Suppressor of Cytokine Signaling 3 Is an Inducible Host Factor That Regulates Virus Egress during Ebola Virus Infection

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ABSTRACT

Ebola virus (EBOV) initially targets monocytes and macrophages, which can lead to the release of proinflammatory cytokines and chemokines. These inflammatory cytokines are thought to contribute to the development of circulatory shock seen in fatal EBOV infections. The VP40 matrix protein is a key viral structural protein that is critical for virion egress. Physical and functional interactions between VP40 and host proteins such as Tsg101 and Nedd4 facilitate efficient release of VP40-driven virus-like particles (VLPs) and infectious virus. Here, we show that host suppressor of cytokine signaling 3 (SOCS3) can also bind to EBOV VP40, leading to enhanced ubiquitinylation and egress of VP40. Indeed, titers of infectious EBOV derived from SOCS3 knockout mouse embryonic fibroblasts (MEFs) were significantly reduced compared to those from wild-type (WT) MEFs at 24 and 48 h postinfection. Importantly, this reduced virus yield could be rescued back to WT levels by exogenously expressing SOCS3. Lastly, we show that SOCS3 expression is induced by EBOV glycoprotein (GP) expression and that VLPs containing EBOV VP40 and GP induced production of proinflammatory cytokines, which induced SOCS3 for negative-feedback regulation. These data indicate that host innate immune protein SOCS3 may play an important role in budding and pathogenesis of EBOV.

IMPORTANCE

The VP40 matrix protein is a key structural protein critical for Ebola virus budding. Physical and functional interactions between VP40 and host proteins such as Tsg101 and Nedd4 facilitate efficient release of VLPs and infectious virus. We reported that host TLR4 is a sensor for Ebola GP on VLPs and that the resultant TLR4 signaling pathways lead to the production of proinflammatory cytokines. Host SOCS3 regulates the innate immune response by controlling and limiting the proinflammatory response through negative-feedback inhibition of cytokine receptors. We present evidence that Ebola virus VLPs stimulate induction of SOCS3 as well as proinflammatory cytokines, and that expression of human SOCS3 enhances budding of Ebola VLPs and infectious virus via a mechanism linked to the host ubiquitinylation machinery.

Ebola and Marburg viruses are emerging human pathogens within the *Filoviridae* family and are considered potential agents of bioterrorism. To date, there are no approved vaccines or therapeutics to prevent or treat filovirus infections in humans. Severe hemorrhagic disease caused by filoviruses are characterized by generalized fluid distribution problems, hypotension, coagulation disorders, and fulminant shock (1). Ebola virus (EBOV) first targets monocytes/macrophages and dendritic cells (DCs), leading to the release of proinflammatory cytokines and chemokines, including tumor necrosis factor (TNF), interleukin-1 β (IL-1 β), macrophage inflammatory protein 1 α (MIP-1 α), and reactive oxygen and nitrogen species (1, 2). An investigation of the interactions between EBOV proteins and host innate immune proteins may provide new insights into EBOV pathogenesis and uncover new strategies to block EBOV replication.

EBOV VP40 is the viral matrix protein that plays a critical role in budding of virus-like particles (VLPs) and infectious virus. Late-budding domains (L domains) of VP40 are known to facilitate interactions with specific host proteins to promote efficient release of VLPs and infectious virus (3–7). For example, the host

ubiquitin ligase neural precursor cell expressed developmentally downregulated protein 4 (Nedd4) interacts with the PPxY-type L domain of EBOV VP40, leading to both VP40 ubiquitinylation and enhanced VLP budding (5, 8). In addition to VP40, the expression of other viral proteins can enhance the efficiency of VLP budding. For example, the EBOV surface glycoprotein (GP) facil-

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itates virus entry and, when coexpressed with VP40, can enhance VLP egress (9) (10).

Filovirus VLPs have proven to be useful tools for providing insights into virus assembly and budding pathways, host interactions that modulate these processes, and the interplay between viral proteins and the host innate immune response relevant to replication and pathogenesis. Several recent studies have reported on host innate immune responses following infection or stimulation of macrophages/monocytes and dendritic cells with EBOV or VLPs, respectively (11, 12). For example, incubating EBOV VP40+GP VLPs with DCs induced IL-6, NF- κ B, and extracellular-signal-regulated kinases 1 and 2 (12, 13). More recently, it was reported that expression of human interferon-stimulated gene 15 (ISG15) impairs the budding of EBOV VP40 VLPs in an L-domain-dependent manner. Indeed, ISG15 inhibited Nedd4 ubiquitin ligase activity, resulting in decreased ubiquitinylation of VP40 and subsequent inhibition of VP40 VLP budding (14, 15). This previously undescribed mechanism of budding inhibition reinforced the importance of the cellular ubiquitinylation machinery for efficient budding and demonstrated that virus budding is a target of innate immune defenses.

Toll-like receptors (TLRs) are essential for initiating the innate response to lipopolysaccharide (LPS) from Gram-negative bacteria, as well as other microbial components of Gram-negative and Gram-positive bacteria, mycoplasma, spirochetes, and fungi. Ten TLRs have been identified in humans, and natural or synthetic ligands for at least nine TLRs have been identified. One candidate cell surface molecule for inducing proinflammatory cytokines is TLR4, which initiates a signal transduction pathway after binding to a ligand that ultimately results in NF- κ B activation (16, 17). We previously reported that VLPs expressing VP40 and GP induce the proinflammatory cytokines mediated by TLR4 (18).

Suppressor of cytokine signaling protein 1 (SOCS1) and SOCS3 regulate innate immune responses by controlling and limiting the proinflammatory response through negative-feedback inhibition of cytokine receptors (19). The expression of SOCS proteins, such as SOCS1 and SOCS3, alters the activity of macrophages and DCs, affecting their ability to differentiate and defend against invading pathogens (20, 21, 30). SOCS1 and SOCS3 are composed of a kinase inhibitory region, a classical Src homology 2 (SH2) domain, and a SOCS box (19, 21). The SOCS box is conserved in all cytokine-inducible SH2-domain-containing protein (CIS)-SOCS family proteins (22, 23). One function of the SOCS box is to recruit components of the ubiquitin-transferase system and coordinate their assembly and ability to ubiquitinylate target proteins. The CIS-SOCS family of proteins, as well as other SOCS-box-containing molecules, can function as E3 ubiquitin ligases.

Here, we present evidence that EBOV VLPs containing VP40+GP, but not VP40 alone, can stimulate induction of SOCS3 and that expression of SOCS3 is important for efficient budding of both VLPs and infectious virus. Indeed, SOCS3 knockout mouse embryonic fibroblasts (SOCS3 KO MEFs), unlike wild-type (WT) MEFs, did not produce virus until 48 h postinfection. The mechanism of the SOCS3-mediated enhancement of egress appears to be linked to the host ubiquitinylation machinery, since the SOCS-box region was critical for the ubiquitinylation of VP40. These results suggest that EBOV GP may induce expression of SOCS3 and that SOCS3 may have important implications for EBOV budding, pathogenesis, and immune evasion strategies.

MATERIALS AND METHODS

Cells and plasmids. The monocytic leukemia cell line THP-1 was grown in RPMI 1640 with 10% fetal calf serum (FCS). HEK293-TLR4/MD2 cells and control HEK293 cells were provided by BEI Resources (Manassas, VA). SOCS3 knockout mouse embryonic fibroblasts (SOCS3 KO MEFs) and C57BL/6J WT mouse embryonic fibroblasts (C57BL/6J MEFs) were provided by Akihiko Yoshimura (Keio University). Human 293T cells, HEK293-TLR4/MD2 cells, HEK293, SOCS3 KO MEFs, and C57BL/6J MEFs were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum. Plasmids expressing EBOV (Zaire) VP40 and GP have been described previously (24). Plasmids expressing, full-length SOCS3 (SOCS3 WT), and SOCS3 mutant Δ C41 were kindly provided by Akihiko Yoshimura (Keio University), and the hemagglutinin (HA)-tagged Ub (Ub-HA) plasmid was obtained from Heinrich Gottlinger (Harvard Medical School, Boston, MA).

IP and Western blotting. Immunoprecipitation (IP) and Western blot analyses were performed as described previously (14). Anti-HA and anti-actin antisera were purchased from Sigma-Aldrich (St. Louis, MO). Anti-SOCS3 antiserum was purchased from MBL (Nagoya, Japan). Anti-VP40 monoclonal antiserum was kindly provided by Gene Olinger (USAMRIID).

Reverse transcription-PCR (RT-PCR). Total RNA was isolated from indicated samples by using an RNeasy miniprep kit (Qiagen, CA). DNase-treated RNA (1 μ g) was reverse transcribed to cDNA with oligo(dT) primers and Superscript III (Invitrogen, Grand Island, NY). The cDNA was then amplified by PCR using primers specific for full-length SOCS3 (5'-CAAGGACGGAGACTTCGATT-3' and 5'-GACTGGGTCTTGA CGTGA-3'), EBOV-NP (5'-TCATGGCAATCCTGCAACA-3' and 5'-TCGGTTGAATCATCCCATTTGT-3'), TNF- α (5'-ACAAGCCTGT AGCCCATG and 5'-AAAGTAGACCTGCCAGACT-3'), and β -actin (5'-ACAATGAGCTGCTGGTGGCT-3' and 5'-GATGGGCACAGTG TGGGTGA-3').

VLP purification. VLPs were produced by transfecting 25 μ g of expression plasmids into 2×10^7 HEK293T cells in 175-cm² flask. The EBOV VP40 expression plasmid was transfected alone or in combination with EBOV GP expression plasmids at equal DNA concentrations. At 48 h posttransfection, cells and cellular debris were pelleted away from the harvested VLP-containing supernatant with a low-speed spin. VLPs were then centrifuged through a 20% sucrose cushion at $120,000 \times g$ in an SW41 rotor for 2 h at 4°C, washed in ice-cold NTE buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA), centrifuged at $120,000 \times g$ for 2 h at 4°C, and suspended in 100 μ l of NTE buffer. Protein content was quantitated using the DC protein assay (Bio-Rad, Hercules, CA).

SOCS3 KO cell infection and titration. SOCS3 KO and C57BL/6J MEFs were infected in 12-well plates with EBOV expressing green fluorescent protein (EBOV-GFP) at a multiplicity of infection (MOI) of 0.1 in 1 ml of serum-free DMEM for 1 h at 37°C in a 5% CO₂ atmosphere. Supernatants from EBOV-GFP-infected SOCS3 KO MEF or C57BL/6J MEF cell lines were serially diluted from 10^{-1} to 10^{-6} , and 100 μ l of each dilution was used to infect confluent Vero E6 cells in a 12-well plate. After infection, 2 ml of Eagle minimal essential medium with 3% fetal bovine serum and 1.5% carboxymethyl cellulose was added to each well. Cells were incubated at 37°C for 4 days to allow for focus formation. Carboxymethyl cellulose was then removed by washing with phosphate-buffered saline, and the cells were fixed in 10% formalin. GFP-expressing infected foci were counted using a fluorescence microscope (25).

Biosafety and biocontainment. All work with infectious EBOV was performed in the BSL-4 (laboratory in the biosafety level 4) facility at the Rocky Mountain Laboratories, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Sample inactivation and removal was performed according to standard operating protocols approved by the local Institutional Biosafety Committee.

FIG 1 Induction of SOCS3 mRNA by VLPs requires EBOV GP. (A) THP-1 cells were either untreated (control), or treated with LPS (1.0 μ g/ml), VP40 VLPs (10 μ g/ml), VP40+GP VLPs (10 μ g/ml), or VP40+GP Δ mucin VLPs for 6 or 12 h. Total RNA was isolated, and RT-PCR was used to detect SOCS3 or actin (control) mRNAs. (B) THP-1 monocytic cells were infected with EBOV at an MOI of 3. At 0, 1, 3, 6, and 12 h postinfection, RT-PCR was used to detect SOCS3, TNF- α , and actin mRNAs. The data are representative of three independent experiments.

RESULTS

SOCS3 mRNA is induced in THP-1 cells stimulated by EBOV VP40+GP VLPs or infectious EBOV. Since SOCS3 is an inducible regulator of the innate immune response, we sought to determine whether EBOV VLPs could trigger induction of SOCS3 mRNA. Briefly, since VLPs induce the host innate immune response (26), THP-1 monocytic cells were untreated (negative control), incubated with purified LPS (1.0 μ g/ml) as a positive control, or treated with VLPs composed of VP40 alone, VP40+GP, or VP40+GP Δ mucin for 6 or 12 h (Fig. 1A). EBOV GP has been shown previously to enhance VLP budding (10, 13), and the GP mucin domain is required for the induction of inflammatory cytokines (13). Endogenous SOCS3 mRNA was detected by RT-PCR (Fig. 1A). Expression of SOCS3 mRNA was not observed in untreated control cells, whereas SOCS3 mRNA was detected in cells treated with LPS at 6 and 12 h posttreatment (Fig. 1A). Low levels of SOCS3 mRNA were observed in cells treated with VLPs

containing VP40 alone at 6 and 12 h (lanes 5 and 6); however, the pattern of SOCS3 mRNA expression in cells treated with VLPs containing VP40+GP (lanes 7 and 8) closely mimicked that observed for the LPS positive control (lanes 3 and 4). Little to no SOCS3 mRNA was observed in cells treated with VP40+GP Δ mucin VLPs (lanes 9 and 10). These results show that induction of SOCS3 mRNA in THP-1 cells treated with EBOV VLPs containing VP40+GP most closely mimic that of the LPS positive control, suggesting that expression of WT EBOV GP is important for induction of SOCS3 mRNA in THP-1 cells.

Next, we assessed whether EBOV (Zaire) infection could also induce SOCS3 mRNA expression. Briefly, THP-1 cells were infected with EBOV at an MOI of 3, and cellular RNA was isolated at 1, 3, 6, and 12 h postinfection (Fig. 1B). SOCS3, TNF- α , and actin mRNA levels were determined by RT-PCR. TNF- α mRNA was detected at all time points, and expression of SOCS3 mRNA was detected at 3, 6, and 12 h postinfection (Fig. 1B). Actin mRNA levels served as a control (Fig. 1B). These results demonstrate that EBOV (Zaire) infection induced the expression of SOCS3 and TNF- α mRNAs in THP-1 cells and together suggest that EBOV GP expressed on the surface of either VLPs or infectious virus is important for inducing SOCS3 mRNA expression.

EBOV-mediated induction of SOCS3 mRNA is dependent on TLR4-MD2. We next sought to determine whether an interaction between EBOV-GP and TLR4 would stimulate the TLR4 signaling pathway leading to upregulation of SOCS3 mRNA. To this end, we utilized HEK293-TLR4/MD2 cells stably expressing a functional TLR4/MD2 complex on their surface. HEK293 cells (TLR4 negative) served as a negative control (Fig. 2). As expected, we observed induction of SOCS3 mRNA levels in HEK293-TLR4/MD2 cells treated with LPS at 6 and 12 h posttreatment, but we observed no induction of SOCS3 mRNAs levels in untreated HEK293-TLR4/MD2 cells or in HEK293-TLR4/MD2 cells treated with VLPs containing VP40 alone (Fig. 2). In contrast, we observed strong induction of SOCS3 mRNA levels in HEK293-TLR4/MD2 cells treated with VP40+GP VLPs (Fig. 2). Signals for SOCS3 mRNAs levels were absent in HEK293 cells under all treatment conditions, and TLR4 mRNA was only detected in HEK293-TLR4/MD2 cells as expected (Fig. 2). Actin mRNA levels served as an internal control (Fig. 2). These results suggest that induction of SOCS3 mRNA by EBOV GP is dependent on expression of the host TLR4-MD2 complex.

Increased expression of SOCS3 protein correlates with enhanced budding of EBOV VP40 VLPs. One mechanism by which

FIG 2 EBOV VP40+GP VLPs induce SOCS3 mRNA mediated by TLR4-MD2. HEK293-TLR4-MD2 and HEK293 cells were either untreated (cont.) or treated with LPS (1.0 μ g/ml), VP40 VLPs (10 μ g/ml), or VP40+GP VLPs (10 μ g/ml) for 6 or 12 h as indicated. Total RNA was isolated, and RT-PCR was used to detect SOCS3, TLR4, and actin. The data are representative of three independent experiments.

FIG 3 EBOV VP40 VLP budding is enhanced by SOCS3 in a dose-dependent manner. Human HEK293T cells were mock transfected or transfected with the indicated plasmids. The total amount of plasmid DNA transfected was held equivalent in all samples by using empty pCAGGS vector. VLPs and cell extracts were harvested at 24 h posttransfection, and proteins were analyzed by Western blotting with the indicated antisera. The data are representative of five independent experiments.

SOCS3 regulates cytokine signaling via protein ubiquitination, a cellular process that also plays a key role in enhancing EBOV VLP budding (5). We therefore sought to determine whether increased expression of SOCS3 protein would enhance budding of EBOV VP40 VLPs. Briefly, human HEK293T cells were mock transfected or transfected with a constant amount of VP40 together with increasing amounts of a SOCS3 expression plasmid (Fig. 3). At 24 h posttransfection, both cell lysates and VLPs were harvested, and proteins were detected and quantified by Western blotting (Fig. 3). The levels of VP40 in cell lysates were equivalent in all samples and, as expected, the levels of SOCS3 in cell extracts increased with increasing amounts of SOCS3 plasmid transfected (Fig. 3; Cells). Actin served as a loading control. Interestingly, the levels of VP40 in VLPs increased by 3- to 4-fold in a dose-dependent manner as the SOCS3 protein levels increased (Fig. 3; VLPs). SOCS3 was also detected in VP40 VLPs in a dose-dependent manner (Fig. 3), suggesting that a VP40-SOCS3 interaction may lead to specific packaging of SOCS3 in budding VLPs. These results suggest that SOCS3 may promote egress of EBOV VP40 VLPs.

SOCS3-mediated ubiquitinylation of VP40 enhances VLP budding. We next sought to investigate the mechanism by which SOCS3 promotes VP40 VLP egress. Since SOCS3 is known to have substrate-specific E3 ubiquitin ligase-like activity, similar to host Nedd4, we hypothesized that SOCS3 may modulate ubiquitinylation of VP40 leading to enhanced release of VP40 VLPs (22, 27). To test this, HEK293T cells were transfected with the indicated combinations of plasmids expressing VP40, SOCS3-WT, and HA-tagged ubiquitin (Ub), and cell lysates were harvested (Fig. 4). Cell lysates were first immunoprecipitated with anti-VP40 antiserum, followed by Western blotting with anti-HA antiserum to detect ubiquitinated forms of VP40 (Fig. 4). Little to no VP40 ubiquiti-

FIG 4 Expression of SOCS3 enhances ubiquitinylation of VP40. Schematic diagram of SOCS3-WT and mutant ΔC41. Both SOCS3-WT and mutant ΔC41 are tagged at their C termini with a *c-myc* epitope tag. The SOCS box is located at the C terminus between amino acids 184 and 225. Human HEK293T cells were transfected with the indicated plasmids. Cell extracts were analyzed by IP/Western blotting or Western blotting as indicated. This is a single gel from which irrelevant lanes were removed. The data are representative of five independent experiments.

nylation was detected in extracts from cells expressing VP40 alone (Fig. 4, lane 1), or VP40+Ub-HA (lane 2). In contrast, an enhanced level of VP40 ubiquitinylation was readily observed in lysates from cells expressing VP40+Ub-HA+SOCS3-WT (Fig. 4, lane 3). To determine whether the ubiquitin ligase activity of the SOCS box region of SOCS3 is important for the observed ubiquitinylation of VP40, we used SOCS box deletion mutant ΔC41 in this assay (Fig. 4) (28). Indeed, VP40 ubiquitinylation was reduced significantly in lysates from cells expressing VP40+Ub-HA+ΔC41 (Fig. 4, lane 4). IP/Western blot analysis of VP40 and detection of SOCS3-WT and ΔC41 by Western blotting are shown as controls (Fig. 4). These data indicate that the SOCS-box activity of SOCS3 is important for interaction with, and ubiquitinylation of VP40, which may lead to enhanced egress of VP40 VLPs.

SOCS3 is required to facilitate efficient budding of infectious EBOV. Results described above demonstrate that SOCS3 en-

FIG 5 SOCS3 is required for efficient production of VLPs and live virus. (A) C57BL/6J MEFs (B6 WT MEFs) or SOCS3 KO MEFs were mock transfected or transfected with EBOV VP40, and cells and VLPs were harvested at 24 h post-transfection. Cell lysates and VLPs were analyzed by Western blotting with anti-VP40 antiserum. RNA was isolated from C57BL/6J MEFs or SOCS3 KO MEFs and analyzed by RT-PCR. The data are representative of three independent experiments. (B) WT MEFs or SOCS3 KO MEFs were infected with EBOV at an MOI of 0.1. Supernatants were collected on days 1, 2, 3, and 4 postinfection, and infectious virus was titrated by focus-forming assay. Two independent experiments were performed in triplicate. (C) WT or SOCS3 KO MEFs were infected with EBOV at an MOI of 0.1, and total cellular RNA was harvested at 24 and 48 h. postinfection and subjected to qRT-PCR to quantify virus infection. The bars represent the averages of two independent experiments, both performed in triplicate.

hances budding of VP40 VLPs, possibly via a ubiquitylation mechanism. We next sought to determine whether SOCS3 knock-out cells were defective not only for budding of VP40 VLPs but also for budding of infectious EBOV. Briefly, C57BL/6J MEFs or SOCS3 KO MEFs were mock transfected or transfected with EBOV VP40 for 24 h, after which the cell lysate and VLPs were harvested and analyzed by Western blotting (Fig. 5A). EBOV

FIG 6 SOCS3 overexpression rescues EBOV production in SOCS3 KO MEF cells. (A) SOCS3 KO MEFs stably overexpressing a WT SOCS3 in *trans* (SOCS-3 WT *trans*) were established by blasticidin selection. SOCS3 expression was verified by RT-PCR. The data are representative of two independent experiments. (B) SOCS-3 KO MEFs stably overexpressing WT SOCS3 (SOCS-3 WT *trans*), SOCS3 KO MEFs, and WT MEFs were infected with EBOV at an MOI of 0.1. Supernatants were collected at 24, 48, 76, and 92 h postinfection, and virus was titrated by a focus-forming assay. The data are representative of three independent experiments.

VP40 was expressed abundantly in both WT and KO cells, whereas budding of VP40 VLPs from SOCS3 KO cells was reduced by approximately 3- to 4-fold compared to that from SOCS3 WT cells (Fig. 5A). As a control, levels of SOCS3 mRNA were readily detected by RT-PCR in WT cells, but not in the KO cells as expected (Fig. 5A).

Next, we quantified egress of infectious EBOV from WT and KO MEFs by infecting cells with EBOV at an MOI of 0.1 and titrating virus using a focus-forming assay of supernatants collected on days 1, 2, 3, and 4 postinfection (Fig. 5B). Measurable virus production from SOCS3 KO MEFs was not detected until days 3 and 4 postinfection (Fig. 5B, white bars). In contrast, EBOV production from SOCS3 WT MEFs was detected on day 1 and increased steadily in a time-dependent manner (Fig. 5B, black bars). The total cellular RNA was harvested at days 1 and 2 postinfection and subjected to real-time qRT-PCR (qRT-PCR) to quantify virus infection (Fig. 5C). No significant difference in EBOV infectivity was observed between WT and SOCS3 KO MEFs (Fig. 5C). These findings demonstrate that expression of host SOCS3 is important for efficient egress of both VLPs and authentic EBOV.

Exogenous expression of SOCS3 rescues budding of infectious EBOV from KO cells. To further confirm the importance of SOCS3 for efficient production of EBOV, we sought to determine whether expression of SOCS3 in *trans* in SOCS3 KO MEFs could restore EBOV production back to levels achieved in SOCS3 WT MEFs. Briefly, we established SOCS3 KO MEFs stably overexpressing WT SOCS3 in *trans* (SOCS3 WT *trans*), and SOCS3 expression in these cells was verified by RT-PCR (Fig. 6A). We in-

fecting SOCS3 WT *trans* cells, SOCS3 KO MEFs and C57BL/6J WT MEFs cells with EBOV at an MOI = 0.1, and then titrated EBOV by focus forming assay from supernatants collected at 24, 48, 76 and 92 h postinfection. Indeed, titers of EBOV from SOCS3 WT *trans* cells were restored back to titers measured from WT MEFs at the early time points of 24 and 48 h, and these titers increased slightly at 72 and 96 h postinfection (Fig. 6B). As before, little to production of EBOV was measurable at 24 and 48 h postinfection from SOCS3 KO MEFs (Fig. 6B, white bars). Together, these data strongly support our main conclusion that SOCS3 is a critical innate immune host factor required for efficient budding of infectious EBOV.

DISCUSSION

Innate immune responses to virus infection provide a critical first line of defense for the host. The interplay between EBOV proteins and the host innate immune system is an area of great interest, since the outcomes of these virus-host interactions can directly influence virus replication, pathogenesis, and immune evasion. EBOV VP35 and VP24 proteins function as interferon (IFN) antagonists, disrupting various steps in the host immune response to infection (29–31). Components of the host innate immune response, including IFN-induced proteins, were shown recently to inhibit the role of EBOV VP40 in viral assembly and budding (14, 15). For example, ISG15 possesses antiviral activity and inhibits budding by disrupting L-domain-mediated virus-host interactions (14, 32). Here, we present evidence of a previously undescribed interaction between an innate immune signal transduction protein, SOCS3, and EBOV VP40. Expression of SOCS3, a key negative regulator of the inflammatory cytokine response, enhanced budding of EBOV VP40-driven VLPs in a dose-dependent manner, and budding of infectious EBOV in cell culture.

SOCS3 contains a critical C-terminal region termed the SOCS box, which possesses E3 ubiquitin ligase activity (19). We were particularly interested in the reported activity of SOCS3 as an adaptor protein that coordinates and organizes ubiquitinylation complexes in the cell, since VP40 ubiquitinylation mediated by host Nedd4 plays a role in viral budding (5). Our results suggest that expression of SOCS3 alone can enhance ubiquitinylation and release of EBOV VP40-driven VLPs. This effect appears to require enzymatically active SOCS3, since ubiquitinylation of VP40 is minimal in the presence of a SOCS-box deletion mutant (Fig. 4). We have shown that EBOV VP40 interacts with SOCS3, and our results suggest that a functional SOCS-box is important for ubiquitinylation of VP40. Although the SOCS-box is known to function as a substrate recognition domain (33, 34), its precise role in mediating interactions between SOCS3 and VP40 or in coordinating a multiprotein ubiquitinylation complex important for budding, awaits further experimentation.

There is growing evidence that the SOCS family of host proteins can be exploited for viral benefit. The beneficial effects of SOCS3 gene expression on viral replication have been described for both hepatitis C virus and influenza A virus (35), and SOCS-box activity can result in viral protein ubiquitinylation (36). Nishi et al. found that SOCS-box activity facilitates human immunodeficiency virus 1 (HIV-1) Gag ubiquitinylation and budding, and SOCS-box deleted SOCS1 reduces HIV-1 budding (34). Consistent with these findings in other viruses, we showed that progeny EBOV titers were reduced significantly in SOCS3 KO MEFs, and virus production was recovered in cells stably overexpressing

SOCS3 (Fig. 5 and 6). These findings suggest that the presence of SOCS3 is necessary for enhanced production of infectious EBOV.

Previous studies have clearly demonstrated that EBOV VLPs containing full-length GP can induce a proinflammatory response marked by the production of cytokines (e.g., IL-6 and IL-8) from monocytes/macrophages and dendritic cells; the early targets of EBOV infection (1, 37). We reported previously that the induction of inflammatory cytokines was due to EBOV GP interacting with TLR4, resulting in SOCS1 induction (18). TLR4 is a cell surface receptor that recognizes LPS, a prototypical activator of NF- κ B and other proinflammatory responses (38–40). TLR4 is highly expressed in cells that respond to LPS, such as peripheral blood leukocytes, macrophages, and monocytes (41). VLPs containing VP40 and GP induced the proinflammatory cytokines IL-6 and TNF- α in dendritic cells similar to the induction seen in response to LPS (13). Our observation of the induction of proinflammatory cytokines by live virus and EBOV VP40+GP VLPs, but not by VP40 VLPs, extend these findings to show that expression of SOCS3 RNA is also enhanced in THP-1 cells (Fig. 1 and 2). We used a HEK293 stable cell line expressing functional TLR4/MD2 to show that EBOV VLPs induced expression of proinflammatory cytokines and SOCS3 in a TLR4/MD2-dependent manner.

We speculate that induction of SOCS3 may benefit EBOV infection, both via enhancement of virus budding and by dampening innate immune signaling, thus allowing the virus to evade host inflammatory and antiviral responses. This strategy of immune evasion has been described for the intracellular parasite *Toxoplasma gondii*, which induces expression of SOCS3 in macrophages, leading to impairment of IFN- γ signaling (42).

EBOV GP interacts with a variety of cell surface molecules to gain entry into cells (43, 44). For example, cell surface molecules, including calcium-dependent lectins (45) and β 1 integrin (46), have been implicated in mediating EBOV entry and, more recently, the TAM family of proteins (Tyro3, Axl, and Mer) were reported to be involved in mediating entry of EBOV (47). Interestingly, TAM receptor signaling was shown in a separate report to induce SOCS3 expression, leading to subsequent negative regulation of surface-expressed TLR receptors and TLR-induced cytokine-receptor cascades (48). Thus, it would be of interest to determine whether EBOV GP-mediated binding to TAM receptors on macrophages or monocytes could stimulate expression of SOCS3, leading to inhibition of inflammation and enhancement of virus budding.

Although SOCS3 has been shown previously to modulate innate immune responses to both DNA and RNA viruses (34, 49–51), a role for SOCS3 in enhancing EBOV VP40 ubiquitinylation leading to efficient VLP egress was previously undescribed. In addition, our findings provide new insights into innate immune interactions with key structural proteins of EBOV and suggest novel mechanisms of action by which SOCS3 may influence pathogenesis and immune evasion. The intriguing interplay between the host innate immune system and EBOV is highlighted by SOCS3, an antagonist of the innate immune response that has a positive effect on EBOV budding, and ISG15, an agonist of the innate immune response that has a negative effect on EBOV VLP budding. Further experiments investigating this interplay will provide fundamental knowledge necessary for the future development of novel antiviral therapies.

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REFERENCES

- Feldmann H, Bugany H, Mahner F, Klenk HD, Drenckhahn D, Schnitler HJ. 1996. Filovirus-induced endothelial leakage triggered by infected monocytes/macrophages. *J Virol* 70:2208–2214.
- Bray M, Geisbert TW. 2005. Ebola virus: the role of macrophages and dendritic cells in the pathogenesis of Ebola hemorrhagic fever. *Int J Biochem Cell Biol* 37:1560–1566. <http://dx.doi.org/10.1016/j.biocel.2005.02.018>.
- Harty RN, Brown ME, McGettigan JP, Wang G, Jayakar HR, Huibregtse JM, Whitt MA, Schnell MJ. 2001. Rhabdoviruses and the cellular ubiquitin-proteasome system: a budding interaction. *J Virol* 75:10623–10629. <http://dx.doi.org/10.1128/JVI.75.22.10623-10629.2001>.
- Craven RC, Harty RN, Paragas J, Palese P, Wills JW. 1999. Late domain function identified in the vesicular stomatitis virus M protein by use of rhabdovirus-retrovirus chimeras. *J Virol* 73:3359–3365.
- Harty RN, Brown ME, Wang G, Huibregtse J, Hayes FP. 2000. A PPxY motif within the VP40 protein of Ebola virus interacts physically and functionally with a ubiquitin ligase: implications for filovirus budding. *Proc Natl Acad Sci U S A* 97:13871–13876. <http://dx.doi.org/10.1073/pnas.250277297>.
- Harty RN, Paragas J, Sudol M, Palese P. 1999. A proline-rich motif within the matrix protein of vesicular stomatitis virus and rabies virus interacts with WW domains of cellular proteins: implications for viral budding. *J Virol* 73:2921–2929.
- Jayakar HR, Murti KG, Whitt MA. 2000. Mutations in the PPPY motif of vesicular stomatitis virus matrix protein reduce virus budding by inhibiting a late step in virion release. *J Virol* 74:9818–9827. <http://dx.doi.org/10.1128/JVI.74.21.9818-9827.2000>.
- Timmins J, Schoehn G, Ricard-Blum S, Scianimanico S, Vernet T, Ruigrok RW, Weissenhorn W. 2003. Ebola virus matrix protein VP40 interaction with human cellular factors Tsg101 and Nedd4. *J Mol Biol* 326:493–502. [http://dx.doi.org/10.1016/S0022-2836\(02\)01406-7](http://dx.doi.org/10.1016/S0022-2836(02)01406-7).
- Yang Z, Delgado R, Xu L, Todd RF, Nabel EG, Sanchez A, Nabel GJ. 1998. Distinct cellular interactions of secreted and transmembrane Ebola virus glycoproteins. *Science* 279:1034–1037. <http://dx.doi.org/10.1126/science.279.5353.1034>.
- Licata JM, Johnson RF, Han Z, Harty RN. 2004. Contribution of Ebola virus glycoprotein, nucleoprotein, and VP24 to budding of VP40 virus-like particles. *J Virol* 78:7344–7351. <http://dx.doi.org/10.1128/JVI.78.14.7344-7351.2004>.
- Bosio CM, Moore BD, Warfield KL, Ruthel G, Mohammadzadeh M, Aman MJ, Bavari S. 2004. Ebola and Marburg virus-like particles activate human myeloid dendritic cells. *Virology* 326:280–287. <http://dx.doi.org/10.1016/j.virol.2004.05.025>.
- Wahl-Jensen V, Kurz SK, Hazelton PR, Schnitler HJ, Stroher U, Burton DR, Feldmann H. 2005. Role of Ebola virus secreted glycoproteins and virus-like particles in activation of human macrophages. *J Virol* 79:2413–2419. <http://dx.doi.org/10.1128/JVI.79.4.2413-2419.2005>.
- Martinez O, Valmas C, Basler CF. 2007. Ebola virus-like particle-induced activation of NF- κ B and Erk signaling in human dendritic cells requires the glycoprotein mucin domain. *Virology* 364:342–354. <http://dx.doi.org/10.1016/j.virol.2007.03.020>.
- Okumura A, Pitha PM, Harty RN. 2008. ISG15 inhibits Ebola VP40 VLP budding in an L-domain-dependent manner by blocking Nedd4 ligase activity. *Proc Natl Acad Sci U S A* 105:3974–3979. <http://dx.doi.org/10.1073/pnas.0710629105>.
- Malakhova OA, Zhang DE. 2008. ISG15 inhibits Nedd4 ubiquitin E3 activity and enhances the innate antiviral response. *J Biol Chem* 283:8783–8787. <http://dx.doi.org/10.1074/jbc.C800030200>.
- Qureshi ST, Gros P, Malo D. 1999. Host resistance to infection: genetic control of lipopolysaccharide responsiveness by Toll-like receptor genes. *Trends Genet* 15:291–294. [http://dx.doi.org/10.1016/S0168-9525\(99\)01782-5](http://dx.doi.org/10.1016/S0168-9525(99)01782-5).
- Beutler B. 2000. Endotoxin, Toll-like receptor 4, and the afferent limb of innate immunity. *Curr Opin Microbiol* 3:23–28. [http://dx.doi.org/10.1016/S1369-5274\(99\)00046-6](http://dx.doi.org/10.1016/S1369-5274(99)00046-6).
- Okumura A, Pitha PM, Yoshimura A, Harty RN. 2010. Interaction between Ebola virus glycoprotein and host Toll-like receptor 4 leads to induction of proinflammatory cytokines and SOCS1. *J Virol* 84:27–33. <http://dx.doi.org/10.1128/JVI.01462-09>.
- Yoshimura A, Naka T, Kubo M. 2007. SOCS proteins, cytokine signaling and immune regulation. *Nat Rev Immunol* 7:454–465. <http://dx.doi.org/10.1038/nri2093>.
- Dalpe A, Heeg K, Bartz H, Baetz A. 2008. Regulation of innate immunity by suppressor of cytokine signaling (SOCS) proteins. *Immunobiology* 213:225–235. <http://dx.doi.org/10.1016/j.imbio.2007.10.008>.
- Yasukawa H, Sasaki A, Yoshimura A. 2000. Negative regulation of cytokine signaling pathways. *Annu Rev Immunol* 18:143–164. <http://dx.doi.org/10.1146/annurev.immunol.18.1.143>.
- Kamizono S, Hanada T, Yasukawa H, Minoguchi S, Kato R, Minoguchi M, Hattori K, Hatakeyama S, Yada M, Morita S, Kitamura T, Kato H, Nakayama K, Yoshimura A. 2001. The SOCS box of SOCS-1 accelerates ubiquitin-dependent proteolysis of TEL-JAK2. *J Biol Chem* 276:12530–12538. <http://dx.doi.org/10.1074/jbc.M010074200>.
- Kamura T, Sato S, Haque D, Liu L, Kaelin WG, Jr, Conaway RC, Conaway JW. 1998. The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. *Genes Dev* 12:3872–3881. <http://dx.doi.org/10.1101/gad.12.24.3872>.
- Licata JM, Simpson-Holley M, Wright NT, Han Z, Paragas J, Harty RN. 2003. Overlapping motifs (PTAP and PPEY) within the Ebola virus VP40 protein function independently as late budding domains: involvement of host proteins TSG101 and VPS-4. *J Virol* 77:1812–1819. <http://dx.doi.org/10.1128/JVI.77.3.1812-1819.2003>.
- Hill-Batorski L, Halfmann P, Neumann G, Kawaoka Y. 2013. The cytoprotective enzyme heme oxygenase-1 suppresses Ebola virus replication. *J Virol* 87:13795–13802. <http://dx.doi.org/10.1128/JVI.02422-13>.
- Warfield KL, Perkins JG, Swenson DL, Deal EM, Bosio CM, Aman MJ, Yokoyama WM, Young HA, Bavari S. 2004. Role of natural killer cells in innate protection against lethal Ebola virus infection. *J Exp Med* 200:169–179. <http://dx.doi.org/10.1084/jem.20032141>.
- Kamura T, Maenaka K, Kotoshiba S, Matsumoto M, Kohda D, Conaway RC, Conaway JW, Nakayama KI. 2004. VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. *Genes Dev* 18:3055–3065. <http://dx.doi.org/10.1101/gad.1252404>.
- Sasaki A, Inagaki-Ohara K, Yoshida T, Yamanaka A, Sasaki M, Yasukawa H, Koromilas AE, Yoshimura A. 2003. The N-terminal truncated isoform of SOCS3 translated from an alternative initiation AUG codon under stress conditions is stable due to the lack of a major ubiquitination site, Lys-6. *J Biol Chem* 278:2432–2436. <http://dx.doi.org/10.1074/jbc.C200608200>.
- Cardenas WB, Loo YM, Gale M, Jr, Hartman AL, Kimberlin CR, Martinez-Sobrido L, Saphire EO, Basler CF. 2006. Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. *J Virol* 80:5168–5178. <http://dx.doi.org/10.1128/JVI.02199-05>.
- Reid SP, Leung LW, Hartman AL, Martinez O, Shaw ML, Carbonnelle C, Volchkov VE, Nichol ST, Basler CF. 2006. Ebola virus VP24 binds karyopherin α 1 and blocks STAT1 nuclear accumulation. *J Virol* 80:5156–5167. <http://dx.doi.org/10.1128/JVI.02349-05>.
- Basler CF, Mikulasova A, Martinez-Sobrido L, Paragas J, Muhlberger E, Bray M, Klenk HD, Palese P, Garcia-Sastre A. 2003. The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3. *J Virol* 77:7945–7956. <http://dx.doi.org/10.1128/JVI.77.14.7945-7956.2003>.
- Okumura A, Lu G, Pitha-Rowe I, Pitha PM. 2006. Innate antiviral response targets HIV-1 release by the induction of ubiquitin-like protein ISG15. *Proc Natl Acad Sci U S A* 103:1440–1445. <http://dx.doi.org/10.1073/pnas.0510518103>.
- Bourette RP, De Sepulveda P, Arnaud S, Dubreuil P, Rottapel R, Mouchiroud G. 2001. Suppressor of cytokine signaling 1 interacts with the macrophage colony-stimulating factor receptor and negatively regulates its proliferation signal. *J Biol Chem* 276:22133–22139. <http://dx.doi.org/10.1074/jbc.M101878200>.

34. Ryo A, Tsurutani N, Ohba K, Kimura R, Komano J, Nishi M, Soeda H, Hattori S, Perrem K, Yamamoto M, Chiba J, Mimaya J, Yoshimura K, Matsushita S, Honda M, Yoshimura A, Sawasaki T, Aoki I, Morikawa Y, Yamamoto N. 2008. SOCS1 is an inducible host factor during HIV-1 infection and regulates the intracellular trafficking and stability of HIV-1 Gag. *Proc Natl Acad Sci U S A* 105:294–299. <http://dx.doi.org/10.1073/pnas.0704831105>.
35. Pauli EK, Schmolke M, Wolff T, Viemann D, Roth J, Bode JG, Ludwig S. 2008. Influenza A virus inhibits type I IFN signaling via NF- κ B-dependent induction of SOCS-3 expression. *PLoS Pathog* 4:e1000196. <http://dx.doi.org/10.1371/journal.ppat.1000196>.
36. Akhtar LN, Benveniste EN. 2011. Viral exploitation of host SOCS protein functions. *J Virol* 85:1912–1921. <http://dx.doi.org/10.1128/JVI.01857-10>.
37. Baize S, Leroy EM, Georges AJ, Georges-Courbot MC, Capron M, Bedjabaga I, Lansoud-Soukate J, Mavoungou E. 2002. Inflammatory responses in Ebola virus-infected patients. *Clin Exp Immunol* 128:163–168. <http://dx.doi.org/10.1046/j.1365-2249.2002.01800.x>.
38. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282:2085–2088. <http://dx.doi.org/10.1126/science.282.5396.2085>.
39. Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med* 189:615–625. <http://dx.doi.org/10.1084/jem.189.4.615>.
40. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 162:3749–3752.
41. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 274:10689–10692. <http://dx.doi.org/10.1074/jbc.274.16.10689>.
42. Zimmermann S, Murray PJ, Heeg K, Dalpke AH. 2006. Induction of suppressor of cytokine signaling-1 by *Toxoplasma gondii* contributes to immune evasion in macrophages by blocking IFN- γ signaling. *J Immunol* 176:1840–1847. <http://dx.doi.org/10.4049/jimmunol.176.3.1840>.
43. Takada A, Robison C, Goto H, Sanchez A, Murti KG, Whitt MA, Kawaoka Y. 1997. A system for functional analysis of Ebola virus glycoprotein. *Proc Natl Acad Sci U S A* 94:14764–14769. <http://dx.doi.org/10.1073/pnas.94.26.14764>.
44. Wool-Lewis RJ, Bates P. 1998. Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines. *J Virol* 72:3155–3160.
45. Simmons G, Rennekamp AJ, Chai N, Vandenberghe LH, Riley JL, Bates P. 2003. Folate receptor alpha and caveolae are not required for Ebola virus glycoprotein-mediated viral infection. *J Virol* 77:13433–13438. <http://dx.doi.org/10.1128/JVI.77.24.13433-13438.2003>.
46. Takada A, Watanabe S, Ito H, Okazaki K, Kida H, Kawaoka Y. 2000. Downregulation of β 1 integrins by Ebola virus glycoprotein: implication for virus entry. *Virology* 278:20–26. <http://dx.doi.org/10.1006/viro.2000.0601>.
47. Shimajima M, Takada A, Ebihara H, Neumann G, Fujioka K, Irimura T, Jones S, Feldmann H, Kawaoka Y. 2006. Tyro3 family-mediated cell entry of Ebola and Marburg viruses. *J Virol* 80:10109–10116. <http://dx.doi.org/10.1128/JVI.01157-06>.
48. Rothlin CV, Ghosh S, Zuniga EI, Oldstone MB, Lemke G. 2007. TAM receptors are pleiotropic inhibitors of the innate immune response. *Cell* 131:1124–1136. <http://dx.doi.org/10.1016/j.cell.2007.10.034>.
49. Zhao DC, Yan T, Li L, You S, Zhang C. 2007. Respiratory syncytial virus inhibits interferon- α -inducible signaling in macrophage-like U937 cells. *J Infect* 54:393–398. <http://dx.doi.org/10.1016/j.jinf.2006.06.005>.
50. Miyoshi H, Fujie H, Shintani Y, Tsutsumi T, Shinzawa S, Makuuchi M, Kokudo N, Matsuura Y, Suzuki T, Miyamura T, Moriya K, Koike K. 2005. Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling (SOCS)-1 gene expression. *J Hepatol* 43:757–763. <http://dx.doi.org/10.1016/j.jhep.2005.03.028>.
51. Mahller YY, Sakthivel B, Baird WH, Aronow BJ, Hsu YH, Cripe TP, Mehrian-Shai R. 2008. Molecular analysis of human cancer cells infected by an oncolytic HSV-1 reveals multiple upregulated cellular genes and a role for SOCS1 in virus replication. *Cancer Gene Ther* 15:733–741. <http://dx.doi.org/10.1038/cgt.2008.40>.