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Efficient Human Cytomegalovirus Replication in Primary Endothelial Cells Is SOCS3 Dependent

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Keywords
SOCS3 · Human primary endothelial cells · Infection · Human cytomegalovirus

Abstract
Background: In immunocompromised patients, human cytomegalovirus (HCMV) infection is a major cause of morbidity and mortality. Suppressor of cytokine signaling (SOCS) proteins are very potent negative regulators of the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways. We hypothesized that HCMV exploits SOCS1 and/or SOCS3 to its advantage. ***Methods:*** All experiments were carried out with primary human lung-derived microvascular endothelial cells (HMVEC). SOCS1 and SOCS3 were silenced by transfecting the cells with siRNA. HCMV was propagated and titered on human lung-derived fibroblasts MRC5. Real-time PCR and Western blot were used to detect mRNA and protein levels, respectively. ***Results:*** The data presented show that an efficient replication of HCMV in HMVEC is dependent on SOCS3 protein. Time course analysis revealed an increase in SOCS3 protein levels in infected cells. Silencing of SOCS3 (siSOCS3) resulted in inhibition of viral immediate early, early, and late antigen production. Consistently, HCMV titers produced by siSOCS3 cultures were significantly decreased when compared to control transfected cultures (siCNTRs). STAT1 and STAT2 phosphorylation was increased in siSOCS3-infected cells when compared to siCNTR-treated cells. ***Conclusion:*** These findings indicate the implication of SOCS3 in the mechanism of HCMV-mediated control of cellular immune responses.

Introduction

Suppressor of cytokine signaling (SOCS) proteins are very potent negative regulators of the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways [1]. Eight members of this family have been reported, SOCS1–SOCS7 and the cytokine-inducible SH2 domain-containing protein [2–5]. SOCS proteins play an important role in maintaining and restoring homeostasis upon cytokine stimulation [1]. They consist of 3 main domains: the N-terminal domain variable in length and sequence; a central SH2 domain, and a C-terminal SOCS

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box domain [5]. The SH2 domain binds to the phosphorylated target proteins whereas the SOCS box binds to the ubiquitin machinery allowing for polyubiquitination and, consequently, degradation of the target proteins in the proteasome. SOCS1 and SOCS3 also contain a kinase inhibitor region which can directly inhibit the kinase activity of the target proteins [1, 6].

The interferons (IFNs) represent a tightly regulated JAK/STAT-dependent defense system against viral infections [7]. SOCS1 and SOCS3 have been investigated for their particular ability to attenuate IFN-mediated responses to viral infections [8]. A number of viruses exploit SOCS1 and SOCS3 [6, 9–12], including members of the Herpesviridae, such as herpes simplex virus (HSV-1) [10–12] and Epstein-Barr virus (EBV) [9]. Upregulation of SOCS1 and SOCS3 proteins by these viruses correlates with an enhanced infectivity and replication capacity, mainly by attenuating the activation and the production of type I IFN, although not exclusively [6].

Human cytomegalovirus (HCMV) is a member of the Herpesviridae family. Although infection with this virus is asymptomatic in immunocompetent individuals, it leads to life-long latency, and seroprevalence in adults reaches 80%. In neonates and immunocompromised patients, including transplant recipients, primary infection or reactivation of this virus is a major cause of morbidity and mortality [13].

HCMV interference with type I and II IFN activation as a means of immune evasion occurs at different stages. The mechanisms are highly dependent on the virus strain and on the cell type of the model investigated [14]. In the well-characterized model of fibroblasts infected with the HCMV Towne strain, interference of type II IFN responses occurs through several mechanisms. Inhibition of STAT1 phosphorylation by src homology region 2 domain-containing phosphatase (SHP2) takes place about 16 h postinfection (p.i.) [15] followed by JAK1 disruption 72 h p.i. [16]. As JAK1 is a shared component of type I and type II IFN pathways, the disruption of this protein affects both pathways. HCMV also targets p48, another key component of the type I IFN pathway [17]. HCMV can inhibit type I IFN-dependent expression of major histocompatibility complex class I, IFN-regulatory factor-1, myxovirus resistance protein A, and 2,5-oligoadenylate synthetase gene expression in fibroblasts and human umbilical vein endothelial cells (HUVEC) [14, 17], although some of these findings are in conflict with others reporting an upregulation of the latter genes in HCMV-infected fibroblasts [18–22]. A virally mediated decrease in STAT2 phosphorylation followed by a reduction in the total amount of this protein was shown in HCMV-infected fibroblasts. Degradation of STAT2 was found to be strain dependent and was not observed for Towne virus [23].

Most of the studies published so far on HCMV infection of adherent cells were carried out in fibroblasts or HUVEC. Being at the interface between blood and tissue, the endothelium has been shown to be a strategic natural site of infection for HCMV in vivo following a primary infection [24–26], and it is believed to act as a viral reservoir [27] involved in viral spread and persistence.

We hypothesized that HCMV exploits SOCS1 or SOCS3 proteins to efficiently replicate in endothelial cells. Since the lung endothelium has been reported to be productively infected during acute HCMV infection in humans [26], we chose to work with primary human lung-derived microvascular endothelial cells (HMVEC) for this study.

To date, only 2 publications have linked the modulation of SOCS expression and HCMV [28, 29]. The studies were carried out in monocytes [28] or monocyte-derived dendritic cells [29]. In both cases, upregulation of SOCS3 correlated with the control of cytokine expression and was thus at least partially involved in a HCMV-mediated mechanism of immunosuppression. In this study, we show for the first time that silencing of SOCS3 leads to inhibited replication of HCMV in HMVEC, and we provide evidence of the implication of SOCS3 in the HCMV-induced inhibition of STAT2 phosphorylation. Based on these findings, we suggest a key role for SOCS3 in the process of HCMV replication in endothelial cells.

Methods

Cells and Viruses

All experiments were carried out with primary HMVEC (Lonza, Basel, Switzerland) between passages 4 and 8, cultured in complete EGM-2 medium (Lonza).

For all experiments, the HCMV strain TB40/E [30], kindly provided by Dr. Christian Sinzger (University of Ulm, Germany), was used. The virus was propagated on human lung-derived fibroblasts MRC5 [31] (LGC Standards, Teddington, UK) cultured in Dulbecco's modified Eagle's medium (Invitrogen, Basel, Switzerland) containing 10% fetal calf serum (A15–101, lot: A10110–2432, PAA) and 2 mM L-glutamine (Invitrogen). Purification of HCMV was carried out by ultracentrifugation over a 15% sucrose cushion (50 mM Tris-HCl, 12 mM KCl, 5 mM Na_2EDTA) at 20,000 *g* for 90 min at 4 °C using an SS-34 rotor (Beckman Coulter, Fullerton, CA, USA). The infectious titers of the stock virus and of the supernatants collected 5 days p.i. were determined by TCID50 assays on MRC5 [32]. For reference, the TCID50s determined for Figure 3d inocula at a multiplicity of infection (MOI) of 1 were 3.9 for siSOCS1 and siCNTR and 2.7 for siSOCS3, which correspond to 7,943 and

501 infectious particles/mL respectively. As control, HCMV was ultraviolet (UV) inactivated (5 min, 30 cm distance from a 30-W, 230-V, 50-Hz UV lamp [Osram, Winterthur, Switzerland]) [33].

HMVEC were infected with either MOI1 or MOI5, as indicated. If not otherwise specified, the infection rate was determined 1 day p.i. by immunofluorescence, as previously described [34], employing an antibody against immediate early (IE) 52-, 72-, and 86-kDa proteins (Ref. 11-003; Argene, Varhiles, France). All cell preparations were tested negative for mycoplasma by 4,6-diamidino-2-phenylindole (D9542; Sigma, Buchs, Switzerland) staining.

In stimulation experiments, cells were incubated for 5 h with either 100 U/mL IFNγ (PeproTech, Rocky Hill, NJ, USA) or 500 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma). All experiments presented in this study were independently repeated at least 2 times; more details about the replicates are mentioned in the figure legends.

Real-Time PCR

Total RNA from cells was isolated using the RNeasy mini kit (Qiagen, Hombrechtikon, Switzerland) with a DNase (RNase-Free DNase Set; Qiagen) treatment step. For reverse transcription of the mRNA, superscript III reverse transcriptase (cat. No. 18080-093; Invitrogen) was used following the manufacturer's instructions. cDNA was analyzed with TaqMan gene expression assays (Applied Biosystem) for SOCS1 (assay ID: Hs00705164_s1), SOCS3 (assay ID: Hs00269575_s1), and GAPDH (assay ID: Hs9999905_m1) using HotStarTaq master mix (Qiagen) supplemented with $MgCl_2$ (1.5 μM). Amplification of cDNA was performed with a real-time thermocycler (iQ5 Cycler; Bio-Rad, Reinach, Switzerland) as follows: 15 min at 95 °C and 45 cycles for 15 s at 95 °C and 60 s at 60 °C. GAPDH was used as housekeeping gene [35] to normalize results; data were analyzed using the iQ5 Optical System Software (Bio-Rad), and results are reported as relative expression levels compared to the untreated controls.

Western Blot

Western blot analysis of SOCS1 (#3950; Cell Signaling), SOCS3 (sc-51699; Santa Cruz Biotechnology, Heidelberg, Germany), STAT1P (Tyr701) (sc-136229; Santa Cruz Biotechnology), STAT1 (sc-346; Santa Cruz Biotechnology), STAT2P (Tyr690) (#4441; Cell Signaling), STAT2 (sc-476; Santa Cruz Biotechnology), β-actin (A5441; Sigma), and of the IE (52-, 72-, and 86-kDa proteins; Ref. 11-003, Argene), early (E) (ICP36, CA006-100; Virusys, Taneytown, MD, USA), late (L) (pp65, vp-c 422; Vector Laboratories, Burlingame, CA, USA) viral antigens were carried out as follows. Cell lysates were prepared by adding 50 μL of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Tween 20, 1 mM EDTA, 0.1% SDS, and protease inhibitor; Calbiochem) to the monolayers and collected allowing lysis for 30 min. Protein concentrations were determined by the NanoDrop 1000 spectrophotometer. All steps were carried out strictly on ice until SDS sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.1% bromophenol dye) was added to the samples which were then boiled at 95 °C for 5 min. Equal amounts of protein from cell lysates were separated by gel electrophoresis and transferred to a polyvinyl membrane (GE Healthcare, München, Germany). After blocking with 5% milk powder (Rapilait; Migros, Switzerland) in 1× Tween 20/Tris-buffered saline (0.1% Tween 20, 25 mM Tris-HCl, pH 7.5, and 150 mM NaCl) for 2 h at room temperature, membranes were incubated with the primary antibody overnight at 4 °C, followed by incubation with an anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (GE Healthcare) for 1 h. The target proteins were detected by enhanced chemiluminescence using the ECL reagent (GE Healthcare). Where indicated, signals were quantified with the ImageJ analysis software.

Cell Transfection

Small interfering RNA (siRNA) targeting SOCS1 mRNA (HSS189479, stealth RNAi, Invitrogen, was used in the experiment shown, and sc-40996, Santa Cruz, was used to confirm the results in repeat experiments) or SOCS3 mRNA (HSS113313, stealth RNAi, Invitrogen, was used in the experiment shown, and sc-41000, Santa Cruz, to confirm the results in repeat experiments) and a scrambled nonspecific sequence (CNTR-D or A sc-44232 and sc-37007, respectively, Santa Cruz) were transfected using Lipofectamin RNAiMax (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded in a 24-well plate; 50 pmol of siRNA were mixed with 1.5 μL Lipofectamin RNAiMax in 100 μL OptiMEM (Invitrogen) and incubated for 10 min at room temperature before adding the mixture to the cells. Except for online suppl. Figure 2 (for all online suppl. material, see www.karger.com/doi/10.1159/000501383), where cells were transfected 1 h p.i., cells were always transfected 1 day before infection.

Flow Cytometry

Cells were harvested by trypsin (Invitrogen) treatment. For IE staining, cells were fixed and permeabilized following the BD Cytofix/Cytoperm™ protocol (Becton-Dickinson), and then incubated with Alexa Fluor 488-conjugated IE1 mAb (8B1.2; Merck Millipore, Darmstadt, Germany). The Alexa Fluor-conjugated mouse IgG (Merck Millipore) was used as an isotype-matched control. Fluorescence intensity was measured on a FACSCanto apparatus (BD Biosciences, Basel, Switzerland), and analysis was performed using the FlowJo software (Tree Star, Ashland, OR, USA).

Availability of Data and Materials

The datasets generated and analyzed during the current study are presented within the paper. Additional information is available from the corresponding author on reasonable request.

Statistical Analysis

The significance of TCID50 values was calculated with one-way ANOVA followed by Tukey's multiple comparison test to compare all data sets; with $*p < 0.05$ and $**p < 0.002$. Statistical analyses were performed using GraphPad Prism v8.

Results

SOCS1 and SOCS3 Are Modulated by HCMV Infection in HMVEC

A time course analysis of SOCS1 and SOCS3 mRNA expression in HMVEC was carried out in cells infected with HCMV at MOI5. The transcription of SOCS1 was increased within 7–12 h p.i. (Fig. 1a), whereas SOCS3

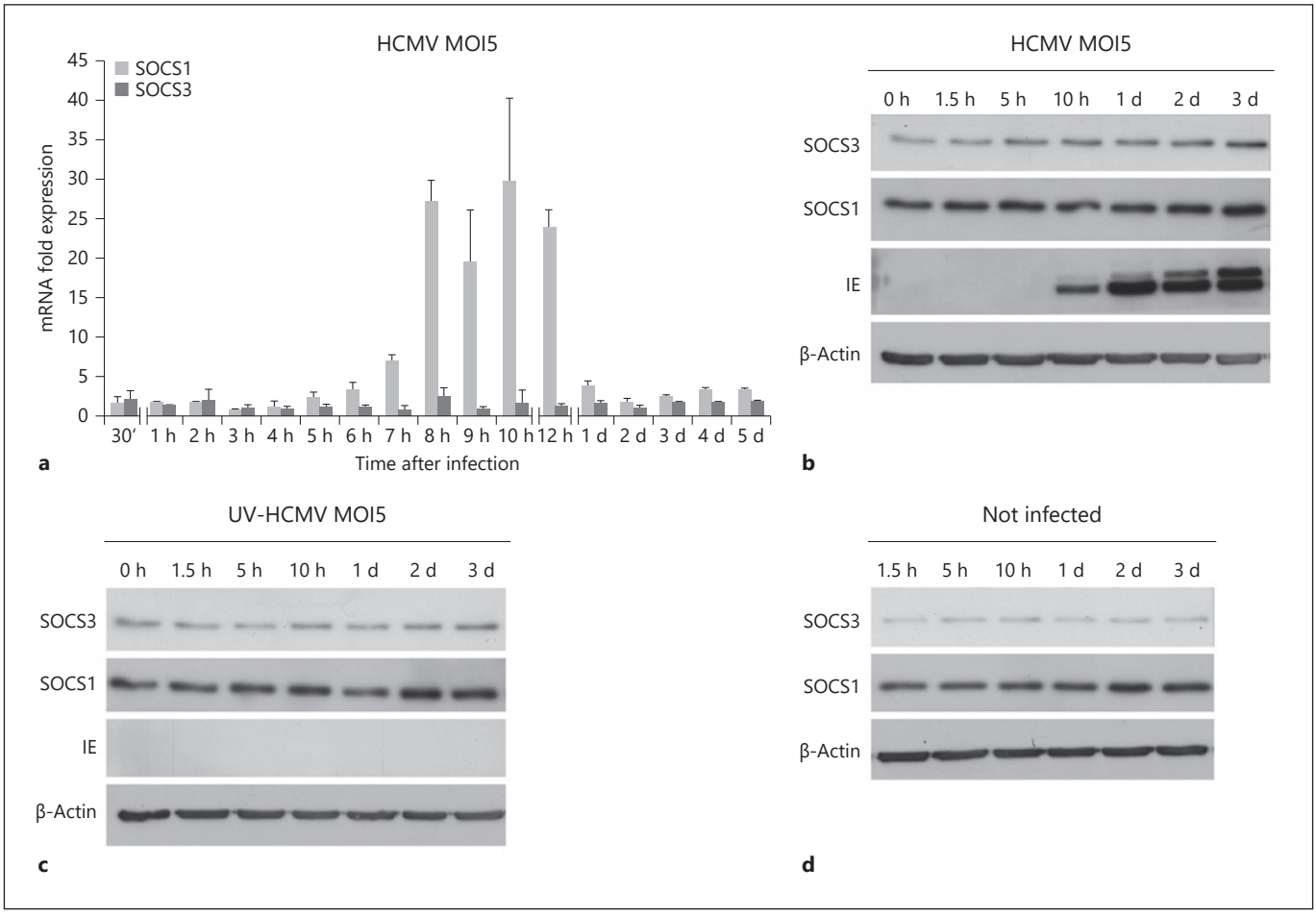

Fig. 1. HCMV infection of HMVEC induces SOCS1 mRNA expression and SOCS3 protein increase. Time course analysis of HCMV-infected HMVEC shows the transcription pattern of SOCS1 and SOCS3 in infected cells. mRNA expression levels are given as fold induction related to the noninfected control and normalized to GAPDH expression. Shown is the mean and standard deviation (SD) of duplicates in 1 representative out of 3 independent experiments (**a**). Western blot analyses of HCMV-infected (**b**), UV-inactivated HCMV-infected (**c**), or non-infected (**d**) HMVEC show the expression patterns of SOCS1 and SOCS3 proteins.

transcription levels were not modulated. Analysis of SOCS1 and SOCS3 protein levels in HCMV-infected HMVEC revealed an increase in SOCS3 protein that started between 5 and 10 h p.i. (Fig. 1b) and continued over time at least until 5 days p.i. (data not shown). In contrast, a constant level of SOCS1 protein was observed suggesting no infection-dependent modulation of this protein. The increase in SOCS3 was dependent on active viral replication since it was not observed in cells infected with UV-inactivated virus (Fig. 1c) or in noninfected cells (Fig. 1d). These results demonstrate a modulation of SOCS1 mRNA and SOCS3 protein levels in HCMV-infected HMVEC and indicate a potential active involvement of these cellular factors in the viral replication process.

Modulation of SOCS1 and SOCS3 in HMVEC Stimulated with IFNγ

The pattern of mRNA and protein expression of SOCS1 and SOCS3 in HMVEC was assessed after stimulation with IFNγ, a strong inducer of SOCS1 and SOCS3 expression. The stimulation resulted in an increased mRNA transcription of these genes, reaching the highest levels 5 h poststimulation (p.s.) for SOCS1 and 1 h p.s. for SOCS3 (Fig. 2a). The increase in mRNA transcription was paralleled by an augmented protein expression of SOCS3, but not of SOCS1 protein, which, in contrast, appeared to be reduced by the stimulus (Fig. 2b). The same pattern was observed in HMVEC stimulated with PMA despite an even stronger upregulation of SOCS1 mRNA expression (online suppl. Fig. 1A and B). Removal of the

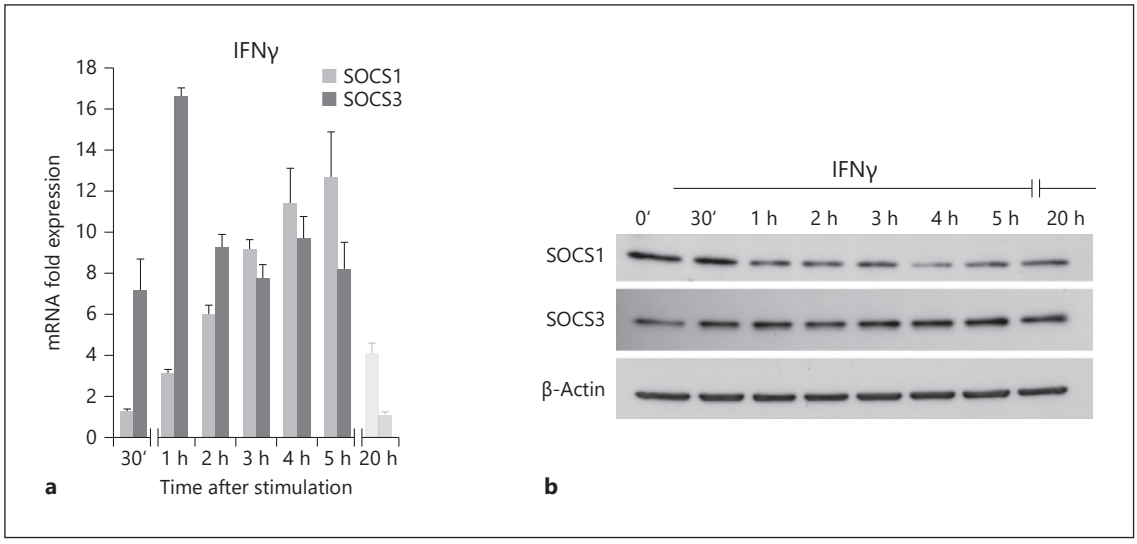

Fig. 2. Modulation of SOCS1 and SOCS3 in stimulated HMVEC. Time course analysis of HMVEC stimulated with 100 U/mL IFNγ is shown for SOCS1 and SOCS3 mRNA (**a**) and protein (**b**) expression. mRNA levels are given as fold induction related to the untreated control and normalized to GAPDH expression (**a**). Shown is the mean and SD of duplicates in 1 representative out of 3 independent experiments. **b** Western blot analysis of SOCS1 and SOCS3 protein expression. At 5 h p.s., cells were washed and incubated in normal medium for 15 additional hours.

stimulus after 5 h followed by culture in normal medium for an additional 15 h reversed the expression of SOCS1 and SOCS3 mRNA and proteins to the baseline level (Fig. 2; online suppl. Fig. 1).

Silencing of SOCS3 Leads to Impaired HCMV Replication in HMVEC

To investigate the role of SOCS1 and SOCS3 in the context of HCMV infection, HMVEC were transfected with siRNA targeting SOCS1 (siSOCS1) or SOCS3 (siSOCS3), or with a scrambled, nonspecific sequence as negative control (siCNTR). HMVEC transfected with either siSOCS1 or siSOCS3 displayed a 40% reduction in the respective target protein when compared to transfection with siCNTR (Fig. 3a). Analysis of infected samples collected 2 (Fig. 3b) or 5 (Fig. 3c) days p.i. revealed a major inhibition of viral antigen production in siSOCS3 cells. Both the E and L viral proteins were particularly affected by SOCS3 silencing.

Titration of cell culture supernatants collected 5 days p.i. showed a significant reduction in the number of infectious particles produced by siSOCS3 cultures compared with siSOCS1 and siCNTR cultures for both MOI1 and MOI5 (Fig. 3d). Interestingly, analysis of IE-expressing cells by immunofluorescence revealed that the percentage of infected cells 1 day p.i. was similar in all 3 culture conditions (Fig. 4a). Further analysis by flow cytometry confirmed that the percentage of IE-expressing cells did not differ between siSOCS1-, siSOCS3-, and siCNTR-treated cells 3 days p.i. (Fig. 4b–d).

The effect of SOCS3 silencing was more prominent during the late phase of infection with a stronger effect on the production of E and particularly L antigens. We obtained a similar reduction in both L viral antigen production and viral titers in supernatants when cultures were transfected with siSOCS3 1 day before infection (Fig. 3b–d) or immediately after infection (online suppl. Fig. 2). These results further point to an active involvement of SOCS3 during HCMV replication in HMVEC.

SOCS3 Silencing Leads to Increased STAT1 and STAT2 Phosphorylation in HCMV-Infected HMVEC

To evaluate the ability of HCMV to control the antiviral cellular defense in HMVEC, analysis of the phosphorylation level of 2 key transcription factors of the type I IFN pathway (STAT1 and STAT2) was carried out. Phosphorylation of STAT1 and STAT2 was induced early upon infection and decreased thereafter, whereas STAT1 and STAT2 protein levels were higher in infected than noninfected cells (Fig. 5a). Analysis of the phosphorylation pattern of STAT1 and STAT2 of infected cultures transfected with the different silencing constructs

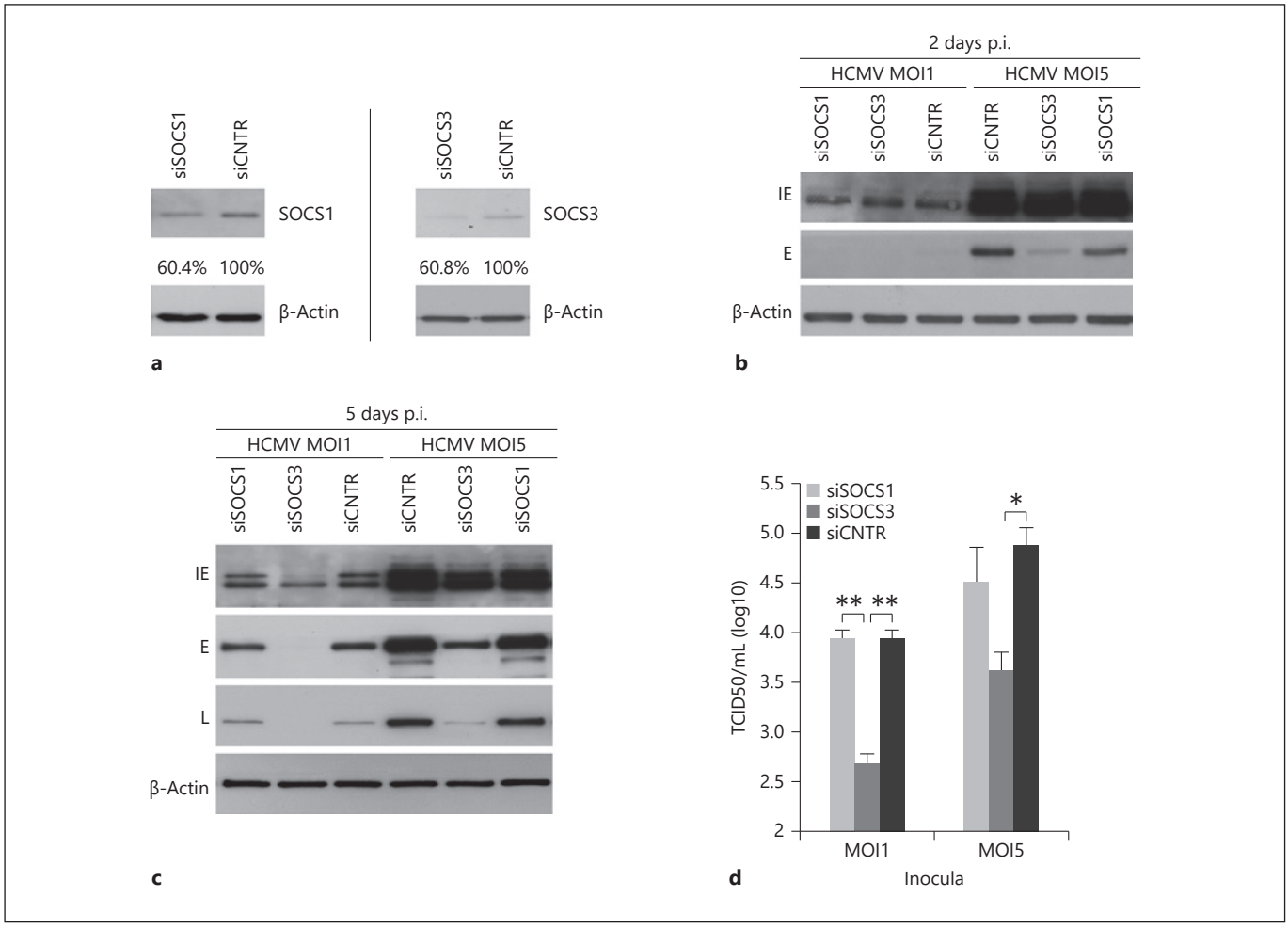

Fig. 3. HCMV infection of HMVEC is impaired upon silencing of SOCS3. HMVEC were transfected with siSOCS1, siSOCS3, and siCNTR, resulting in a reduction in target protein expression of about 40% for both siSOCS1 and siSOCS3 cultures as compared to siCNTR (**a**). Western blot analysis shows the expression pattern of IE, E, and L viral antigens in the 3 conditions (siSOCS1, siSOCS3, and siCNTR) 2 days p.i. (**b**) and 5 days p.i. (**c**). **d** Titration of supernatants collected 5 days p.i. in siSOCS1-, siSOCS3-, and siCNTR-infected cultures for both MOI1 and MOI5 inocula. Means and SD of 2 independent experiments (* $p < 0.05$, ** $p < 0.002$).

(siSOCS1, siSOCS3, or siCNTR) 2 and 5 days p.i. (Fig. 5b and c, respectively) revealed that at both time points, phosphorylation of STAT1 reached the highest level in siSOCS3 followed by siSOCS1 cells, whereas in siCNTR cultures STAT1P level was remarkably lower. STAT2 phosphorylation was increased 1.64- and 1.70-fold in siSOCS3 cultures 2 and 5 days p.i., respectively, compared with the siCNTR cultures. Phosphorylation of STAT2 in siSOCS1 cultures did not differ from siCNTR cultures (Fig. 4b, c).

At 2 day p.i., the total amount of STAT2 protein was increased 1.4 times in siSOCS3 cultures compared to siCNTR. At the same time point, the protein expression level of STAT2 in siSOCS1 cultures was slightly higher (1.2-fold) than siCNTR. Interestingly, STAT2 protein expression was similar in both siSOCS1 and siSOCS3 cultures 5 days p.i. (1.3-fold) compared to the siCNTR cultures, although phosphorylation took place only in the siSOCS3 culture.

These results indicate the induction of an antiviral mechanism in HCMV-infected cultures through inhibition of SOCS3 expression.

Discussion

In this study, we demonstrated that HCMV requires SOCS3 protein for an efficient replication in HMVEC. Silencing of SOCS3 but not SOCS1 markedly reduced the

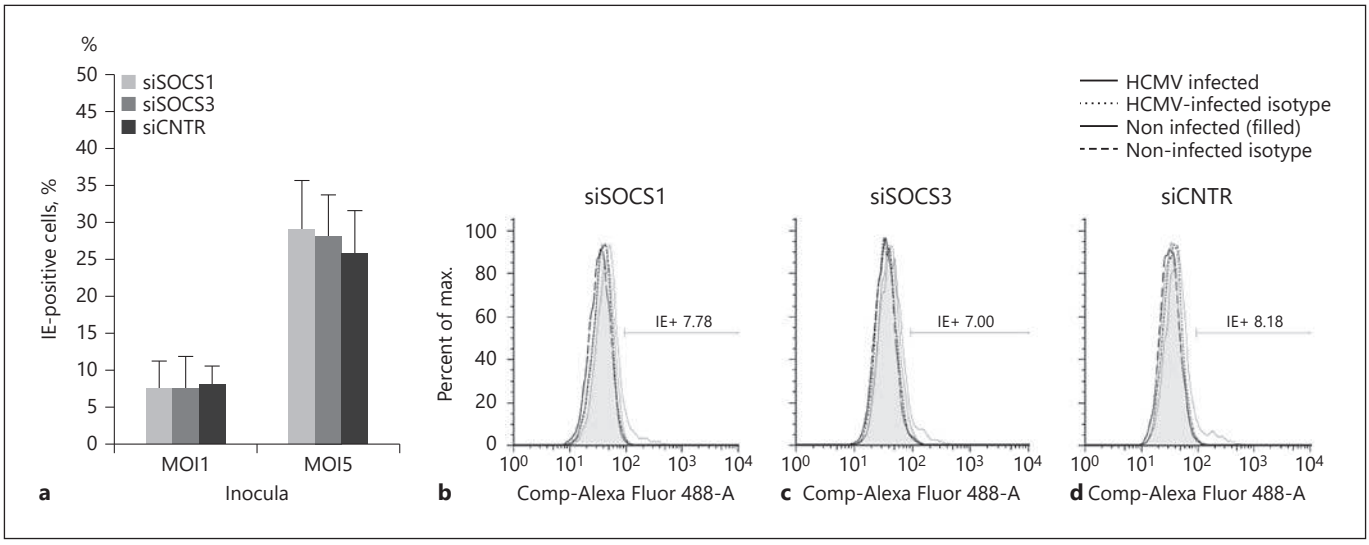

Fig. 4. Percentage of IE-expressing cells is the same in siSOCS1, siSOCS3, and siCNTR cultures 1 and 3 days p.i. HMVEC transfected with siSOCS1, siSOCS3, or siCNTR were infected with HCMV at either MOI1 or MOI5. Percent infected cells 1 day p.i. based on IE staining and immunofluorescence analysis (**a**). Bars represent the mean and SD of duplicates in 1 representative out of 3 experiments. Flow-cytometric analysis of IE expression 3 days p.i. in cells inoculated with MOI1 is shown for siSOCS1 (**b**), siSOCS3 (**c**), and siCNTR (**d**) cultures.

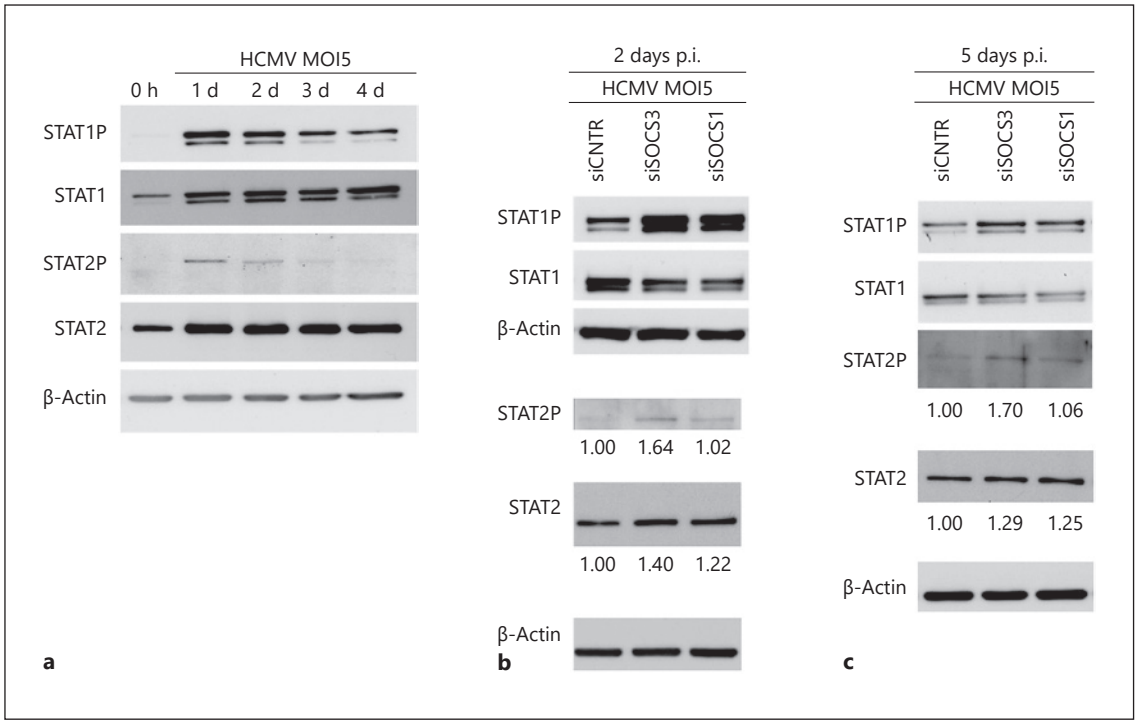

Fig. 5. Increased phosphorylation of STAT2 in siSOCS3 HCMV-infected HMVEC. Time course analysis of HCMV-infected HMVEC shows the phosphorylation pattern and total amount of STAT1 and STAT2 (**a**). HMVEC transfected with siSOCS1, siSOCS3, or siCNTR were infected with HCMV. Shown is the phosphorylation level and total protein levels of STAT1 and STAT2 at either 2 days p.i. (**b**) or 5 days p.i. (**c**). The numbers below the Western blots indicate the fold changes in the protein or phosphorylation levels of STAT2, with siCNTR normalized to 1.00.

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replication capacity of HCMV. This observation adds a new evasion mechanism to the armamentarium of HCMV. SOCS3 mRNA transcription was not modulated by HCMV, but a constant increase in the protein was observed during infection. This increase was dependent on active replication of the virus since it was not observed in UV-inactivated HCMV-infected cultures, where HCMV can enter the cell but is replication incompetent. Modulation of the half-life of SOCS3 has been shown to be dependent on the binding of the ubiquitin machinery to the SOCS box with considerable extension of the half-life of the protein through stabilization of the complex [36, 37]. Moreover, SOCS3 is the only SOCS protein containing a 35 amino acid [38] (PEST: proline [P], glutamate [E], aspartate [D], serine [S], and threonine [T]) motive, which has been described to be recognized as a site for protein degradation [39]. For SOCS3, the PEST motive was reported to be responsible for the cellular turnover of this protein. In fact, removing PEST drastically increased the half-life of SOCS3 without compromising its activity [38]. The exact mechanism behind the observed SOCS3 increase in our model remains open and may involve inhibition of protein degradation. We suggest that SOCS3 may be stabilized by the infection through binding, by viral or virally modulated cellular factors, of the key regions (i.e., PEST, SOCS box-ubiquitin machinery complex) involved in turnover regulation of the protein, resulting in an elongated half-life and, therefore, in an increase in SOCS3 in infected cells.

A time course analysis of SOCS1 expression in HCMV-infected cells revealed an upregulation of SOCS1 mRNA transcription between 7 and 12 h p.i., whereas no modulation of SOCS1 was observed at the protein level. This apparent discrepancy between the mRNA and the protein expression of SOCS1 was also found in IFNγ-stimulated HMVEC. A similar phenomenon has been reported for vesicular stomatitis virus (VSV). Cells infected with VSV exhibited an upregulation of SOCS1 mRNA, but protein levels were decreased [40]. This observation was attributed to the negative posttranscriptional regulation of SOCS1 translation by microRNA-155 (miR-155). The relevance of miR-155 as a regulator of SOCS1 has been published by several groups [40–43]. Expression of miR-155 is induced by toll-like receptor (TLR) signals (TLR2, TLR3, TLR4, and TLR9) and by various types of cellular stimulation (IL-1, TNFα, PMA, IFNβ, and IFNγ), indicating that this SOCS1 modulator is expressed in the context of inflammation [44–48]. Moreover, EBV and Kaposi's sarcoma-associated herpes virus, 2 members of the herpesvirus family, were found to induce expression of miR-155 [49] or to directly express an ortholog of miR-155 [50], respectively. Hypothetically, a similar mechanism may be responsible for our observations of SOCS1 mRNA and protein expression in HMVEC.

The importance of SOCS1 and SOCS3 for HCMV replication in HMVEC was assessed by silencing experiments. Upon silencing of SOCS3, HCMV showed a limited replication capacity in HMVEC. Here, a modulation of E and L viral antigen production was observed, showing a strong decrease in L antigen and a partial reduction in E antigen, whereas IE antigen production was not much impacted. In accordance, the HCMV titers of supernatants collected from siSOCS3 cultures were significantly reduced in comparison with siSOCS1 and siCNTR cultures, further indicating a profound involvement of SOCS3 in the viral replication process. Together, these results suggest SOCS3 plays a role relatively late during infection. In line with this, cells transfected immediately after infection also showed a similar reduction in both viral antigens and infectious viral particles produced. Moreover, the ratio of infected cells detected 1 and 3 days p.i., as based on IE detection, was the same in all the 3 conditions (siSOCS1, siSOCS3, and siCNTR).

These results reinforce the indication that SOCS3 plays a role only later after infection, since the virus can enter and infect the same number of cells in all 3 conditions.

Analysis of the activation pattern of infected cultures revealed an increase in STAT1 phosphorylation for siSOCS1 and, especially, for siSOCS3 cultures as compared to siCNTR. It has been published that STAT1 phosphorylation is controlled by the virus through SHP2 [15]. Indeed, the fact that in all 3 conditions (siSOCS1, siSOCS3, and siCNTR), STAT1P decreased between 2 and 5 days p.i. suggests that the virus can at least partially control the phosphorylation of STAT1 independently from SOCS1 and SOCS3. Nevertheless, the phosphorylation of STAT1 in siSOCS3 cultures was always higher when compared to the other cultures despite the limited silencing level obtained in these cells. This indicates that, in our setting, SOCS3 may at least partially contribute to the virally mediated process of STAT1 phosphorylation control.

We only observed an increased phosphorylation of STAT2 in HCMV-infected cultures that were treated with siSOCS3. It was previously shown that HCMV can inhibit the phosphorylation of STAT2 in TB40/E-infected fibroblasts and induce degradation of STAT2 protein [23]. In our setting, we did not observe any decrease in total STAT2 protein levels, but we observed a decreased phosphorylation of STAT2 during infection. Although

similar levels of STAT2 were detected in both siSOCS1 and siSOCS3 cultures 5 days p.i., only siSOCS3 cells showed an increased STAT2 phosphorylation. Moreover, in the SOCS3 cultures, the level of phosphorylation slightly increased between 2 and 5 days p.i., indicating no viral control of STAT2P in siSOCS3 culture. These results strongly suggest a role for SOCS3 in the virally mediated process that leads to STAT2 dephosphorylation. For another herpesvirus, EBV, it was shown that infection of primary human monocytes upregulates SOCS3. SOCS3 was indicated as a key factor for suppression of IFNα secretion and favored a state of type I IFN irresponsiveness by decreasing STAT2 phosphorylation [9].

Depending on the cell type and on the HCMV strain, the mechanisms involved in the immune escape process may vary resulting in a complex multistep process [14]. In our model, we demonstrate that reduction in SOCS3 leads to restoration of STAT2 phosphorylation and to a higher phosphorylation level of STAT1. The fact that HCMV-infected siSOCS3 cultures showed increased phosphorylation of both these transcription factors indicates a potentially high antiviral activation level for this culture which may be responsible for the impaired HCMV infection.

Conclusion

In summary, this is the first study to show an essential role of SOCS3 during HCMV replication in primary human endothelial cells. Accumulation of SOCS3 protein was observed in infected cells, and a correlation between SOCS3 and the ability of the virus to efficiently replicate was provided. A SOCS3-dependent control of STAT2 and to a lower extent of STAT1 phosphorylation in HCMV-infected culture was also shown. These findings add a novel aspect to the biology of HCMV and potentially identify SOCS3 as a novel target protein to control HCMV viral replication.

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Statement of Ethics

Not applicable.

Disclosure Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

O.S. and N.J.M. conceived the basic concept and designed the experiments. O.S. and A.T. performed the experiments and analyzed the data. A.-L.M., L.D., R.F.S., M.K.J.S., and N.J.M. contributed to the data analysis and development of new ideas. O.S., N.J.M., and G.M.W. wrote the paper. All authors critically revised it.

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