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RSV replication is attenuated by counteracting expression of the suppressor of cytokine signaling (SOCS) molecules

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ABSTRACT

Human RSV causes an annual epidemic of respiratory tract illness in infants and in elderly. Mechanisms by which RSV antagonizes IFN-mediated antiviral responses include inhibition of type I IFN mRNA transcription and blocking signal transduction of JAK/STAT family members. The suppressor of cytokines signaling (SOCS) gene family utilizes a feedback loop to inhibit cytokine responses and block the activation of the JAK/STAT signaling pathway. To evaluate the potential of SOCS molecules to subvert the innate immune response to RSV infection, eight SOCS family genes were examined. RSV infection up-regulated SOCS1, SOCS3, and CIS mRNA expression in HEp-2 cells. Suppression of SOCS1, SOCS3 and CIS by short interfering ribonucleic acid (siRNA) inhibited viral replication. Furthermore, inhibition of SOCS1, SOCS3, or CIS activated type I IFN signaling by inducing STAT1/2 phosphorylation. These results suggest that RSV infection escapes the innate antiviral response by inducing SOCS1, SOCS3 or CIS expression in epithelial cells.

Introduction

Human respiratory syncytial virus (RSV), a major respiratory viral pathogen, is an enveloped and negative-strand ribonucleic acid (RNA) paramyxovirus that causes an annual epidemic of respiratory tract illness in infants and in elderly and high-risk adults (Falsey, 2005; Sigurs et al., 2000). Severe RSV infection in early age causes long-term morbidity and mortality by increasing the risk for recurrent wheezing and asthma throughout childhood (Sigurs et al., 2005; Stein et al., 1999). Respiratory epithelial cells, the principal target for RSV infection, are the first defense in the innate immune response to this virus. In general, type I interferon(IFN), IFN-α/β, is induced by viruses or double-stranded RNA in epithelia and activates signal transduction pathways of IFN-stimulated genes (ISGs) (Decker et al., 2002). To inhibit type I IFN expression, a number of viruses have evolved mechanisms that interfere with either the induction of these cytokines or the function of antiviral IFN-induced proteins (Decker et al., 2002; Bode et al., 2003; Bossert and Conzelmann, 2002; Gotoh et al., 2001). The RSV nonstructural proteins, NS1 and NS2, have been demonstrated to block the antiviral effects of type I IFN (Bossert and Conzelmann, 2002; Spann et al., 2004; Spann et al., 2005; Valarcher et al., 2003). RSV infection interferes with type I IFN signaling by specifically inhibiting signal transducer and activator of transcription (STAT) 1/2 phosphorylation and by degrading STAT2, thus providing a molecular mechanism for viral evasion of the host innate immune response (Lo et al., 2005; Ramaswamy et al., 2004; Zhang et al., 2005.

The suppressor of cytokine signaling (SOCS) family members are important molecules that inhibit cytokine signaling (Naka et al., 1997; Nicola et al., 1999; Starr et al., 1997). The SOCS gene family includes eight members (SOCS1 to SOCS7 and CIS) which bind to tyrosine kinases of Janus family kinase (JAK) elements, resulting in reduction of JAK enzymatic activity and inhibition of tyrosine phosphorylation of JAK/STAT factors (Hansen et al., 1999; Krebs and Hilton, 2000), (Krebs and Hilton, 2001). According to recent studies, several virus accessory proteins such as hepatitis C virus core (Bode et al., 2003) and herpes simplex virus 1 UL41 (Yokota et al., 2005; Yokota et al., 2004) induced SOCS3 expression. Also, expression of SOCS1 by cardiac myocytes blocked enterovirus-induced activation of STAT-responsive genes (Yasukawa et al., 2003).

We therefore hypothesized that RSV escapes from the host type I IFN antiviral responses by modulating expression of the SOCS genes.

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To test this hypothesis, we examined SOCS and CIS expression in HEp-2 cells infected with RSV. In addition, we defined the effect of specific SOCS family members on RSV infection by silencing their gene expression by utilizing short interfering RNA (siRNA). Our results demonstrate that RSV escaped type I IFN antiviral activity by up-regulating SOCS1, SOCS3, and CIS, and that silencing the expression of these genes by siRNA inhibited viral replication. These results suggest that targeting SOCS gene family members could be an important therapeutic target in preventing RSV-induced disease.

Results

RSV infection up-regulated epithelial cell mRNA expression of SOCS1, SOCS3 and CIS

To examine SOCS expression in response to RSV infection, RT real-time PCR was performed to screen mRNA expression of SOCS1–7 and CIS in RSV-infected HEp-2 cells. At an moi of 3, SOCS1, SOCS3 and CIS mRNA were rapidly induced following virus infection (Fig. 1). In the early stage of RSV infection, SOCS1, SOCS3 and CIS expression increased 9.6-fold, 13.8-fold and 7.3-fold compared to uninfected cells ($p<0.01$), respectively. The peaks of expression were 1–2 h after infection and decreased to the levels of uninfected cells by 24 h. RSV infection nonsignificantly increased SOCS2 mRNA expression (2.6-fold) compared to uninfected cells (Fig. 1). RSV infection did not increase HEp-2 cell mRNA levels of SOCS4, SOCS5, SOCS6 or SOCS7 (data not shown). Incubation with UV-inactivated virus did not induce a significant change in the HEp-2 cell mRNA expression of any of the SOCS members, implying that viral gene expression is required for increased SOCS expression. From these results, we conclude that RSV replication is essential for the induction of SOCS1, SCOS3 and CIS, and the increase of these SOCS members could regulate RSV replication in vitro epithelial cell culture.

SiRNA against SOCS1, SOCS3 and CIS suppressed each SOCS expression

To determine the role of SOCS expression during RSV infection, siRNA for SOCS1 (si-SOCS1), SOCS3 (si-SOCS3) and CIS (si-CIS) were used to inhibit gene and protein expression of these molecules. At first, the siRNA inhibition efficacy was determined by the suppression of GFP expression in HEp-2 cells by performing a co-transfection experiment with a GFP-expression plasmid and its siRNA(si-GFP) at a concentration of 20 nM. By microscopic evaluation, the number of GFP expressing cells was decreased greater than 80% compared to the control without siRNA, and this silencing effect lasted at least 10 days (data not shown). Next, in order to evaluate the efficacy of si-SOCS1, si-SOCS3 and si-CIS (si-SOCSs), HEp-2 cells were independently treated with si-SOCS at 20 nM for 24 h, then cells were stimulated with IFNα for 1 h . The expression of each SOCS mRNA was suppressed over 90% by each siRNA (Fig. 2A). In protein level, the induction of each SOCS protein was decreased by each siRNA specifically at the time point of 6 h after stimulation with IFNα in si-SOCS transfected HEp-2 cells (Fig. 2B). In induction with RSV infection, si-SOCSs were independently transfected into HEp-2 cells 24 h prior to RSV infection to determine the suppressive efficacy of the siRNA against its respective SOCS gene. At the time point that was 48 h after transfection and 24 h post RSV infection, HEp-2 mRNA expression of SOCS1, SOCS3, and CIS was suppressed in a dose dependent fashion by each of the siRNA. We found that specific siRNA transfection at a concentration of 20 nM reduced SOCS1, SOCS3 and CIS mRNAs by 31.8%, 29.4% and 25.8% compared to that of no-siRNA control, respectively (Fig. 2C). While treatment with si-GFP at any concentration had no effect on the expression of SOCS1, SOCS3, and CIS, the treatment with the specific si-SOCS decreased that gene's expression at 100 nM and 20 nM compared to that of the si-GFP control ($p<0.01$).

Suppression of SOCS1, SOCS3 and CIS inhibited RSV replication in HEp-2 cells

After transfection of each si-SOCS or si-GFP to the HEp-2 cells, cells were challenged with RSV at moi 1. The virus titers in the cell culture supernatant were determined by a plaque-forming assay 24 h post infection. The virus titers were decreased in a dose dependent fashion for each si-SOCS (Fig. 3). The virus titer was suppressed over 80% by si-SOCS1, si-SOCS3, or si-CIS at a concentration of 20 nM compared to that of no-siRNA control. While the treatment with si-GFP at any concentration did not decrease viral replication, the treatment with si-SOCS decreased viral replication at 100 nM and 20 nM compared to that with si-GFP control ($p<0.05$). These results suggest that induction of SOCS1, SOCS3, and CIS by RSV infection is a mechanism by which the virus increases viral titers.

Inhibition of SOCS1, SOCS3 and CIS activated IFN signal pathway

The SOCS gene family utilizes a feedback loop to inhibit cytokine responses and block the activation of the JAK/STAT signaling pathway (Krebs and Hilton, 2001). To determine the mechanism by which the siRNAs for SOCS1, SOCS3, and CIS mediate reductions in viral titers of RSV-infected HEp-2 cells, we first examined the association between the concentration of each si-SOCS and JAK/STAT activation. Real-time PCR was performed to evaluate mRNA levels for 2′–5′OAS1 in HEp-2 cells 48 h after transfection. Poly I:C was used as a positive control for an inducer of type I IFN signaling (Schaefer et al., 2005). Expression of 2′–5′OAS1 increased in a dose dependent fashion in parallel with the concentration of siRNA administered for SOCS1, SOCS3, and CIS (Fig. 4A). However, si-GFP also induced the expression of 2′–5′OAS1 at a concentration of 100 nM, but the expression of 2′–5′OAS1 was the same level as the control when the siRNA concentration was decreased to 20 nM. These results suggest that inhibition of SOCS1, SOCS3 and CIS promote expression of IFN-inducible genes and that a high concentration of siRNA might activate JAK/STAT1 signaling by the nature of being double-stranded RNA (Shen et al., 2004; Sledz et al., 2003). The later experiments were performed with 20 nM at the concentration of siRNA.

Next, we examined the effect of each si-SOCS on IFN-inducible gene, 2′–5′OAS1, mRNA in RSV-infected cells (Fig. 4B). In RSV-infected HEp-2 cells, si-SOCS1 ($p<0.05$) or si-SOCS3 ($p<0.05$) at a concentration of 20 nM each significantly increased 2′–5′OAS1 mRNA expression, compared to si-GFP control. These results suggest that cells treated with si-SOCS are released from the negative regulation of SOCS on JAK/STAT1 signal transduction.

si-SOCS activated type I IFN-induced STAT1/2 phosphorylation

It has been reported that RSV infection not only specifically inhibits type I IFN-induced phosphorylated STAT1 and STAT2 (p-STAT), but also selectively inhibits STAT2 expression (Lo et al., 2005; Ramaswamy et al., 2004; Zhang et al., 2005). To further assess the role of si-SOCS in regulating JAK/STAT signaling, immunoblot analysis was performed to check the expression of STAT1/2 and their phosphorylated isoforms (Fig. 5). Since STAT phosphorylation is an early response to type I IFN signaling, we examined the time course of STAT phosphorylation in response to RSV infection. Poly I:C, a strong type I IFN inducer as a consequence of binding to toll-like receptor 3 (TLR3), was used as a positive control (Fig. 5A). There was no effect of si-SOCS1 or si-SOCS3 beside si-CIS on the basal levels of total STAT1 (tSTAT1) to si-GFP-treated cells. Also, there was no effect of si-SOCS1, si-SOCS3, or si-CIS on the basal levels of total STAT2 (sSTAT2) compared to si-GFP-treated cells (Figs. 5B, C, D, E, F, H). While total STAT2 of si-GFP treated cells was decreased at 4 h post RSV infection (Figs. 5B, H), total STAT2 of si-SOCS3 treated cells was increased (Figs. 5D, H). The cells treated with si-SOCS1, si-SOCS3, or

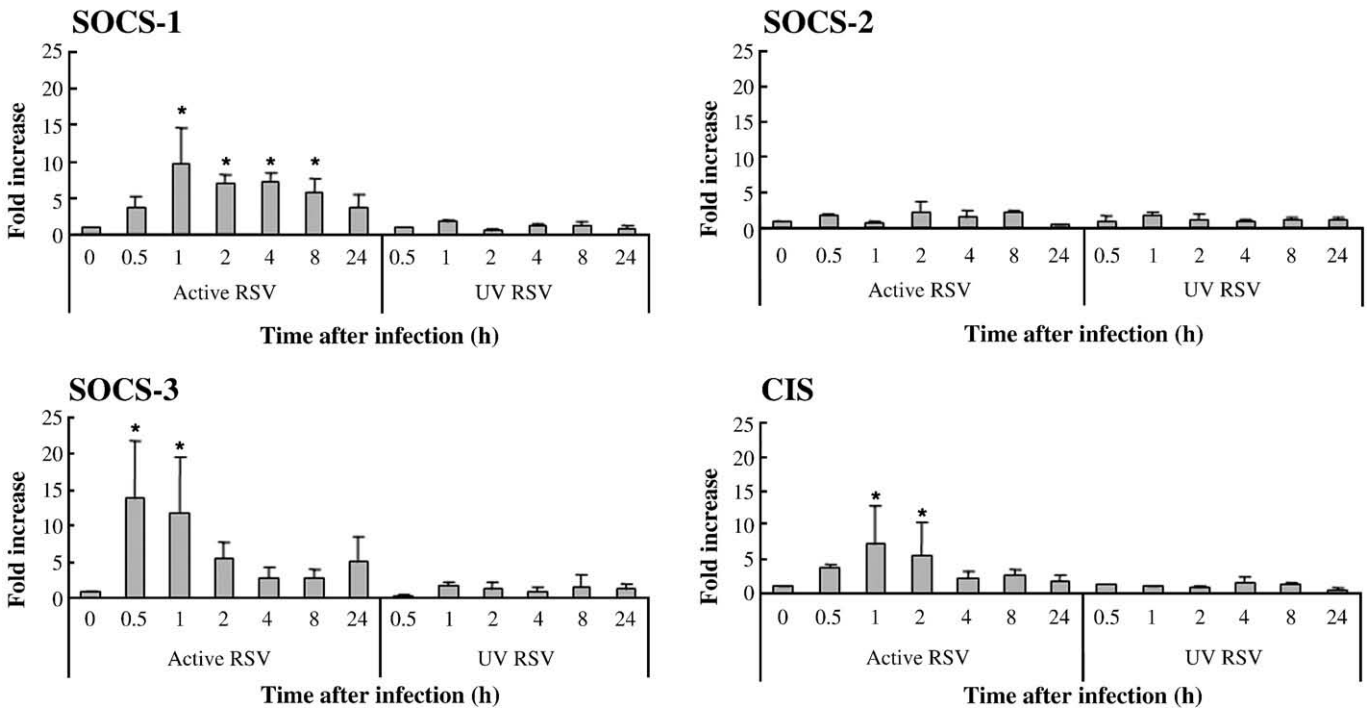

Fig. 1. RSV induced SOCS1, SOCS3 and CIS mRNA expression in the HEp-2 cells. HEp-2 cells were infected with purified RSV at moi 3 or treated with the same amount of UV-inactivated RSV. Total RNA was extracted at the indicated times for RT real-time PCR. Panels A, B, C, D show the fold increase in mRNA levels of SOCS1, SOCS2, SOCS3 and CIS expression, respectively, following RSV infection compared to the value at time 0 (uninfected cells). Data is expressed as the mean ± SE of three independent experiments. *$p < 0.01$ versus time zero.

CIS had an increased basal level of STAT1/2 phosphorylation compared to si-GFP treated cells (Figs. 5B, C, D, E, G, I). STAT1/2 phosphorylation was observed in all groups treated with si-SOCS at the designated time points after RSV infection, while there was a faint signal of STAT1/2 phosphorylation in the group treated with si-GFP (Figs. 5B, G, I). RSV infection resulted in a further up-regulation of pSTAT1 in si-SOCS3 treated cells (Fig. 5D), while RSV infection resulted in suppression of pSTAT1 in si-CIS treated cells in a time dependent fashion (Figs. 5E, G). Interestingly, si-SOCS1, si-SOCS3 and si-CIS had no effect on RSV infection-induced STAT2 phosphorylation (Figs. 5B, C, D, I) while poly I:C suppressed STAT2 phosphorylation (Fig. 5A). These results indicate that inhibition of SOCS1, SOCS3 or CIS induce both STAT1 and STAT2 phosphorylation; furthermore, si-SOCS1, si-SOCS3, and si-CIS could inhibit RSV replication by STAT1/2 phosphorylation.

Discussion

A main focus of these studies was to understand how virus subverts the IFN-inducible antiviral response (Decker et al., 2002; Horvath, 2004). Some RNA and DNA viruses have been reported to inhibit the innate immune response (Bode et al., 2003; Bossert and Conzelmann, 2002; Zhang et al., 2005; Yokota et al., 2004). Previous studies have elucidated some mechanisms of how paramyxoviridae family, including RSV, could attenuate type I IFN-induced antiviral activity (Gotoh et al., 2001). We have already reported that RSV infection of macrophage-like U937 cells results in increased expression of SOCS1, SOCS3, and CIS mRNA, while phosphorylation of STAT1 and STAT2 was shown to be decreased (Zhao et al., 2007). In this work we provide experimental evidence that RSV infection resulted in up-regulation of SOCS1, SOCS3, and CIS in HEp-2 cell originated from human epithelial cells. Furthermore, suppression of these SOCS genes by using siRNA inhibited RSV replication in vitro.

Signaling by several cytokines which activate the JAK/STAT pathway has been blocked by SOCS family proteins via a negative feedback regulation mechanism (Hansen et al., 1999; Krebs and Hilton, 2000; Narazaki et al., 1998). Virus-induced expression of the SOCS molecules efficiently counteracts the cellular response which leads to IFN-inducible antiviral gene expression (Bode et al., 2003; Brand et al., 2005). For example, overexpression of the human hepatitis virus C core protein results in induction of SOCS3 mRNA expression in human hepatic cells and is associated inhibition of IFN-α-induced tyrosine phosphorylation and activation (Bode et al., 2003; Kawaguchi et al., 2004). Herpes simplex virus type 1 infection induces SOCS3 protein, which is mainly responsible for the suppression of type I IFN signaling and its products. Transfection of antisense oligonucleotides for SOCS3 suppressed HSV-1 replication in a human amnion cell line (Yokota et al., 2004). Additionally, cardiac myocyte-specific transgenic expression of SOCS1 inhibited the expression of STAT-responsive genes as a result of enterovirus infection and there was an associated increase in viral replication (Yasukawa et al., 2003). Furthermore, SOCS1 inhibition using dominant-negative SOCS1 in the cardiac myocytes increased resistance to the acute cardiac injury caused by enteroviral infection.

In our experiments, we screened all eight SOCS members (SOCS1–7, and CIS), and found that RSV infection rapidly induced SOCS1, SOCS3 and CIS mRNA expression. In the supernatant of RSV-infected HEp-2 cells, INF-β was detected since 12 h post infection. Maximal induction of INF-beta was at 24 h post infection (data not shown). RNA expression of SOCS-1, SOCS-3, and CIS was induced in the very early stage of infection within 1 h. Oshansky et al. (2009) reported RSV surface proteins induce SOCS-1, and SOCS-3 in the very early stage of infection via the Toll-Like Pathway (TLR) in fully-differentiated primary normal human bronchial epithelial (NHBE) cells within 15 min. There are some reports that SOCS proteins can also be induced through TLR stimulation independent of type I IFN (Baetz et al., 2004; Dalpke and Heeg, 2003; Dalpke et al., 2001; Nakagawa et al., 2002). Furthermore, RIG-I, an intracellular receptor that binds to dsRNA in the cytosol and can induce type I IFN independent of TLR signaling (Yoneyama et al., 2004), may be associated with signaling following RSV infection. These indicate

that RSV infection affect SOCS negative regulation of the type I IFN response, an attribute associated with cross-talk between the TLR and JAK/STAT pathways. Our results also indicated SOCS expression in the very early stage of infection was induced not by IFN, but by RSV surface protein.

SOCS1 binds to JAKs to inhibit catalytic activity (Nicola et al., 1999; Alexander et al., 1999). Others have found that overexpression of SOCS1 completely abolished mRNA expression of the IFN-α inducible antiviral genes 2′–5′OAS1 and MxA (Brand et al., 2005; Vlotides et al., 2004). SOCS3 inhibits JAK activation through its N-terminal domain associated with an activated receptor (Nicholson et al., 2000). Expression of SOCS3 mainly inhibits 2′–5′OAS1 mRNA transcription (Vlotides et al., 2004). The CIS gene was induced by STAT5 in response to erythropoietin and growth factor (Erickson-Miller et al., 2000; Wang and Jiang, 2005; Yoshimura et al., 1995), but how it regulates IFN signal transduction is still unclear. STAT1 is a main pathway of many interferon-stimulated genes in response to IFNs (Sledz et al., 2003; Bromberg et al., 1996). IFN-activated STAT2 contributes to IFN-inducible biological responses that are independent of IFN-stimulated gene factor 3 (ISGF3) activity by forming a heterodimer with STAT1 that will bind to chromatin and determine transcriptional activation that contributes to IFN-inducible biological responses (Brierley and Fish, 2005). Under the condition of RSV infection, inhibition of SOCS1, SOCS3 or CIS by using each siRNA independently promoted expression of STAT1/2 and their phosphory-

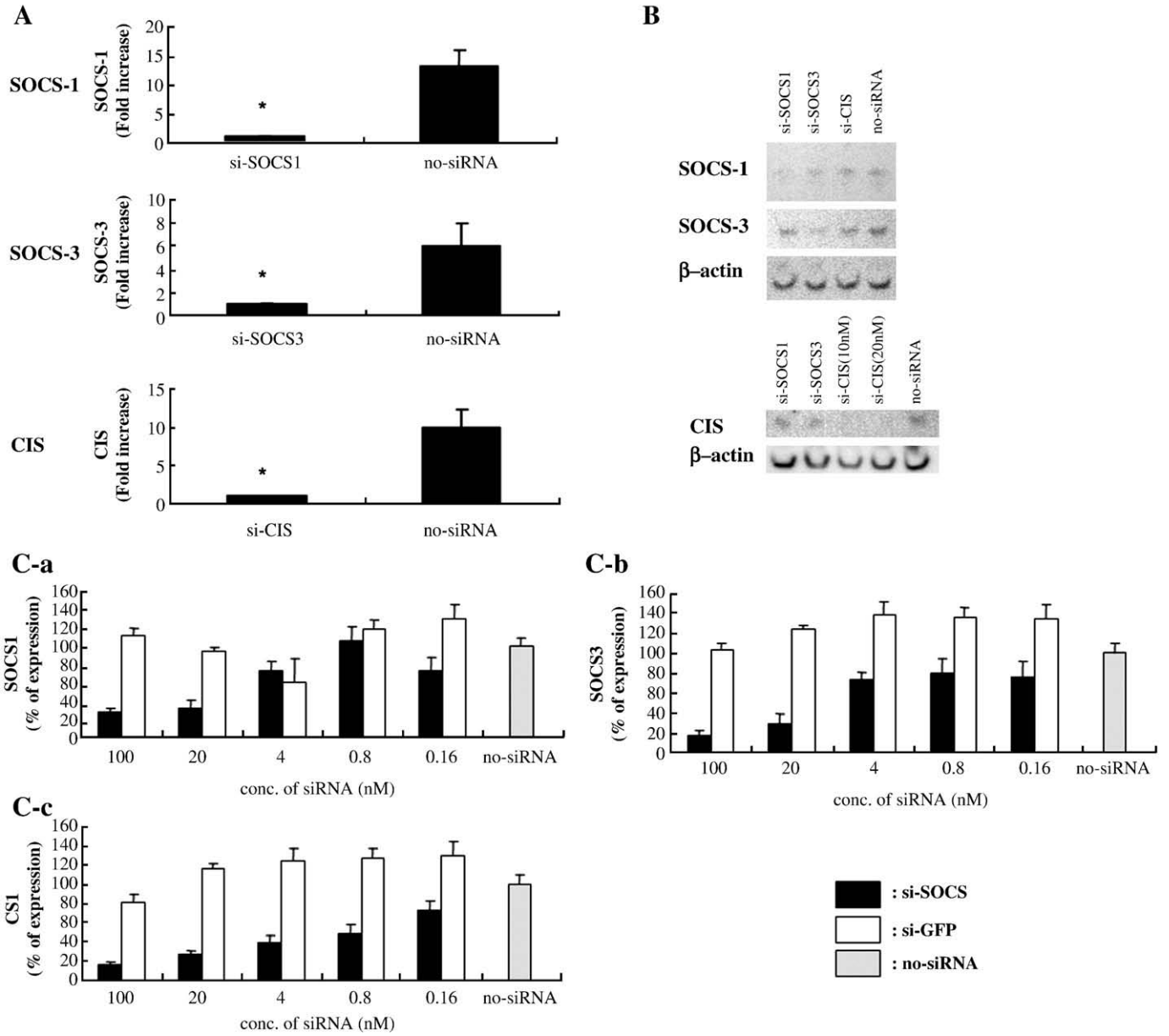

Fig. 2. The effect of si-SOCSs on SOCS1, SOCS3, and CIS gene and protein expression. (A) HEp-2 cells were transfected independently with siRNA, si-SOCS1, si-SOCS3 and si-CIS at 20 nM. After 24 h, cells were stimulated with IFNα for 1 h , RNA was then extracted and real-time RT-PCR was performed to evaluate SOCS expression. The data was then normalized using the delta/delta Ct method with expression of GAPDH. *$p<0.01$ versus no-siRNA. (B) For protein analysis, cells were treated with siRNA at 20 nM for 24 h, followed by IFNα treatment. At 6 h following IFNα treatment, cell lysates were harvested for western blotting analysis. After transferring blots, the membranes were incubated with primary antibodies (1:200 for SOCS1 and CIS, 1:100 for SOCS3, 1:10,000 for β-actin) and secondary antibodies (1:5000). (C) siRNA specific for SOCS1, SOCS3, or CIS mRNA; si-SOCS1(a), si-SOCS3(b), or si-CIS(c), was transfected into HEp-2 cells to silence these SOCS family members, and siRNA for GFP(si-GFP) was used as a control. To assess efficacy of target gene inhibition, cells were seeded on 96-well plates and treated with siRNA at indicated doses for 24 h, followed by virus infection at moi 1. At 24 h after infection, Taqman PCR was performed to quantify the mRNA level of each SOCS family members. The black bar indicated the treatment with each si-SOCS, the white bar indicated the treatment with si-GFP. Data were normalized to GAPDH mRNA levels and shown as the percentage of that of no-siRNA (gray bar). *$p<0.01$ versus si-GFP control at the same concentration.

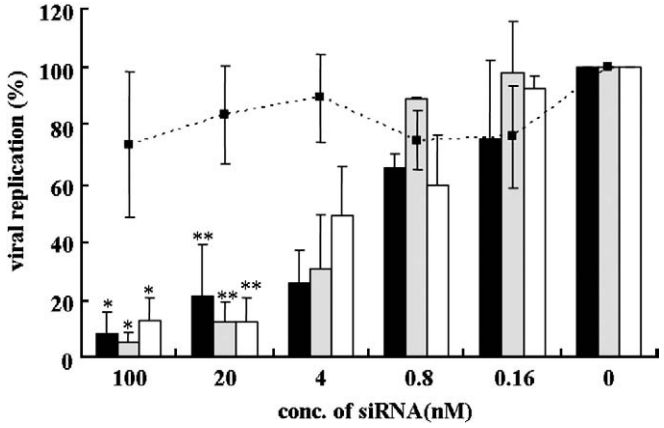

Fig. 3. The inhibitory effect of various concentrations of siRNA on RSV-replication. si-SOCSs were independently transfected into HEp-2 cells, and siRNA for si-GFP was used as a control. HEP-2 cells were seeded on 96-well plates and treated with siRNA at indicated doses for 24 h, followed by virus infection at moi 1. The virus titers that were in supernatant were determined by a plaque-forming assay 24 h post infection. The transfection with si-SOCS1, si-SOCS3, si-CIS, or si-GFP is indicated by a black bar, gray bar, white bar, or a dotted line, respectively. *$p<0.01$, and **$p<0.05$ versus si-GFP control at the same concentration.

lation, while RSV infection without si-SOCS treatment did not induce sufficient expression and phosphorylation of STAT1/2. These results indicate that SOCS1, SOCS3, and CIS block the expression and phosphorylation of STAT1/2, providing a possible mechanism for virus evasion of the host cell antiviral response.

An important clinical aspect of RSV infection is the link between severe RSV disease in childhood and the development of allergic asthma later in life (Sigurs et al., 2000). There is evidence to suggest that the ability to signal through STAT1 might be critically important in protecting against RSV-induced Th2 immune responses and airway disease. For instance, RSV infection in the absence of STAT1-dependent IFN signaling in mice fundamentally changes the immune response to RSV infection. In wild type (WT) mice on a BALB/c background, RSV infection resulted in a predominant Th1 immune response with high levels of IFN-γ in the lung. However, RSV infection in STAT1 knock-out (KO) mice of the same genetic background resulted in a mixed Th1/Th2 response with high levels of Interleukin-4, Interleukin-5, Interleukin-13, and even higher levels of IFN-γ than seen in the WT mouse (Durbin et al., 2002; Johnson et al., 2005). RSV infection increased airway obstruction, airway hyperresponsiveness and airway epithelial mucus production in STAT1 KO mice (Hashimoto et al., 2005), while these features were not present in RSV-infected WT mice. Others have also shown that inducing Type I IFN expression can prevent Th2 immune responses as a result of RSV infection. For instance, administration of cytosine phosphate-guanine-oligodeoxynucleotides (CpG-ODN) which bind TLR9 and induce production of IFN-α, β, inhibit RSV-enhanced allergic sensitization in guinea pigs by increasing lung IFN-γ/IL-5 ratios and significantly reducing airway T-cells and eosinophils (Tayyari et al., 2005). Therefore, increasing STAT1-mediated signaling by inhibiting SOCS expression might be a therapeutic target to prevent severe RSV bronchiolitis in infancy. This possibility is supported by the finding that silencing SOCS1 enhances antigen presentation by dendritic cells and augments cellular immunity with induction of IFN-γ secretion in CD8 T cells (Shen et al., 2004). Therefore, treatment with siRNA against SOCS1 might be a relevant intervention in the context of post-bronchiolitis allergic sensitization in children.

Taken together, we conclude that RSV evades the innate antiviral response by inducing SOCS1, SOCS3 and CIS in infected epithelial cells. We found that suppression of SOCS resulted in inhibition of virus replication. Meanwhile, silencing of SOCS molecules possibly provides a new anti-virus strategy in the early stage of infection by activating innate immunity to antiviral response.

Materials and methods

Cells and virus

HEp-2 cells were maintained in Eagle's minimal essential medium supplemented with glutamine, amphotericin, gentamicin, penicillin G, and 10% fetal bovine serum (10%FBS-MEM). The RSV A2 strain was originally provided by Dr. Robert Chanock at the National Institutes of Health (Bethesda, MD). Master stocks and purified virus working stocks of RSV were prepared as previously described (Graham et al., 1988; Schwarze et al., 2000) and snap frozen until use. In order to remove bioactive substances in the supernatant, ultra-centrifuged purified RSV was used in this study. Briefly, cells and supernatant were harvested, the cells were disrupted by ultrasonic manipulation, and the suspension was clarified by centrifugation (8000 ×*g*, 20 min). The supernatant was layered over 30% sucrose in STEU buffer (sodium chloride 0.1 M, Tris 0.01 M, ethylenediaminetetraacetic acid [EDTA] 0.001 M, and urea 1 M, all obtained from Sigma [St. Louis, MO]) and centrifuged (100,000 ×*g*, 1 h, 10° C). The pellet was resuspended in 1.2 ml phosphate-buffered saline (PBS), aliquoted and frozen at 70 °C (Schwarze et al., 2000). RSV stocks were titered by methylcellulose plaque assay. Inactivated virus was prepared by exposing the viral stock to 254-nm ultraviolet light for 15 min on ice. UV-inactivation RSV was verified that inactivated virus could not make CPE in HEp-2 cell, and could not replicate determined by real-time quantitative PCR.

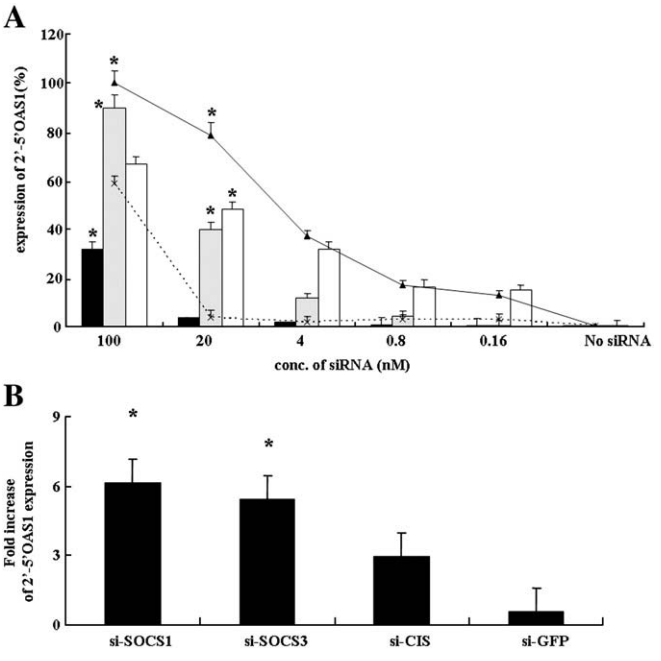

Fig. 4. Inhibition of SOCS1, SOCS3 and CIS induced the expression of IFN associated genes. (A) Effect of si-SOCS on the expression of 2′–5′OAS1 gene was compared with si-GFP in non-infected HEp-2 cells determined by real-time PCR. Poly I:C was used as a positive control (see Materials and methods). The final concentrations of poly I:C were 14 μg, 2.8 μg, 0.56 μg, 0.11 μg and 0.02 μg/100 μl/well. Data were normalized to GAPDH mRNA level and shown as a percentage of poly I:C at the highest concentration (14 μg/100 μl/well) of transfected cells (adjusted as 100%). The transfection with si-SOCS1, si-SOCS3, si-CIS, si-GFP, or poly I:C is indicated by a black bar, gray bar, white bar, dotted line, or a solid line, respectively. (B) The effect of si-SOCS on the expression of 2′–5′OAS1 gene was compared with si-GFP in RSV-infected HEp-2 cells. Cells were treated with siRNA at a concentration of 20 nM for 24 h, followed by virus infection at moi 1. 24 h post viral infection, 2′–5′OAS1 were assayed by real-time PCR. *$p<0.05$ versus si-GFP control.

Unless otherwise mentioned, virus infection was performed at a multiplicity of infection (moi) 1.

Real-time polymerase chain reaction

Taqman PCR reagents for SOCS1–7 and CIS were purchased from ABI (Applied Biosystems, Foster City, CA). To determine activation of the pathway leading to the induction of the type I IFN, expression of 2′–5′ oligoadenylate synthetase 1 (2′–5′OAS1) was examined by Interferon Response Watcher kit using the SYBR Green method (Takara, Shiga, Japan). Total cell RNA was extracted by IsoGene (NipponGene, Tokyo, Japan). One microgram of total RNA was used for the reverse transcription (RT) reaction with Supercript III (Invitrogen Life Technologies, Carlsbad, CA). Taqman PCR for SOCS1–7 and CIS was performed according to the Taqman Master Mix reagents kit protocol supplied by the manufacturer (Applied Biosystems, Foster City, CA). Thermal Cycler conditions: the reactions were incubated for 2 min at 50 °C followed by 10 min denaturation at 95 °C; then the reactions were run for 40 cycles of denaturation for 15 s at 95 °C and an extension for 1 min at 60 °C per cycle using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The real-time polymerase chain reaction (PCR) data was analyzed using the standard curve method or delta/delta Ct method. For the standard curve method, at the begining, the maximum expression of each SOCS gene in RSV-infected cell was checked. Then, standard curves in each SOCS genes were made by serial 5 times dilution with c-DNA for each SOCS gene. For RSV, GAPDH, standard curves were made in the same way. Finally, data were standardized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems, Foster City, CA), and presented as a relative value. For delta/delta Ct method, Ct value of GAPDH is used for normalization of target gene expression.

Induction of SOCS genes by IFNα

IFNα is a well known potent SOCS inducer. To induce SOCS gene or protein, HEp-2 cells were stimulated by IFNα-2b (INTRON®, Schering-Plough Co, Kenilworth, NJ) of 2000 IU/ml at the final concentration.

Target siRNA design and transfection

We designed siRNA against to SOCS1, SOCS3, CIS and GFP (Green Fluorescent Protein) mRNA using a program provided by RNAi Co. (Tokyo, Japan, http://design.rnai.jp/sidirect/index.php) (Table 1). This program can select highly effective and selective siRNA sequences using novel guidelines that were established through an extensive study of the relationship between siRNA sequences and RNA interfering activity to avoid off-target gene silencing (Naito et al., 2004). The siRNA against Green Fluorescent Proteins (GFP) was selected as a negative control for silencing human genes. One day prior to transfection, 1×10^5 or 1×10^4 of HEp-2 cells were seeded into 24-well or 96-well plates, respectively (Wu et al., 2004). Once cells were 50% confluent, siRNA was transfected into the HEp-2 cells. For siRNA transfection, cationic lipid complexes were prepared according to the instruction of Stealth™ RNAi-Lipofectamine™ 2000 (Invitrogen Co., CA). For 24-well plate, siRNA and 0.8 μl of Lipofectamine2000 were incubated in 100 μl of Opti-MEM I Reduced Serum Medium (Opti-MEM) (Gibco, Grand Island, N.Y.) at room temperature for 15 min, then added with 10% FBS-MEM without antibiotics resulting in 500 μl at the final volume. For 96-well plate, siRNA and 0.2 μl of Lipofectamine 2000 were incubated in 25 μl of Opti-MEM at room temperature for 15 min, then added with 10% FBS-MEM without antibiotics resulting in 125 μl at the final volume. The cells were transfected with the siRNA/Lipofectamine2000 complex at a volume of 400 μl/well for 24-well plates and 100 μl/well for 96-well plates.

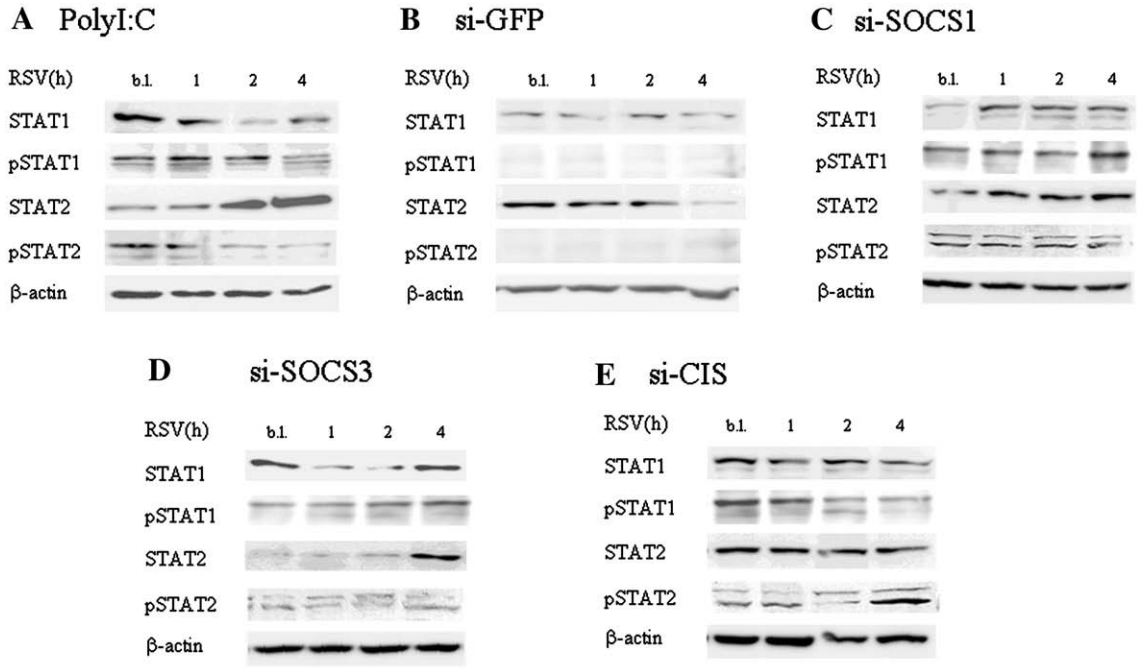

Fig. 5. si-SOCS activated STAT1/2 phosphorylation. Cells were treated with siRNA at a concentration of 20 nM for 24 h, followed by virus infection at moi 1. The basal level (b.l.) meant a non-RSV-infected cell, just before infection. At 1, 2, and 4 h following RSV infection, cell lysates were harvested for western blotting analysis. After transferring blots, the membranes were incubated with primary antibodies (1:500 for STAT1/2 and pSTAT1/2, 1:10,000 for β-actin) and secondary antibodies (1:10,000). Poly I:C (26 μg/ml in 12-well plates) (A) and si-GFP (B) were used as positive and negative controls. The immunoblotting images from cells treated with si-SOCS1 (C), si-SOCS3 (D) and si-CIS (E) were shown. The results shown are representative of three independent experiments. The data was analyzed by densitometer, then normalized to β-actin signal. The graph of total STAT1 (tSTAT1) (F), phosphorylated STAT1 (pSTAT1) (G), total STAT2 (tSTAT2) (H), and phosphorylated STAT2 (pSTAT2) (I) were shown. Data is expressed as the mean ± SE of three independent experiments. Histograms show the fold increases in densitometry of each protein compared to b.l. of si-GFP that is taken as one. Basal level comparison among group: $*p<0.05$ versus b.l. in si-GFP. Comparison within the group: $**p<0.05$ versus b.l. in the same group.

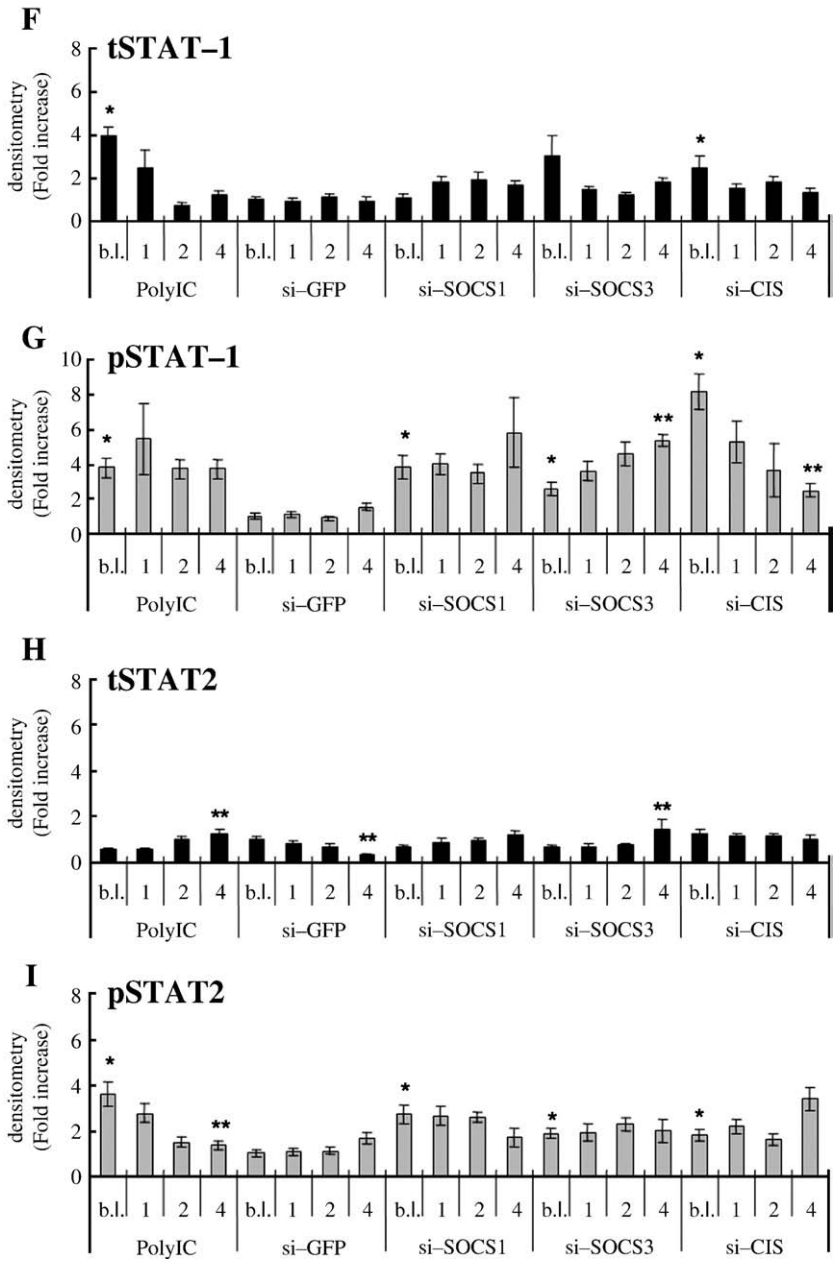

Fig. 5 (*continued*).

The final concentrations of siRNA used in the experiments were 100 nM, 20 nM, 4 nM, 0.8 nM, 0.16 nM, and 0 nM. The transfected cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h until RSV inoculation.

To determine the inhibition efficacy of the siRNA, co-transfections of pFB-hrGFP (Stratagene, La Jolla, CA) and siRNA for GFP (si-GFP) were performed using Lipofectamine 2000 according to the manufacturer's protocol. Briefly, 0.4 μg of pFB-hrGFP was co-transfected with

Table 1
siRNA duplexes against human SOCS genes and GFP.

Oligo name	Location	siRNA	Nucleotide sequence (5′–3′)	Genbank accession no.
SOCS1	800–822	Sense strand	CGCCGUGCACGCAGCAUUAAC	NM_003745.1
		Antisense strand	UAAUGCUGCGUGCACGGCGGG	
SCOC3	1334–1356	Sense strand	CCUGGUGGGACGAUAGCAACC	NM_003955.3
		Antisense strand	UUGCUAUCGUCCCACCAGGAC	
CIS	657–679	Sense strand	CCAAUGUACGCAUUGAGUAUG	NM_013324.4
		Antisense strand	UACUCAAUGCGUACAUUGGUG	
GFP	509–531	Sense strand	CUGAACAGCGGCAAGUUCUAC	AY613996
		Antisense strand	AGAACUUGCCGCUGUUCAGGC	

20 nM si-GFP to the HEp-2 cells. Forty-eight hours after transfection, the cells were observed under fluorescence microscopy (Zeiss, Oberkochen, Germany) and the transfection efficiency was determined.

Western blot analyses

Cells were lysed in a buffer [20 mM Tris–HCl (pH 7.4), 0.5% sodium deoxycholate, 10% glycerol, 150 mM NaCl, 2 mM Ethylene Diamine Tetra-Acetic acid (EDTA), 50 mM β-glycerophosphate, 2 mM Na_3VO_4, 10 mM NaF, 1 mM Dithiothreitol (DTT), 1 mM Phenylmethylsulfonyl Fluoride (PMSF), and 0.1% protease inhibitor cocktail (Roche, Penzberg, Germany)] and incubated for 30 min on ice, then centrifuged at 15,000 rpm for 15 min. The protein concentrations of the supernatant were determined with DC protein assay kit (Bio-Rad, Hercules, CA). Ten micrograms of protein or 20 μg of protein were applied into each well to analyze STAT protein or SOCS protein, respectively. The sample was electrophoresed on a 4.75% stacking/10% separating sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidine difluoride (PVDF) membrane (Immobilone; Millipore, Schwalbach, Germany). The membranes were blocked with 5% nonfat milk powder in 50 mM Tris–HCl (pH 7.6), 0.15 M NaCl, and 0.1% Tween 20 (TBS-T) for 1 h, then incubated with the following primary monoclonal (mAb) or polyclonal (pAb) antibodies: goat anti-human SOCS1 pAb (sc-7005, Santa Cruze, CA), rabbit anti-human SOCS3 pAb (C204, IBL, Takasaki Japan), goat anti-human CIS pAb (sc-1529, Santa Cruz, CA), mouse anti-human STAT1 and STAT2-pAb (Santa Cruz, CA), rabbit anti-human p-STAT1 and p-STAT2-pAb (Santa Cruz, CA), and mouse anti-β-actin mAb (Sigma, Saint Louis, MO) in dilution buffer with agitation at 4 °C overnight. After washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h. Protein bands were visualized using ECL plus Western detection reagents (Amersham-Pharmacia Biotech, Buchinghamshire, England), and imaged with Image Reader LAS-3000 (Fuji Film Tokyo, Japan), then the density of each band was measured using the program, NIH image.

2′–5′ OAS-1 gene expression analysis

The HEp-2 cells were seeded into 96-well plates at a density of 1×10^4/well. Total RNA was extracted from the following two groups at either: a) 48 h after transfection, or b) 24 h post RSV infection which occurred 24 h after siRNA transfection. Polyinosine–polycytidylic acid (poly I:C) (Takara Co., Shiga, Japan) was selected as a positive control as an inducer of type I IFN and IFN-inducible genes. The final concentrations of poly I:C were 14 μg, 2.8 μg, 0.56 μg, 0.11 μg and 0.02 μg/100 μl/well. Expression of 2′–5′OAS1 mRNA was quantified by real-time PCR with SYBR Green I as described above.

Enzyme-linked immunoassays

Culture supernatants were harvested from culture media in duplicates. IFN-β concentration was determined using a commercial sandwich enzyme-linked immunosorbent assay kit (PBL Biomedical Laboratories, Piscataway, NJ).

Statistics

The results were expressed as mean ± standard error (SE). The paired Student's *t*-test was used to determine differences between groups and these were deemed to be significant if $p<0.05$.

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