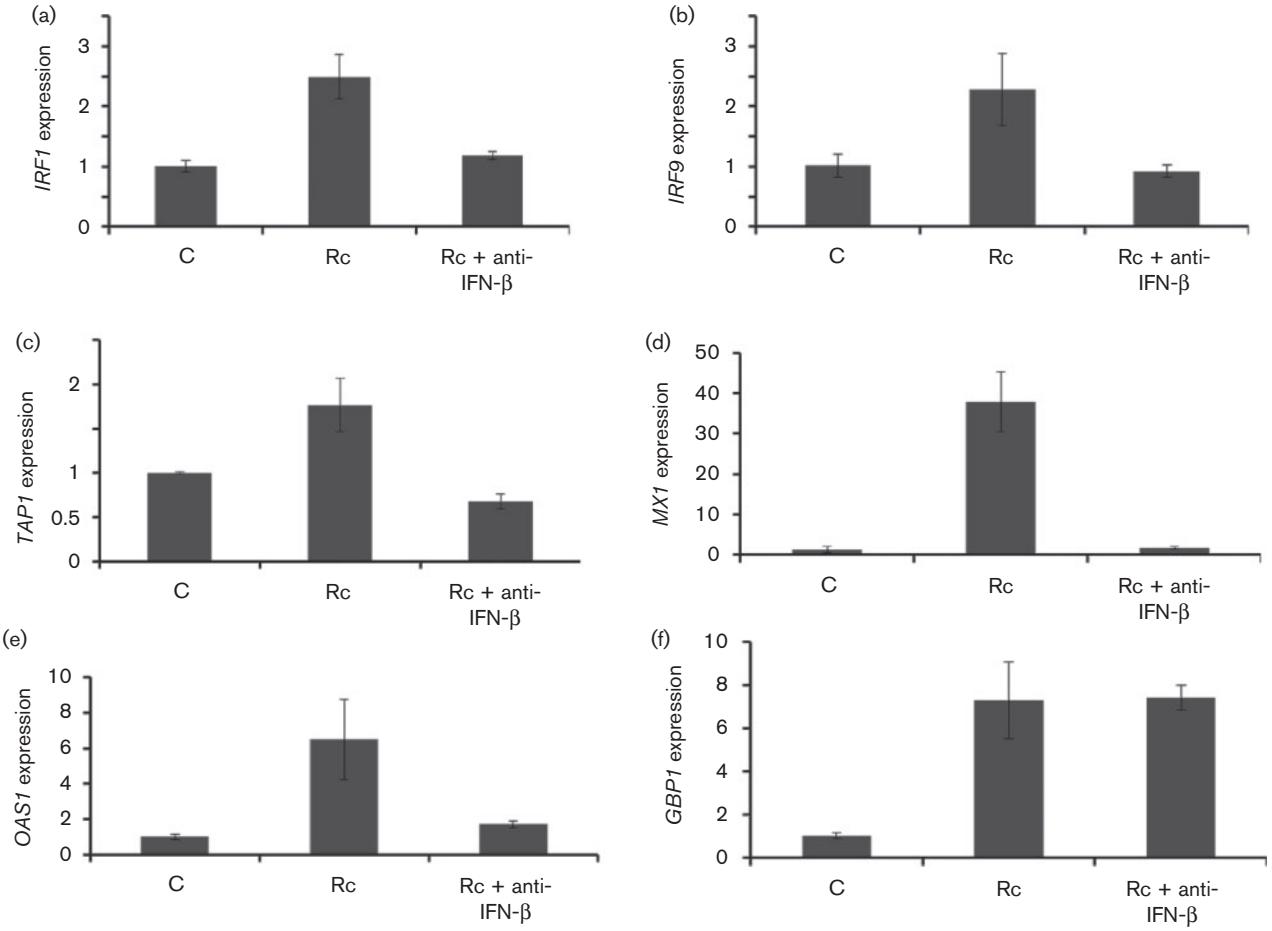

Fig. 3. Transcriptional status of IFN-stimulated genes in HMECs after *R. conorii* infection. Endothelial cells were infected with *R. conorii* for 72 h in the absence (Rc) and presence of an antibody to neutralize the autocrine/paracrine effects of IFN-β (Rc + anti-IFN-β). Simultaneously processed uninfected cells (C) were used to analyse the basal level of expression. The bar diagrams depict the expression levels of IFN-stimulated genes, namely *IRF1* (a), *IRF9* (b), *TAP1* (c), *MX1* (d), *OAS1* (e) and *GBP1* (f), as determined by quantitative RT-PCR and normalized to *GAPDH* (housekeeping gene) by the $\Delta\Delta C_t$ method. The data represent the mean ± SE from at least three separate observations in relation to baseline expression in uninfected controls which was given a value of 1 to facilitate comparison between experimental groups.

strongly increased after IFN stimulation. We have also demonstrated that isopeptidase activity of UBP43 leads to increased accumulation of ISG15–protein complexes in endothelial cells subjected to RNA interference during rickettsial infection and accumulation of free ISG15 and/or proteins conjugated to ISG15 exerts inhibitory effects on *R. conorii* replication (Colonne *et al.*, 2011b). Therefore, UBP43 apparently performs a dual role as an ISG15 protease as well as a negative regulator for IFN-β signalling in *R. conorii*-infected host endothelium.

SOCS1 is one of the major prototypical proteins belonging to the suppressor of cytokine signalling family. It binds to JAK proteins via an SH2 domain and blocks the phosphorylation of STAT1, preventing its dimerization and nuclear translocation, a critically important step for IFN signalling (Dai *et al.*, 2006; Qin *et al.*, 2006). Intracellular pathogens specifically induce SOCS proteins during host invasion to antagonize IFN signalling, leading to the dampening of immune responses. For instance, hepatitis C virus infection has been shown to induce SOCS1 and SOCS3 expression, resulting in the inhibition of STAT1 and STAT3 phosphorylation and subsequent interference with T-cell functions (Yao *et al.*, 2005). Our findings clearly demonstrate that *R. conorii*-infected endothelial cells display increased SOCS1 transcription and, interestingly, only partial inhibition of SOCS1 expression following IFN-β neutralization. It is well established that the promoter region of the *SOCS1* gene contains putative STAT1 binding sites (Naka *et al.*, 1997; Saito *et al.*, 2000). Therefore, activated STAT1 apparently contributes to only partial induction of SOCS1 expression. This may further explain the increased transcriptional activation of *SOCS1* in cells experiencing *UBP43* knockdown because UBP43 depletion strongly increased STAT1

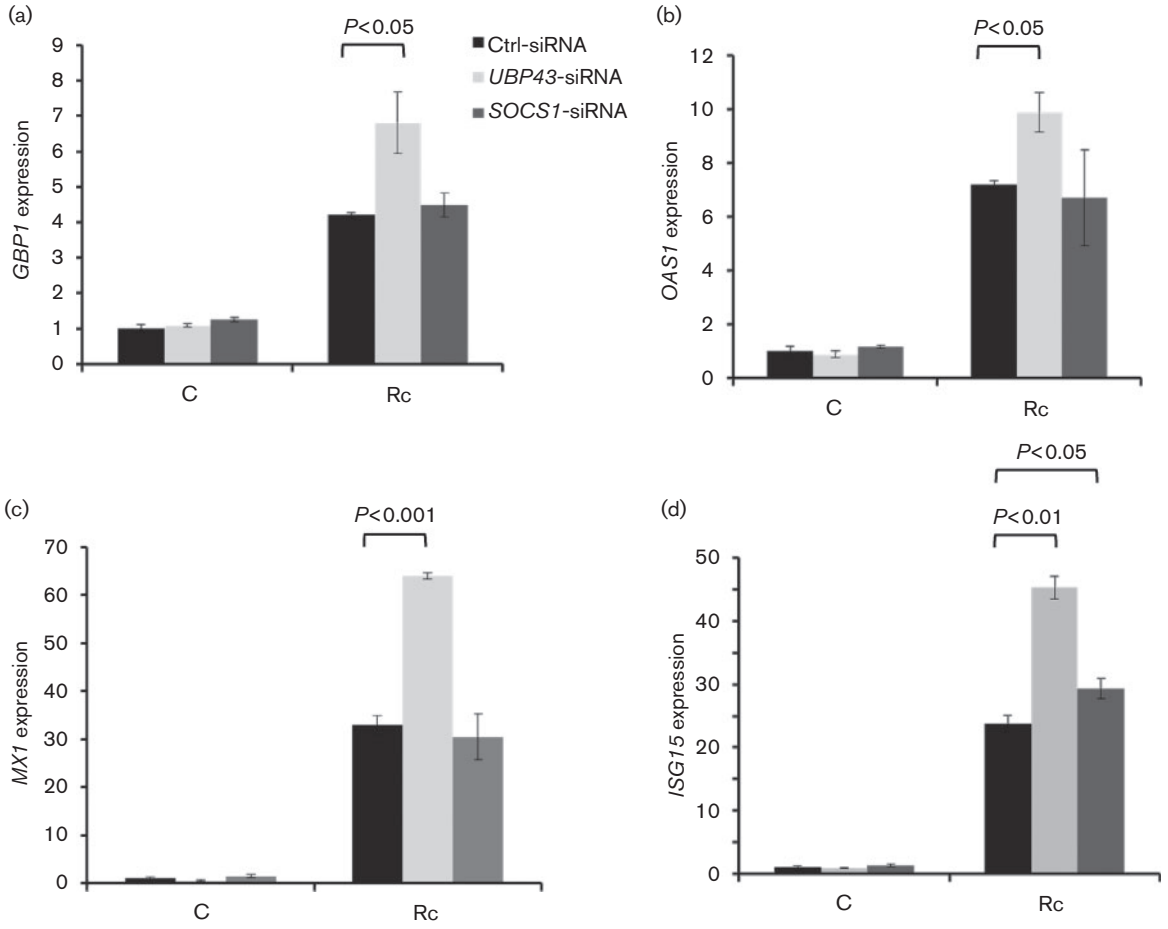

Fig. 4. Effect of *SOCS1* and *UBP43* knockdown on transcriptional activation of IFN-stimulated genes in *R. conorii*-infected HMECs. Endothelial cells were transfected with gene-specific siRNA sequences directed against *SOCS1* or *UBP43* prior to infection with *R. conorii* for 72 h. The expression levels (relative to *GAPDH*) of *GBP1* (a), *OAS1* (b), *MX1* (c) and *ISG15* (d) in uninfected controls (C) and infected HMECs (Rc) were analysed by quantitative RT-PCR. Expression of all of these IFN-stimulated genes was negatively regulated by UBP43, with the exception of *ISG15*, which was negatively regulated by both UBP43 and SOCS1. The datasets represent mean ± SE from a minimum of three independent observations performed in duplicate.

phosphorylation in infected cells. It is also possible that induced SOCS1 expression is a component of rickettsial strategy to subdue and subvert IFN signalling in host endothelium and that the levels of accumulation of SOCS1 in infected host cells may correlate with the virulence potential of the invading pathogen and the severity of resulting disease. Although the magnitude of SOCS1 induction independent of IFN-β is about two-fold, it is expected to have significant implications since SOCS proteins are potent, tightly regulated inhibitors known for their capacity to exert biological effects even in relatively small quantities (Chen *et al.*, 2000). Considering that interference with *SOCS1* yields no adverse effect on STAT1 phosphorylation, it apparently functions through a mechanism independent of inhibitory feedback regulation of STAT1 activation. SOCS1 may, however, negatively regulate a specific subset of host genes, for example *ISG15*, via other mechanism(s) independent of direct inhibition of IFN-β signalling in *R. conorii*-infected endothelium. Another important consideration in this context is that anti-rickettsial host responses activated by IFN-γ may also be adversely affected because SOCS1 is capable of inhibiting IFN-γ mediated immune responses (Alexander *et al.*, 1999), which have been implicated in rickettsial clearance *in vivo* (Walker *et al.*, 2001). Therefore, further detailed studies are currently being performed to investigate the potential impact of SOCS1 induction on the host immune responses and rickettsial replication in both *in vitro* and *in vivo* murine models relevant to the pathogenesis of human rickettsiosis. Also, the identity of pathogen-associated molecular patterns or rickettsial effectors responsible for the induction and/or subversion of host cell signalling mechanisms is the subject of further detailed investigations in our laboratory. Production of IFN-β and expression of downstream genes

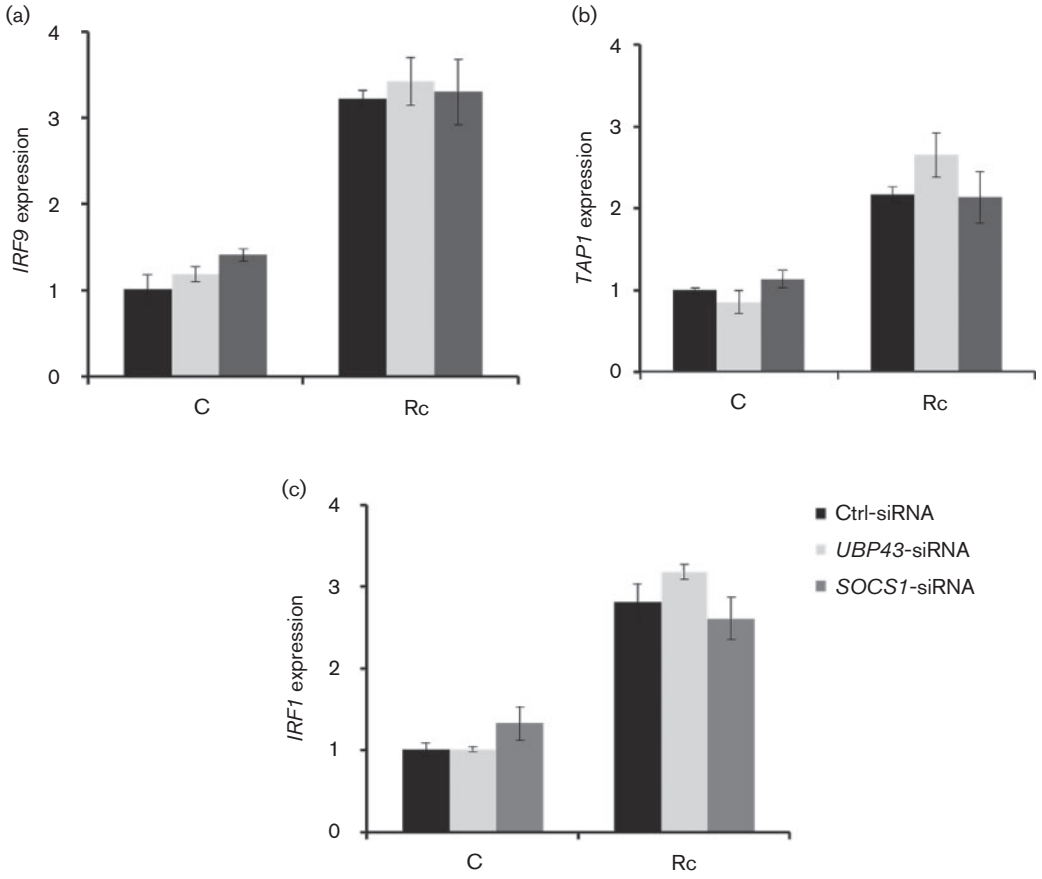

Fig. 5. Effect of *SOCS1* and *UBP43* knockdown on transcriptional activation of IFN-stimulated genes in *R. conorii*-infected HMECs. Endothelial cells were transfected with gene-specific siRNA sequences directed against *SOCS1* or *UBP43* and then infected with *R. conorii* for 72 h. Expression levels (relative to *GAPDH*) of *IRF9* (a), *TAP1* (b) and *IRF1* (c) in uninfected controls (C) and infected HMECs (Rc) were analysed by quantitative RT-PCR. Notably, expression of none of these IFN-stimulated genes was significantly altered by either *UBP43* or *SOCS1* knockdown. The datasets represent mean ± SE from a minimum of three independent observations performed in duplicate.

by host endothelium likely constitutes an essential first line of defence against invading and intracellular rickettsiae and offer a unique strategic opportunity to identify potential rickettsial effectors and/or pathogen recognition mechanisms involved in switching on the upstream signalling mechanisms leading to such responses.

In addition to *UBP43* and *SOCS1*, this study also identifies transcriptional activation of *OAS1*, *IRF1*, *IRF9*, *MX1* and *TAP1*, and provides evidence for the dependence of these genes on IFN-β production and resultant autocrine/paracrine signalling from infected cells. Expression of *GBP1*, on the other hand, seems to occur independently of the host IFN-β signalling as the presence of IFN-β-neutralizing antibody during the infection has no effect on its expression. *GBP1* is an IFN-γ-inducible gene known for antimicrobial effects against intracellular bacteria, for example *Chlamydia* and *Listeria* infections (Tietzel *et al.*, 2009; Kim *et al.*, 2011). However, it can also be induced by type I IFNs, IL-1β and TNF (Decker *et al.*, 1991; Guenzi *et al.*, 2001, 2003). Although the detailed molecular mechanism of this activation is not yet established, IFN-β may indirectly regulate endothelial *GBP1* activation, possibly via activated STAT1, because *GBP1* expression displays significant increase after *UBP43* knockdown, which is associated with increased STAT1 phosphorylation. Nevertheless, the potential involvement of GBP1 in anti-rickettsial activity has not been explored and requires detailed investigation.

Among other IFN-stimulated genes, *OAS1* activates latent RNase, which degrades viral RNA, thereby inhibiting viral replication (Coccia *et al.*, 1990; Lin *et al.*, 2009). So far, there is no published evidence to suggest that OAS1 has anti-bacterial functions. Therefore, some of the IFN-stimulated genes may simply represent a somewhat generalized response to IFN-β, instead of specific cellular responses of the host to intracellular rickettsiae. Another downstream gene, *MX1*, codes for a member of the large GTPase family of proteins, which is mainly induced by

IFNα/β during viral infections (Staeheli *et al.*, 1986; Grimm *et al.*, 2007). However, the exact mechanisms of MX1 protein function also remain largely unknown. In our system, the expression of both OAS1 and MX1 was negatively regulated by UBP43 but not by SOCS1, because increased STAT1 activation due to *UBP43* interference enhances transcriptional activation of both *OAS1* and *MX1* genes. TAP1 facilitates translocation of peptides from cytosol into endoplasmic reticulum and participates in the expression of MHC class I molecules on the cell surface and antigen presentation to cytotoxic T lymphocytes during microbial infections (Van Kaer *et al.*, 1992; de la Salle *et al.*, 1994). Interestingly, the *TAP1* gene can be induced by both IFN-γ and IFN-β via formation of STAT1 homodimers, which bind to GAS elements on the gene promoter (Min *et al.*, 1998).

Initially characterized for its role in the transcriptional activation of type I IFN genes, IRF1 acts as a transcription factor by binding to the IFN-β gene promoter to regulate its transcription and that of several other downstream IFN-stimulated genes implicated in promoting anti-bacterial and antiviral innate immunity (Miyamoto *et al.*, 1988; Harada *et al.*, 1989). As another key transcription factor, IRF9, associates with phosphorylated STAT1/STAT2 heterodimers to form the ISGF3 complex, which translocates to the nucleus and binds to regulatory IFN-stimulated response elements (ISREs) to induce the expression of specific IFN-stimulated genes (Levy *et al.*, 1989; Fu *et al.*, 1992). We find that although *IRF1, IRF9* and *TAP1* are induced during *R. conorii* infection in an IFN-β dependent manner, the expression of these genes is refractory to interference with UBP43 or SOCS1 response. Thus, *R. conorii*-induced STAT1 activation is apparently sufficient to trigger the expression of *IRF1*, *IRF9* and *TAP1* genes, and potentiation of STAT1 phosphorylation as a consequence of *UBP43* knockdown essentially has no effect on their transcriptional activation. On the contrary, *ISG15* is negatively regulated by both SOCS1 and UBP43. Since *ISG15* is a STAT1-dependent gene, increased STAT1 activation following *UBP43* knockdown could trigger a strong transcriptional activation of *ISG15* expression. Importantly, *ISG15* represents the only IFN-stimulated gene identified in this study to be regulated by SOCS1, as evidenced by significant increase in its expression in cells subjected to *SOCS1* knockdown. Since STAT1 activation remains unaffected by *SOCS1* interference, the inhibitory effects of SOCS1 on *ISG15* expression may be attributed to other as-yet-unidentified STAT1-independent mechanism(s).

To summarize, distinct patterns of the regulation of IFN-stimulated genes by SOCS1 and UBP43 may represent host cell-specific mechanisms of innate immune responses against *Rickettsia* infection. Elucidation of the roles of these newly identified IFN-stimulated genes and their negative regulators in anti-rickettsial host defence is an important area of future scientific enquiry. Considering the emergence of severe forms/strains of *R. conorii* with fatal outcomes despite treatment with antibiotics, understanding the pathogen-induced regulation of IFN signalling is expected to provide new insights into novel virulence mechanisms exploited by infectious rickettsiae during the onset and progression of resultant clinical syndromes.

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