MicroRNA-98 and let-7 Regulate Expression of Suppressor of Cytokine Signaling 4 in Biliary Epithelial Cells in Response to Cryptosporidium parvum Infection

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Expression of the cytokine-inducible Src homology 2 (CIS) protein and suppressors of cytokine signaling (SOCS) proteins represents an important element of host cell reactions in response to infection. We have demonstrated previously that Cryptosporidium parvum infection down-regulates microRNA-98 (miR-98) and let-7 to induce CIS expression in biliary epithelial cells. We report here that down-regulation of miR-98 and let-7 also coordinates epithelial expression of SOCS4 after C. parvum infection. Targeting of the SOCS4 3′ untranslated region by miR-98 or let-7 resulted in translational repression. Functional manipulation of miR-98 caused reciprocal alterations in SOCS4 protein expression. Transfection of miR-98 precursor abolished C. parvum–stimulated SOCS4 up-regulation. Moreover, expression of SOCS4 in epithelial cells showed an inhibitory effect on phosphorylation of signal transducers and activators of transcription proteins induced by C. parvum. These data suggest that miRNAs play an important role in the coordinated regulation of CIS and SOCS expression in epithelial cells in response to C. parvum infection.

Epithelial cells play a critical role in the initiation, regulation, and resolution of both innate and adaptive immune reactions in response to microbial infection of the gastrointestinal tracts. These epithelial cells express pathogen-recognition receptors, including Toll-like receptors (TLRs). TLRs recognize pathogens and activate a set of adaptor proteins (eg, myeloid differentiation protein 88 [MyD88]) leading to the activation of a variety of intracellular regulatory signals, including nuclear factor kB (NF-κB), the Janus kinase (JAK) signaling pathway, and the signal transducers and activators of transcription (STAT) signaling pathway [1, 2]. Activation of these signaling pathways initiates a series of host cell defense reactions against pathogens, including parasites [3]. Such epithelial defense responses are finely controlled and reflect a delicate balance between effector functions and their potential to cause damage to healthy tissues [4–6].

To perform a fine-tuning of immune responses, epithelial cells have developed multiple strategies for the feedback regulation of intracellular signaling pathways. Several endogenous proteins have recently been identified to counterregulate TLR and cytokine signaling cascades and promote resolution of inflammation, such as Toll-interacting protein and A20 [4–6]. The cytokine-inducible Src homology 2 (CIS) protein and the suppressors of cytokine signaling (SOCS) proteins constitute a family of intracellular molecules that have emerged as key physiological regulators of cytokine responses in many cell types [7]. The best-characterized SOCS family members are CIS and SOCS1–3, which function in a classical, negative-feedback loop and inhibit cytokine signaling by interacting with the JAK and...
The protozoan parasite Cryptosporidium parvum is a causative agent of human gastrointestinal disease worldwide [13, 14]. Humans are infected by ingesting C. parvum oocysts; oocysts then excyst in the gastrointestinal tract, releasing infective sporozoites. The released sporozoite infects epithelial cells and forms a vacuole in which the organism remains intracellular but extracytoplasmic [5, 15]. Because of the minimally invasive nature of C. parvum infection, innate immune responses are critical to the host’s defense against infection [16, 17]. C. parvum sporozoites can also travel up the biliary tract to infect biliary epithelial cells, particularly in patients with AIDS [16]. Infection of human biliary epithelial cells by C. parvum in vitro mimics parasitic apical invasion and epithelial innate immune responses in vivo [18]. The invasion of epithelial cells in vitro by C. parvum results in the rapid expression of antimicrobial peptides (eg, β-defensins) and the inflammatory chemokines [19, 20]. How epithelial cells finely balance the inflammatory reactions and antimicrobial immune responses during C. parvum infection is still unclear.

MicroRNAs (miRNAs) are a newly identified class of endogenous small regulatory RNAs that mediate either messenger RNA (mRNA) cleavage or translational suppression, resulting in gene suppression [4–6]. More than 700 miRNAs have been identified in humans, and it has been predicted that miRNAs control 20%–30% of human genes [4–6]. The importance of miRNA-mediated posttranscriptional mechanisms extends beyond simply determining the rate of mRNA translation and degradation. Each miRNA has multiple predicted targets, and many of the targets are functionally related [21, 22]. Various miRNAs can also target the same mRNA molecule, resulting in coordinated expression [23]. Thus, miRNAs can coordinate the regulation of multiple miRNAs and allow cells to respond with unusual ability to environmental cues. Studies have revealed key roles for miRNAs in diverse regulatory pathways, including control of timing in cell development and differentiation, apoptosis, cell proliferation, and, more recently, immune regulation [10, 15, 24–27]. Induction of miR-155 during the macrophage inflammatory response suggests its potential involvement in the regulation of inflammation [15, 25, 28].

We recently demonstrated that miR-98 and let-7 regulate translation of CIS in human biliary epithelial cells in response to C. parvum infection or lipopolysaccharide stimulation [29]. We show here that C. parvum infection induces SOCS4 expression in biliary epithelial cells. C. parvum–induced expression of SOCS4 involves miRNA-mediated translational gene repression. In addition, C. parvum infection increases phosphorylation of STAT3 and STAT6 in epithelial cells, and induction of SOCS4 appears to provide an inhibitory effect on C. parvum–stimulated STAT phosphorylation. Thus, miRNA-coordinated regulation of CIS and SOCS4 expression has been identified in epithelial cells after C. parvum infection.

METHODS

C. parvum and infection model. C. parvum oocysts of the Iowa strain were purchased from a commercial source (Bunch Grass Farm). H69 cells are SV40-transformed human biliary epithelial cells originally derived from normal liver harvested for transplant [29, 30]. Nonimmortalized normal human biliary epithelial cells (HIBEpiCs) were obtained from ScienCell Research Laboratories. Before infecting cells, oocysts were treated with 1% sodium hypochlorite on ice for 20 min, followed by extensive washing with Dulbecco’s modified Eagle medium (DMEM)–F12. Infection was done in a culture medium (DMEM-F12) containing viable C. parvum oocysts (oocysts with host cells at a 5:1 to 10:1 ratio), as described elsewhere [29, 30].

Plasmids. The functionally defective dominant negative (DN) mutant of TLR4 was obtained from M. F. Smith (University of Virginia). MyD88-DN (a DN mutant of MyD88) was a gift of J. Tschopp (University of Lausanne). H69 cells stably transfected with TLR4-DN or MyD88-DN plasmid constructs were obtained as described elsewhere [18, 31]. The HuSH 29mer short hairpin RNA–SOCS4 and control constructs were purchased from OriGene.

Western blot analysis. Whole cell lysates were obtained with the M-PER Mammalian Protein Extraction reagent (Pierce) plus several protease inhibitors (1 mmol/L phenylmethylsulfonylfuoride, 10 μg/mL leupeptin, and 2 μg/mL pepstatin). To detect STAT tyrosine phosphorylation, cells were harvested in lysis buffer containing phosphatase inhibitors (1 mmol/L Na3VO4 and 1 mmol/L NaF). Antibodies to SOCS4 (Invitrogen), phosphor-STAT1 (Tyr705), phosphor-STAT3 (Tyr705), phosphor-STAT6 (Tyr641), STAT1, STAT3, STAT6 (all from Cell Signaling), and actin (Sigma-Aldrich) were used. Densitometric levels of SOCS4 signals were quantified, normalized to actin, and expressed relative to levels in uninfected cells.

Northern blot analysis. Total cellular RNAs were obtained using TRIzol reagent (Invitrogen) and run on a 15% Tris, borate, and ethylenediaminetetraacetic acid (EDTA) urea gel (90 mmol/L Tris, 64.6 mmol/L boric acid, and 2.5 mmol/L EDTA [pH 8.3]; Invitrogen). A locked nucleic acid digoxigenin–labeled probe of miR-98 (Exiqon) was hybridized using UltraHyb reagents (Ambion), in accordance with the manufacturer’s instructions, and small nuclear RNA RNU6B was blotted for control [24, 29].

Real-time polymerase chain reaction. Comparative real-time polymerase chain reaction (PCR) was performed using the SYBR Green PCR Master mix (Applied Biosystems). The
primer sequences for amplification of human SOCS4 were 5′-GTATCGGAAAGCTCAAGTTA-3′ (forward) and 5′-TTCCAGACAGTTTCTCTTG-3′ (reverse); the primer sequences for amplification of interleukin 8 (IL-8) were 5′-GGGCCATGTTGCAAACTC-3′ (forward) and 5′-GGAGAACCCACCACGAAAGAA-3′ (reverse); and the primer sequences for amplification of GAPDH were 5′-TGACCCACCACTGGTTAGC-3′ (forward) and 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse). Cycle threshold (Ct) values were analyzed using the comparative Ct (ΔΔCt) method; the amount of target was obtained by normalizing to the endogenous reference (GAPDH) and was expressed relative to the control (uninfected cells) [24].

miRNA precursors and antisense oligonucleotides to miRNAs. To manipulate the cellular function of miR-98 and let-7 in H69 cells, we used specific antisense oligonucleotides to miRNAs (anti-miRs) to inhibit miRNA function and specific miRNA precursors to increase miRNA expression, as described elsewhere [24, 26]. Anti-miRs (Anti-miR miRNA inhibitors; Ambion) are commercially available, chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNAs [32]. For experiments, H69 cells were grown to 90% confluence and treated with anti-miRs or precursors to miR-98 and let-7 (0–30 nmol/L; Ambion), using the Lipofectamine 2000 reagent (Invitrogen). Nonspecific anti-miR and precursor (Ambion) were used as controls.

Luciferase reporter constructs and luciferase assay. Complementary 37-base pair DNA oligonucleotides containing the putative miR-98/let-7 target site within the 3′ untranslated region (UTR) of human SOCS4 were synthesized with flanking SpeI and HindIII restriction enzyme digestion sites (sense, 5′-ctagTACAAAGGTAGAAAGTCTCATACTACCTACCTTCTCTTTACCTTGTA-3′; antisense, 5′-agtCTAAAGATGAGTAGATGAGACTTTTCTACCTTACCTTGTA-3′) and cloned into the multiple-cloning site of the pMIR-REPORT Luciferase vector (Ambion). Another pMIR-REPORT Luciferase construct containing a mutant 3′ UTR (TACTACCTTCATCTCAGAGGTAGTATGAGACTTTTCTA CCGGAATC-3′) was also generated as a control. We then transfected cultured cells with each reporter construct, as well as anti-miR-98/let-7 or miR-98/let-7 precursor. Luciferase activity was measured and normalized to the control β-galactosidase level, as described elsewhere [29, 31].

**RESULTS**

**Induction of SOCS4 protein expression in biliary epithelial cells by C. parvum infection in a TLR4/MyD88-dependent manner.** When H69 cells were exposed to *C. parvum* for up to 48 h, a significant increase in SOCS4 protein content was detectable in cells 12–48 h after *C. parvum* infection (Figure 1A). An increase in SOCS4 protein levels was also detected in HIBEpiCs 24 and 48 h after exposure to *C. parvum* (Figure 1B). Activation of TLR4/MyD88 signaling has been demonstrated previously in biliary and intestinal epithelial cells during *C. parvum* infection [18, 29, 33]. To test whether TLR signals are involved in *C. parvum*-induced SOCS4 expression, we analyzed the expression of SOCS4 in H69 cells stably transfected with TLR4-DN or MyD88-DN [32]. No increase in the SOCS4 protein level was found in TLR4-DN–transfected or MyD88-DN–transfected cells after *C. parvum* infection, compared with that in noninfected cells (Figure 1C).

**No change in SOCS4 mRNA levels in biliary epithelial cells after *C. parvum* infection.** When HIBEpiCs and H69 cells were exposed to *C. parvum* for up to 12 h, no significant change in SOCS4 mRNA levels was detected by real-time PCR (Figure 2A). No change in SOCS4 mRNA was detected in TLR4-DN–transfected or MyD88-DN–transfected cells after *C. parvum* infection (Figure 2B). As a positive control, we analyzed IL-8 transactivation, a TLR4/NF-κB–dependent process induced by *C. parvum* in epithelial cells [34]. A significant increase in IL-8 mRNA expression was confirmed in H69 cells 8 and 12 h after exposure to *C. parvum* (Figure 2C).

**Targeting of the SOCS4 3′ UTR by miR-98 and let-7, resulting in translational suppression.** The inconsistency between SOCS4 mRNA level and protein content in H69 cells and HIBEpiCs after *C. parvum* infection suggested the involvement of posttranscriptional regulation. To test whether miRNA-mediated posttranscriptional gene regulation is involved in this process, we used the MicroRNA.org database (http://www.microrna.org) [35] to screen those miRNAs expressed in H69 cells, on the basis of our previous microarray analysis [36]. We found that the miR-98 and let-7 family has complementarity to the SOCS4 3′ UTR. We identified one potential binding site for the miR-98 and let-7 family in the SOCS4 3′ UTR, extending between 1825 and 1850 (Figure 3A).

To test the potential targeting of SOCS4 mRNA by miR-98 and/or let-7, we generated pMIR-REPORT luciferase constructs containing the SOCS4 3′ UTR with the putative let-7 and miR-98 binding site (Figure 3B). In addition, constructs with the TACTACCTTCATCTCAGAGGTAGTATGAGACTTTTCTACCGGAATC mutation at the putative binding sites were generated as controls (Figure 3B). We then transfected H69 cells with these reporter constructs and assessed luciferase activity 24 h after transfection. As shown in Figure 3B, a significant decrease in luciferase activity was detected in cells transfected with the SOCS4 3′ UTR construct containing the potential binding site, compared with that in cells transfected with the mutant control vector. No change in luciferase activity was observed in cells transfected with the mutant SOCS4 3′ UTR construct, suggesting that endogenous translational repression of the construct with the SOCS4 3′ UTR occurred. In addition, anti–miR-98 and anti–let-7 markedly increased SOCS4 3′ UTR–associated luciferase reporter translation (Figure 3B). In contrast, miR-98 and let-7 precursors significantly decreased luciferase activity (Figure 3B). These data
Figure 1. Induction of expression of suppressor of cytokine signaling 4 (SOCS4) protein in biliary epithelial cells by *Cryptosporidium parvum* infection in a Toll-like receptor 4 (TLR4)/myeloid differentiation protein 88 (MyD88)-dependent manner. A and B, Analysis of SOCS4 protein in H69 cells and nonimmortalized normal human biliary epithelial cells (HIBEpiCs). Cells were exposed to *C. parvum* oocysts for up to 48 h, after which Western blot analysis was performed for SOCS4. Up-regulation of SOCS4 protein was detected in both H69 cells (A) and HIBEpiCs (B) after *C. parvum* infection. C, Analysis of SOCS4 protein in H69 cells stably transfected with TLR4-DN (dominant negative) or MyD88-DN. Cells were exposed to *C. parvum* for 24 h. *C. parvum*-stimulated expression of SOCS4 protein was not detected in cells transfected with either TLR4-DN or MyD88-DN. The Western blots shown in panels A–C are representative of 3 independent experiments; densitometric levels of SOCS4 signals were quantified, normalized to actin, and expressed relative to levels in uninfected cells. *P < .05 for the comparison with nontreated controls (t test).

suggest that miR-98 and let-7 target the SOCS4 3′ UTR, resulting in posttranscriptional suppression.

**Reciprocal alterations in SOCS4 protein expression due to manipulation of miR-98 function.** To test whether miRNA-mediated translational repression of SOCS4 is directly relevant to SOCS4 protein expression, we treated H69 cells with anti-miR-98 or miR-98 precursor for 72 h and then measured SOCS4 protein expression by Western blot analysis. Transfection of H69 cells with miR-98 precursor caused a dose-dependent decrease in SOCS4 protein content (Figure 4A). In con-
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Involvement of TLR4/MyD88-dependent down-regulation of miR-98 and let-7 in C. parvum–induced SOCS4 protein expression. To further test whether miRNA-mediated SOCS4 translational repression is involved in C. parvum–induced SOCS4 expression, we measured expression of miR-98 and let-7 in H69 cells after exposure to C. parvum for up to 48 h. Consistent with results from our previous studies using a Northern blot probe that recognizes both miR-98 and let-7 [29, 31], decreased expression of miR-98 and let-7 was detected in cells 8–48 h after C. parvum infection (Figure 5A). C. parvum–repressed expression of miR-98 and let-7 was not identified in cells stably transfected with TLR4-DN or MyD88-DN (Figure 5B), confirming the involvement of TLR4/MyD88 signaling.

To further test whether miRNA-mediated SOCS4 translational repression is indeed involved in C. parvum–induced SOCS4 protein expression, we transfected H69 cells with various doses of miR-98 precursor for 48 h and then exposed the cells to C. parvum for 24 h, after which Western blot analysis was performed for SOCS4. miR-98 precursor significantly inhibited C. parvum–induced up-regulation of SOCS4 protein in H69 cells in a dose-dependent manner (Figure 6A). No significant change in SOCS4 mRNA levels was found in miR-98 precursor–treated cells 24 h after C. parvum infection, compared with levels in cells treated with control precursor or in the nontreated control cells (Figure 6B). In addition, cells treated with either miR-98 precursor or control precursor followed by exposure to C. parvum for various periods displayed similar cellular levels of SOCS4 mRNA (Figure 6C). Taken together, these data suggest that C. parvum infection decreases expression of miR-98 and let-7 through activation of the TLR4/MyD88 signaling pathway to regulate SOCS4 protein expression in biliary epithelial cells.

Possible effect of up-regulation of SOCS4 on C. parvum–induced STAT phosphorylation due to feedback loop. Activation of STAT signaling has been reported previously in parasite infection [37]. Expression of SOCS4 and SOCS5 provides a feedback loop to the STAT signaling pathway during septic peritonitis [38]. We tested whether expression of SOCS4 influences STAT phosphorylation in H69 cells after C. parvum infection. We first used a specific small interfering RNA (siRNA) to knock down SOCS4 expression in H69 cells and then measured levels of phosphorylated STAT1, STAT3, and STAT6. Although all 3 STATs showed a basal level of phosphorylation in cells transfected with a control siRNA, a significant increase in phosphorylation of contrast, a dose-dependent increase in SOCS4 protein level was identified in H69 cells treated with anti–miR-98 (Figure 4B). However, no significant change in SOCS4 mRNA level was found between the control cells and the cells treated with miR-98 precursor (Figure 4C) or anti–miR-98 (Figure 4D), suggesting that miR-98 does not induce degradation of SOCS4 mRNA.

Possible effect of up-regulation of SOCS4 on C. parvum–induced STAT phosphorylation due to feedback loop. Activation of STAT signaling has been reported previously in parasite infection [37]. Expression of SOCS4 and SOCS5 provides a feedback loop to the STAT signaling pathway during septic peritonitis [38]. We tested whether expression of SOCS4 influences STAT phosphorylation in H69 cells after C. parvum infection. We first used a specific small interfering RNA (siRNA) to knock down SOCS4 expression in H69 cells and then measured levels of phosphorylated STAT1, STAT3, and STAT6. Although all 3 STATs showed a basal level of phosphorylation in cells transfected with a control siRNA, a significant increase in phosphorylation of

Figure 2. No effect of Cryptosporidium parvum infection on suppressor of cytokine signaling 4 (SOCS4) messenger RNA (mRNA) levels in biliary epithelial cells. A, Analysis of SOCS4 mRNA in H69 cells and nonimmortalized normal human biliary epithelial cells (HIBEpiCs). Cells were exposed to C. parvum oocysts for up to 12 h, after which real-time polymerase chain reaction analysis for SOCS4 mRNA was performed. No significant change in SOCS4 mRNA expression in H69 cells or HIBEpiCs in response to C. parvum infection was observed. B, Expression of SOCS4 mRNA in TLR4-DN (dominant negative) and MyD88-DN H69 cells in response to C. parvum infection. C, C. parvum–induced expression of interleukin 8 (IL-8) mRNA in H69 cells. IL-8 mRNA levels are presented as cycle threshold values normalized to GAPDH and are expressed relative to those in uninfected cells. *P < .05 for the comparison with nontreated controls (t test).
Figure 3. Targeting of the suppressor of cytokine signaling 4 (SOCS4) 3′ untranslated region (UTR) by microRNA-98 (miR-98) and let-7, causing translational suppression. A, Schematic of SOCS4 messenger RNA (mRNA). The schematic shows one potential binding site in the 3′ UTR for miR-98 and let-7. B, Binding of miR-98 and let-7 to the potential binding site in the SOCS4 3′ UTR, resulting in translational suppression. Reporter constructs containing the potential binding site of miR-98 and let-7 within the SOCS4 3′ UTR or the mutant sequence were generated. H69 cells were transiently cotransfected with the reporter construct and either let-7 or miR-98 precursor oligonucleotide or their antisense for 24 h. Luciferase activity was then measured and normalized to the control β-galactosidase (β-gal) level. Data are means ± standard deviations for 3 independent experiments. Ctrl, control; Mut, mutant; WT, wild type. * for the comparison with controls (t test); # for the comparison with SOCS4 3′ UTR–transfected cells (t test).

STAT3 and STAT6, but not STAT1, was detected in cells treated with the SOCS4 siRNA by Western blot analysis (Figure 7A). Knockdown of SOCS4 protein by the siRNA was confirmed by Western blot analysis. No significant change in the total level of each tested STAT was detected after SOCS4 siRNA treatment. Moreover, increased phosphorylation of STAT3 and STAT6 was found in cells after exposure to C. parvum for 12 h. In contrast, after exposure to C. parvum for 24 h, cells showed a significant decrease in STAT3 and STAT6 phosphorylation (Figure 7B). Accordingly, a higher level of SOCS4 protein was detected in cells exposed to C. parvum for 24 h, compared with that in cells exposed to C. parvum for 12 h (Figure 7B).

DISCUSSION

Our results provide the first evidence, to our knowledge, that suggest that miR-98 and let-7 play an important role in C. parvum–induced expression of SOCS4 in host epithelial cells. Using an in vitro model of human cryptosporidiosis employing human biliary epithelial cells, we found that C. parvum infection induces SOCS4 expression through posttranscriptional gene regulation involving activation of the TLR/MyD88 pathway. Targeting of the SOCS4 3′ UTR by miR-98 and let-7 results in translational suppression. C. parvum–induced SOCS4 expression in biliary epithelial cells involves negation of miR-98
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Figure 4. Reciprocal alterations in suppressor of cytokine signaling 4 (SOCS4) protein expression as a result of the manipulation of microRNA-98 (miR-98) function. A and B, Induction of a dose-dependent decrease or increase, respectively, in SOCS4 protein expression in H69 cells by transfection of miR-98 precursor or anti–miR-98. H69 cells were treated with various doses of miR-98 precursor (A) or anti–miR-98 (B), after which Western blot analysis for SOCS4 was performed. A nonspecific precursor (precursor-Ctrl) and a nonspecific anti-miR (anti–miR-Ctrl) were used as controls. A representative Western blot from 3 independent experiments is shown for panels A and B. Actin was also blotted to ensure equal loading. Densitometric levels of SOCS4 signals were quantified, normalized to actin, and expressed relative to those in non–anti-miR–treated cells. C and D, No effect of miR-98 precursor or anti–miR-98 transfection on SOCS4 messenger RNA (mRNA) levels. H69 cells were exposed to miR-98 precursor or anti–miR-98, after which real-time polymerase chain reaction analysis for SOCS4 mRNA was performed. * for the comparison with nontreated cells (t test).

We have demonstrated previously that miR-98 and let-7 regulate CIS protein expression via translational repression in epithelial cells [29]. Several lines of evidence from this study support that miR-98 and let-7 also target the SOCS4 3′ UTR, resulting in suppression of translation in biliary epithelial cells. First, complementarity of miR-98 and let-7 to the SOCS4 3′ UTR was identified using the MicroRNA.org database (http://www.microrna.org) for miRNA target prediction [35]. A significant decrease in luciferase activity was detected in cells transfected with the pMIR-REPORT luciferase construct containing the SOCS4 3′ UTR with the putative miR-98 and let-7 binding site. This translational repression is likely mediated by miR-98 and let-7, because precursors and anti-miRs specific to miR-98 or let-7 caused reciprocal alterations in SOCS4 3′ UTR–associated luciferase activity. Second, transfection of cells with anti–miR-98 induced significant expression of SOCS4 protein. In contrast, miR-98 precursor decreased cellular SOCS4 protein content. Finally, transfection of cells with miR-98 precursor did not induce degradation of SOCS4 mRNA. Therefore, miR-98 and let-7 regulate both SOCS4 and CIS protein expression via translational repression in epithelial cells, implicating that miR-98 and let-7 can coordinately regulate expression of CIS and SOCS4.

Microbe-induced up-regulation of CIS and SOCS expression has predominantly been demonstrated in immune cells. Up-regulation of CIS and SOCS proteins has previously been reported in infected epithelial cells [39]. In the present study, we found that C. parvum infection increased SOCS4 expression in human biliary epithelial cells via activation of the TLR4/MyD88 pathway. Interestingly, cellular SOCS4 mRNA levels showed no significant change after C. parvum infection. Instead, activation
Figure 5. Decrease in expression of microRNA-98 (miR-98) and let-7 after Cryptosporidium parvum infection in a Toll-like receptor 4 (TLR4)/myeloid differentiation protein 88 (MyD88)–dependent manner. A, Decrease in miR-98 and let-7 expression in H69 cells after C. parvum infection, as assessed by Northern blot analysis. RNU6B (U6) was used as a loading control. Densitometric levels of miR-98 and let-7 signals were quantified, normalized to U6, and expressed relative to those in uninfected cells. B, TLR4/MyD88 dependency of C. parvum–induced down-regulation of miR-98 and let-7 expression. No decrease in miR-98 and let-7 expression was detected in H69 cells stably transfected with TLR4-DN (dominant negative) or MyD88-DN after exposure to C. parvum, as assessed by Northern blot analysis.

Figure 6. Abolishment of Cryptosporidium parvum–stimulated suppressor of cytokine signaling 4 (SOCS4) protein expression by transfection of microRNA-98 (miR-98) precursor. A, Inhibition of C. parvum–induced expression of SOCS4 protein in H69 cells by transfection of miR-98 precursor. Cells were transfected with the miR-98 precursor and then exposed to C. parvum, after which Western blot analysis for SOCS4 was performed. A representative Western blot from 3 independent experiments is shown in panel A. Densitometric levels of SOCS4 signals were quantified, normalized to actin, and expressed relative to those in uninfected cells. B and C, No effect of miR-98 precursor transfection on SOCS4 messenger RNA (mRNA) levels in cells after C. parvum infection. H69 cells were exposed to miR-98 precursor for 48 h and then exposed to C. parvum for 24 h, after which real-time polymerase chain reaction analysis for SOCS4 mRNA was performed (B). Similar SOCS4 mRNA levels were also detected in cells treated with miR-98 precursor after exposure to C. parvum for 2–8 h. * for the comparison with uninfected cells (t test); # for the comparison with infected cells (t test).
Figure 7. Suppression of Cryptosporidium parvum–induced signal transducers and activators of transcription (STAT) phosphorylation by suppressor of cytokine signaling 4 (SOCS4). A, Increases in phosphorylation of STAT3 and STAT6, but not STAT1, in H69 cells after knockdown of SOCS4 by small interfering RNA (siRNA). Cells were transfected with short hairpin RNA against SOCS4 for 72 h, after which Western blot analysis for tyrosine-phosphorylated STATs (pSTATs), total STATs, SOCS4, and actin was performed. B, Correlation of STAT3 and STAT6 phosphorylation with levels of SOCS4 expression in H69 cells after C. parvum infection. H69 cells were exposed to C. parvum oocysts for 12 or 24 h, after which Western blot analysis for tyrosine-phosphorylated STATs, nonphosphorylated STATs, SOCS4, and actin was performed. Densitometric levels of SOCS4 signals were quantified, normalized to actin, and expressed relative to those in SOCS4 siRNA−/H11002 cells. Densitometric levels of phosphorylated STAT3 and STAT6 signals were quantified, normalized to total STAT3 and STAT6, and expressed relative to those in SOCS4 siRNA− cells (A) or uninfected cells (B). *P < .05 for the comparison with SOCS4 siRNA− cells (A) or uninfected cells (B) (t test); #P < .05 for the comparison with cells infected for 12 h (B) (t test).
Expression of SOCS4 in biliary epithelial cells may represent an important feedback mechanism to finely control host cell reactions in response to *C. parvum* infection. CIS and SOCS proteins have classically been shown to be negative regulators of cytokine signaling. Each CIS or SOCS protein has 2 major domains: an SH2 domain and a SOCS box [7]. Although the SH2 domain binds to the phosphorylated tyrosine residues in the substrates, the E3 activity of CIS and SOCS proteins can cause ubiquitination of the substrates for proteasome degradation [41]. Thus, CIS and SOCS proteins function as E3 ubiquitin ligases and mediate the degradation of activated cytokine-signaling complex, resulting in negative feedback regulation [7]. In the present study, we demonstrated that SOCS4 may be involved in the regulation of *C. parvum*-induced STAT phosphorylation. Inhibition of SOCS4 by siRNA interference promoted phosphorylation of STAT3 and STAT6 in biliary epithelial cells. Decreased STAT3 and STAT6 phosphorylation was detected in cells 24 h after *C. parvum* infection, which correlated with a higher level of expression of SOCS4 protein in infected cells. SOCS4-mediated suppression of STAT phosphorylation may be associated with increased degradation of phosphorylated STATs. A recent study by Watanabe et al [38] demonstrated that expression of SOCS4 and SOCS5 increases degradation of phosphorylated STATs, providing a negative feedback loop to regulate STAT signaling during septic peritonitis. Although it is presently unclear what the underlying molecular mechanisms are, it is possible that SOCS4 may facilitate ubiquitination-associated degradation of phosphorylated STATs induced by *C. parvum*.

In conclusion, our data indicate that *C. parvum* induces SOCS4 expression in human biliary epithelial cells via negative of miRNA-mediated translational suppression of SOCS4. Moreover, expression of SOCS4 in biliary epithelial cells is involved in the regulation of STAT phosphorylation in response to *C. parvum* infection. Thus, miRNA-mediated posttranscriptional gene regulation coordinates CIS and SOCS4 expression in biliary epithelial cells in response to *C. parvum* infection and is involved in the regulation of epithelial antimicrobial responses. It will be of interest to extend these studies to determine the role played by miRNAs in epithelial anti-*C. parvum* immunity in vivo.

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