RESEARCH ARTICLE



A respiratory syncytial virus persistent-infected cell line system reveals the involvement of SOCS1 in the innate antiviral response

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HEp-2 cells persistently infected with respiratory syncytial virus (RSV) are a heterogeneous mixture of viral antigen-positive and -negative variants; however, the mechanism through which viral replication becomes latent remains unclear. In this study, we investigated the potential mechanism by which RSV escapes from innate immune surveillance. Persistent-infected RSV HEp-2 cells were isolated and cell clones were passaged. The RSV-persistent cells produced viruses at a lower titer, resisted wild-type RSV re-infection, and secreted high levels of interferon- β (IFN- β), macrophage inflammatory protein-1α (Mip-1α), interleukin-8 (IL-8), and Rantes. Toll-like receptor 3 (TLR3), retinoic acid inducible gene-I (RIG-I), and suppressor of cytokine signaling 1 (SOCS1) levels were upregulated in these cells. The silencing of *TLR3* mRNA decreased the expression of SOCS1 protein and the secretion of cytokines. RSV-persistent cells are in an inflammatory state; upregulation of SOCS1 is related to the TLR3 signaling pathway, which could be associated with the mechanism of viral persistence.

KEYWORDS respiratory syncytial virus (RSV); suppressor of cytokine signaling 1 (SOCS1); interferon; toll-like receptor 3 (TLR3); RSV-persistent cell line

INTRODUCTION

Respiratory syncytial virus (RSV) is the leading viral pathogen associated with bronchiolitis and lower respiratory tract disease in infants and young children worldwide (Psarras et al., 2004). There is a strong association between RSV infection in infancy and the subsequent development of recurrent wheezing and airway hyperresponsiveness later in life (Sigurs et al., 2000). Among adults, the greatest impact of RSV is associated with chronic obstructive pulmonary disease (COPD) (Falsey et al., 2006). Furthermore, numerous studies have in-

Received: 18 April 2015, Accepted: 12 June 2015 Published online: 19 June 2015 # These authors contributed equally. Correspondence: Phone: +86-27-67813139 Email: zhao_wh2004@hotmail.com, wb001553@whu.edu.cn ORCID: 0000-0002-9454-6041 dicated that RSV infections can become persistent and latent (Hobson et al., 1997; Matsuse et al., 2000; Riedel et al., 2008). This may contribute to the pathogenesis of chronic wheezing and asthma in children who have experienced acute RSV bronchiolitis and may serve as a reservoir for RSV transmission, which would ostensibly lead to reinfection (Schwarze et al., 2004; Tripp, 2004; Sikkel et al., 2008).

The RSV RNA genome has been characterized from a high percentage of patients with stable COPD, and was associated with elevated markers of inflammation at low copy numbers (Seemungal et al., 2001; Borg et al., 2003). Persistent RSV infection has been established *in vitro* in several types of cells (Bangham and McMichael, 1986; Valdovinos and Gomez, 2003). These data suggest that although RSV usually causes acute infection, this virus may become latent or persistent both *in vivo* and *in vitro*, which suggests the potential for persistent low-grade RSV infection in this population. In the acute infection

phase, RSV replication evokes retinoic acid inducible gene-I (*RIG-I*) to mediate the early antiviral response, and Toll-like receptor 3 (TLR3) to mediate chemokine expression in airway epithelial cells (Le Goffic et al., 2007; Liu et al., 2007; Oshansky et al., 2009). To avoid clearance, the virus adopts certain strategies to subvert the host's antiviral response, including upregulation of suppressor of cytokine signaling (SOCS) proteins, which negatively regulate interferon (IFN)-dependent signaling pathways (Vlotides et al., 2004; Pothlichet et al., 2008; Hashimoto et al., 2009). TLR3 is an important factor for the activation of innate immunity and mediates responses to the synthetic analog of viral double-stranded RNA, polyriboinosinic polyribocytidylic acid (poly I:C), which has been used extensively in experimental studies to mimic viral infections (Stowell et al., 2009; Jeanette et al., 2013). SOCS proteins can be induced directly by TLR3 stimulation, and do not directly interfere with its signaling, but rather avoid overshooting activation by regulating paracrine IFN- β signaling (Dalpke et al., 2008; Prêle et al., 2008). Some latent infectious viruses such as hepatitis C and enterovirus result in persistent infection through the induction of SOCS1 and SOCS3 (Bode et al., 2003; Yasukawa et al., 2003). Therefore, SOCS proteins play an important role in the balanced activation of innate immunity during viral infection.

In present study, we established epithelial cell lines that were persistently infected with RSV (hereafter termed RSV-persistent cultures or cells) and evaluated the putative role of SOCSs in the regulation of the antiviral response and inflammatory status.

MATERIALS AND METHODS

Virus purification and cell cultures

HEp-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown as monolayers in minimal essential medium (MEM; Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 50 mg/ mL gentamicin, and 50 ng/mL amphotericin B. For viral purification, RSV A2 (ATCC VR-1400) was propagated in HEp-2 cells grown in MEM supplemented with 2% heat-inactivated FBS (MEM-FBS). After the maximum cytopathic effect (CPE) was achieved, the cells were harvested and disrupted by sonication in the same culture medium. Viral purification was performed as previously described (Bangham and McMichael, 1986). Pellets containing the purified virus (pRSV) were resuspended in 10 mmol/L phosphate buffered saline containing 15% sucrose and stored in aliquots at -80 °C.

Isolation of RSV-persistent cultures

To establish RSV-persistent cultures, HEp-2 cells

grown to 50% confluence were seeded into 100-mm Petri tissue culture dishes and exposed to RSV at a multiplicity of infection (MOI) of 3. The cultures were refreshed with 5% FBS in MEM every three days to remove the floating cells. On day 14 after RSV infection, most of the cells had died, and the few surviving cells remained attached and grew as clonal sheets on day 18. After these surviving cells grew into monolayers, the cells were isolated using clone cylinders (CLS31666-125EA, Sigma-Aldrich, USA), trypsinized for collection, and then seeded in 96-well plates at a diluted density of one cell per well for clonal selection; this process was repeated three times. Finally, eight RSV-surviving clones (named A-H) were obtained and designated as "passage 0". A frozen stock was then created after three months of sequential passages. The viral titers were determined through a standard methylcellulose plaque assay (Hashimoto et al., 2009). The clone A was used for further studies.

Reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR) analyses

The total cellular RNA was extracted and reverse-transcribed using the cloned AMV Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA). The cDNA was PCR-amplified with primer sets for the RSV N gene (sense, 5'-CAAATACAAAGATGGCTCT-3', and antisense, 5'-ACATACCTATTAACCCAGT-3'), the human RIG-I gene (sense, 5'-GTGGAATCACGG ATTAGC-3', and anti-sense, 5'-TTGTCTGGCATC TGGAAC-3'), the human TLR3 gene (sense, 5'-GAT GCTCCGAAGGGTGG-3', and anti-sense, 5'-AGG GTTTGCGTGTTTCC-3'), and the human GAPDH gene (sense, 5'-TGATGACATCAAGAAGGTGG-3', and antisense, 5'-TTACTCCTTGGAGGCCATGT-3'). The qPCR reactions were performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Duplicate cycle threshold (CT) values were analyzed using the comparative CT ($2\Delta\Delta$ CT) method (Applied Biosystems, Foster City, CA, USA). The relative amount of target mRNA ($2\Delta\Delta CT$) was obtained by normalizing to the endogenous GAPDH level. The RT-PCR primer sets were as follow: for IFN-B, forward; 5'-AACAAGTGTCTCCTCCAAA-3', reverse; 5'-TCTTCCAGGACTGTCTTCA-3', for 2,5-OAS1, Forward; 5'-AAGGTGGTAAAGGGTGGCT-3', reverse; 5'-GTGCTTGACTAGGCGGATG-3'; MxA forward 5'-GTTTCCGAAGTGGACATCGCA-3' and reverse 5'-GAAGGGCAACTCCTGACAGT-3'; the thermal cycler conditions were as follows. The reactions were denatured for 2 min at 95 °C and then run for 40 cycles of denaturation for 30 s at 95 °C, annealed for 30 s at 62.5 °C. The products were separated on 2% agarose gels and

visualized through ethidium bromide staining.

Immunofluorescence microscopy

The cells were fixed, permeabilized in cold acetone, exposed to 5% nonfat milk to block nonspecific antibody binding, and then incubated with a specific goat anti-RSV F protein polyclonal antibody conjugated to fluorescent isothiocyanate (FITC) at a concentration of 1:500 (Chemicon International Inc., Temecula, CA, USA). The RSV antigen-positive cells (green fluorescence) were counted from five different randomly selected fields on two slides from each group and observed under fluorescence microscopy (Olympus, Japan).

Enzyme-linked immunosorbent assay (ELISAs)

The medium of tenth-generation RSV-persistent cells was refreshed when the cells grew to 60% confluence, and the supernatants were collected 8 h later to determine the IFN- β concentration through ELISA. For acute infection, RSV A2 was inoculated into cells grown to 70% confluence at MOI 3, and the supernatants were collected 8 h later for testing. For poly I:C, HEp-2 cells grown to 70% confluence were pretreated with poly I:C at a final concentration of 10 µg/mL, and the supernatants were collected 8 h later for testing. The concentrations of cytokines in duplicate were determined using a commercial sandwich enzyme-linked immunosorbent assay kit (PBL Biomedical Laboratories, Piscataway, NJ, USA) according to the manufacturer's protocol.

Short-interfering RNA (siRNA)-mediated targeted gene silencing

For siRNA transfection, cationic lipid complexes were prepared according to the instructions for Stealth[™] RNAi-Lipofectamine[™] 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). HEp-2 cells were transfected with 50 nmol/L human siRNA (Invitrogen Co., Shanghai, China) 24 h before RSV infection using Stealth[™] RNAi-Lipofectamine[™] 2000. Briefly, 50 nmol/L siRNA was added to a mixture of antibiotic-free MEM-FBS and Stealth[™] RNAi-Lipofectamine[™] 2000, and the mixture was incubated for 10 min at room temperature. For transfection, 400 µL of this mixture was added to 24-well plates. The cells were then subjected to qPCR or western blot analysis. Control siRNA was purchased from Invitrogen. The sequences for the human SOCS1 (NM 003745), TLR3 (NM 003265), and RIG-I (NM 014314) siRNAs were as follows: human SOCS1 siRNA, 5'-GCAUCCGCGUGCACUUUCAdTdT-3'; human TLR3, 5'-GCUCGAUCUUUCCUA CAACdTdT-3'; and human RIG-I siRNA, 5'-GCAAGAUCUUACUCA GAGAdTdT-3'. The final concentration of siRNA was 20 nmol/L in all experiments. The transfected cells were placed in a 5% CO_2 incubator at 37 °C.

Western blotting

The cells were lysed in buffer containing 20 mmol/ L Tris-HCl (pH 7.4), 0.5% sodium deoxycholate, 10% glycerol, 150 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/ L glycerophosphate, 2 mmol/L Na₃VO₄, 10 mmol/L NaF, 1 mmol/L DTT, 1 mmol/L PMSF, and 0.1% protease inhibitor cocktail (Roche, Penzberg, Germany). The protein concentrations were determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). The proteins were electrophoresed on SDS polyacrylamide gels and electroblotted onto polyvinyldifluoride membranes (Immobilon, Millipore, Schwalbach, Germany). The membranes were incubated with primary antibodies: goat anti-human SOCS1 pAb, rabbit anti-human SOCS-3 pAb, mouse anti-human STAT1/2 pAb, rabbit anti-human phosphorylated STAT (pSTAT) 1/2 (Santa Cruz, CA, USA), rabbit anti-human RIG-I pAb, rabbit anti-human TLR3 pAb (Cell Signaling Technology, MA, USA), and mouse anti-human β -actin mAbs (Sigma-Aldrich). The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin antibody for 1 h. The antibody-reactive protein bands were visualized by enhanced chemiluminescence (ECL) and western blotting detection reagents (Amersham, Buckinghamshire, UK) and documented with an Image Reader. The density of each band was measured using the NIH image program.

Statistical analysis

The results are expressed as the means \pm SE, the significance of the data was analyzed using paired Student's t tests; p < 0.05 was considered significant.

RESULTS

Characterization of RSV-persistent cells

Using the clone cylinders and three rounds of limited diluted selection, eight RSV-surviving clones (A–H) were obtained, and the RSV *N* gene was detected in each "passage 0" culture through qPCR. The number of *N* gene copies of each clone ranged from 2.5×10^2 to 3.5×10^5 copies/mL. During the following months of sub-cloning and passaging, these RSV-surviving clones exhibited two distinct development profiles. Five clones (A, B, E, G, and H) grew slowly with a moderate CPE. In contrast, clones C, D, and F lost their viral infection characteristics; *i.e.*, these clones showed no morphological signs of infection, did not produce detectable viral particles, and did not present any viral antigens and *N* genes (not detected by immunofluorescence or qPCR) (Figure 1A).

From the sub-clones with the same characteristics of RSV persistence, clone A was picked up for subsequent analyses, and 10 generations of RSV-positive sub-clones were consecutively isolated by seeding half of the cells



in each dilution per well on 96-well plates. After each round of selection, a single cell always grew and differentiated into two distinct states: one that maintained the RSV-persistent characteristics and another that had lost RSV infection characteristics. The RSV-persistent cells grew slowly and formed syncytia. In contrast, neither CPE nor viral antigen could be observed in the clones that lost RSV-infected characteristics. To further confirm that RSV-persistent cells differentiated into two distinct types, the viral antigen (F protein) was detected using



Figure 1. (A) The protocol for the isolation of RSV-persistent cells and quantification of RSV N gene copies. Clones A-H (passage 0) were seeded on 12-well plates at a density of 1 × 105/mL. Seventy-two hours after plating, the total cellular RNA was extracted, and qPCR was performed to quantify the RSV N gene copies. The qPCR products were visualized on 2% agarose gels. (B) Sub-cloning of clone A and characterization. Ten rounds of limiting dilution were performed, and each generation was examined for F antigen, N gene, and CPE. Representations of RSV-persistent cells under fluorescent microscopy. White arrow: RSV positive sub-clone A; yellow arrow: RSV negative sub-clone A. an immunofluorescence assay. In each generation of the RSV-persistent sub-clone A, 40%–50% of the cells were RSV F antigen-positive and the remaining cells were RSV antigen-negative (Figure 1B).

The cells with RSV-persistent characteristics exhibited a periodic cell crisis after growing to almost 80% confluence with a severe CPE. Therefore, the RSV-persistent cells were always passaged at 70% confluence to avoid severe CPE. No CPE can be observed in RSV negative sub-clone A (Figure 2A). However, when re-infected with wild-type RSV A2, the RSV-persistent cells were resistant to the re-infection (Figure 2B). These results



Figure 2. (A) Characteristics of RSV-persistent cells. The top row shows the clone A, at day 14 (surviving cells), day 18 (cell sheet), and day 21 (monolayer) post RSV infection. The middle row shows that the RSV positive sub-clone A (RSV-persistent cells) grew with a moderate CPE, cell fusion at 80% confluence, and cell crisis. The bottom row shows that the RSV negative sub-clone A lost its viral infection characteristics and exhibited "normal" cell morphology during passages 1 to 3, respectively (20× objective, scale bar 100 µm). (B) Detection of RSV F antigens after RSV A2 re-infection. From left to right, RSV F antigen (green), natural light (red filter), and merge, respectively (20× objective, scale bar 100 µm). Quantification is shown in the histogram on the right.



suggest that the RSV-persistent clone A is heterogeneous with a mixture of variants, and that a single RSVpersistent cell can divide into an RSV-persistent population and an RSV-free population, in which the former is a mixture of RSV antigen-positive and -negative cells, and the latter consists of cells that have lost their RSV infection characteristics.

Viral replication in RSV-persistent cultures

To analyze RSV propagation and virulence during the persistent state, the tenth generations of the RSV-positive sub-clone A (RSV-persistent cells) and RSV negative subclone A were selected for testing. Cells at 60% confluence were transferred to fresh 2% MEM-FBS medium, and the supernatants of the cultures were collected for plaque assays at various time points. The viral yields of sub-clone A with RSV were relatively low and ranged from 1×10^3 to 1.4×10^4 plaque-forming units (pfu)/mL over the next six days. In contrast, the viral yields of RSV negative sub-clone A reached a peak of 1.5×10^6 pfu/mL on day 3 when re-infected with wild-type RSV A2 at MOI 1 (Figure 3), which is the same result obtained with primary RSV A2 infection in HEp-2 cells. The original HEp-2 cells were then inoculated with the supernatants of the RSV-persistent sub-clone A at MOI 1. CPE was observed at 48 h post-infection, and the viral titer increased to 1 \times 10⁶ pfu/mL. When RSV-persistent cells were re-infected with RSV A2 at the same MOI used to establish acute infection, the viral propagation and cell morphology remained unchanged. These results demonstrate that after their development into distinct cell clones, the RSVpersistent cells retained a low rate of replication with the ability to be infective and virulent as well as the ability to resist RSV re-infection, whereas the other type of clone lost its RSV infection ability, became morphologically "normal", and was unable to defend itself against RSV infection.

Antiviral gene expression levels and negative regulation in RSV-persistent cells

Because viral replication induces antiviral gene expression mainly through the IFN pathway, it could be speculated that this signaling activation is blocked by some negative regulatory mechanism. Therefore, the expression levels of IFN- β were detected using ELISA and the negative regulatory molecules SOCS1 and SOCS3 were detected using western blot.

The RSV-persistent cells were found to secrete more IFN- β (340 ± 110 pg/L) compared to cells with acute RSV infection (160 ± 70 pg/L). Previous studies have confirmed that RSV lytic infection causes transient expression of SOCS1 and SOCS3 within 24 h and that this expression decreases to the basic level after this time period (Hashimoto et al., 2009). In this study, a western

blot analysis was performed 8 h after the RSV A2 infection and the medium of the RSV-persistent culture was refreshed. SOCS1 expression significantly increased in the RSV-persistent, acute infection, and poly I:C-treated cells. In contrast, SOCS3 was only weakly expressed in the RSV-persistent cells compared with strong expression observed in the cells with RSV acute infection (Figure 4A). The qPCR results demonstrated that both acute and persistently infected cells displayed up-regulation of either *RIG-I* or *TLR3* mRNA, although a higher level of TLR3 mRNA expression was observed in the RSVpersistent cells (Figure 4B). Viral replication up-regulated the expression of innate immunity-related molecules and activated the IFN- β signaling pathway, as observed in RSV acute infection, and the negative regulatory molecule SOCS1 was also upregulated, which may contribute to impairment of the host's innate antiviral response.

Upregulation of SOCS1 is related to TLR3 activation in RSV-persistent cells

To clarify whether SOCS1 expression is modulated by RIG-I or TLR3 activation, RNA interference was performed to inhibit the target genes. The tenth generation of RSV-persistent cells were transferred to fresh medium



Figure 3. Viral replication in RSV-persistent cultures. The tenth generations of RSV-persistent cells, RSV-negative sub-clone A, and HEp-2 cells were seeded into 12-well plates, and exposed to RSV A2 at a MOI of 1 (re-infection), respectively. In addition, HEp-2 cells were infected with the supernatants of RSV-persistent cells (positive sub-clone A) at MOI 1. Supernatants of all above four situations, together with RSV persistent cells, were collected for a plaque assay at the indicated times.



Figure 4. (A) Determination of IFN-β concentration and SOCS1 and SOCS3 expression in RSV-persistent cells. The RSV-persistent cells were seeded on 24well plates, and the medium was refreshed when cells grew to 70% confluence. After 8 h of culture, duplicate supernatants were collected for the determination of IFN-β concentration by ELISA, and the cellular proteins were harvested for a western blot assay. For RSV acute infection, HEp-2 cells grown to 70% confluence were inoculated with RSV at a MOI of 3 for 8 h, and the supernatants and cellular proteins were collected. HEp-2 cells pretreated with poly I:C (10 µg/mL) for 8 h were used as the IFN-ß induction controls. The SOCS1 and SOCS3 protein levels were optimized to a value of 1 relative to the poly I:C-treated cells. Data are shown as the relative density compared to the internal control β -actin of three independent experiments. *p < 0.05vs. untreated cells (mock). (B) RIG-I and TLR3 mRNA expression in RSV-persistent cells. The medium was refreshed when the cells grew to 70% confluence. The total cellular RNA was extracted for the qPCRassay after 8 h of culture. The data were normalized to GAPDH mRNA levels and are shown as the mean ± SE of three independent experiments. *p < 0.05 vs. acute infection.

and treated with siRNAs against RIG-I or TLR3 at a final concentration of 20 nmol/mL for the indicated times. The cellular extracts were collected for western blot analysis. Inhibition of TLR3 significantly decreased SOCS1 expression at 12 h after siRNA transfection in RSVpersistent cells (Figure 5A), whereas inhibition of RIG-I had no effect on the expression of SOCS1 (Figure 5B). Furthermore, the mRNA levels of *IFN-\beta* and its inducible genes MxA and 2,5-OAS1 were detected using RT-PCR. As shown in Figure 5C, the expression of *IFN-\beta*, MxA, and 2.5-OAS1 mRNAs decreased over time when TLR3 was silenced, whereas no change was observed when *RIG-I* was silenced (Figure 5D). These data indicate that the expression of SOCS1 is related to activation of the TLR3 signaling pathway in RSV-persistent cells, and also contributes to activation of the IFN-\beta-inducible antiviral response.

SOCS1 modulates STAT1/2 phosphorylation in **RSV-persistent cells**

The upregulation of SOCS1 could be related to the balance between innate antiviral immunity and viral replication in RSV-persistent cultures. To clarify the function of SOCS1 on JAK/STAT signaling transduction in RSV-persistent cells, SOCS1 was silenced using siR-NA at a final concentration of 20 nmol/mL for 24 h, and the cellular extracts were collected to detect the level of STAT1/2 and pSTAT1/2 by western blot. As shown in Figure 6A, the RSV-persistent sub-clone A expressed low levels of phosphorylated STAT1 and STAT2. Silencing of SOCS1 significantly increased the phosphorylation levels of both STAT1 and STAT2. This result implies that upregulation of SOCS1 impairs the innate antiviral response by decreasing pSTAT1 and pSTAT2. Effects of SOCS1 and TLR3 on the expression of chemokines were also observed. RSV-persistent cells were treated with siRNAs against SOCS1 or TLR3 for 24 h, respectively, and the culture supernatants were collected and analyzed using ELISA. No changes in Mip-1 α , Rantes, and IL-8 levels were detected in the SOCS1-silenced cells compared to untreated persistent cells, whereas inhibition of TLR3 sharply decreased the secretion of Mip-1 α , Rantes, and IL-8 (Figure 6B). Therefore, despite the fact that SOCS1 inhibits endogenous IFN- β signaling but does not affect TLR3 signaling in chemokine activation, RSVpersistent cells avoid the excessive expression of IFN- β and maintain an adaptable antiviral response.

DISCUSSION

In this study, we used purified RSV to infect human epithelial cells and established persistent virus-infected clones. The results demonstrate that RSV-persistent cells are heterogeneous and contain different variants:





Figure 5. Expression of SOCS1 and SOCS3 in RSV-persistent cells. Cells of the RSV-persistent cells were grown to 70% confluence and pretreated with short interfering RNA (siRNA; 20 pmol/mL) for the indicated times; the mismatched siRNA were assigned as controls (mock). The cellular proteins were then harvested for a western blot assay. (A) Expression of SOCS1 in RSV-persistent cells with *TLR3* inhibition. (B) Expression of SOCS1 in RSV-persistent cells with *TLR3* inhibition. (B) Expression of SOCS1 in RSV-persistent cells with *RIG-I* inhibition. Data are normalized to the internal control β -actin and are shown as the relative density compared to time zero as 1. All data shown are the means ± SE of three independent experiments. *p < 0.05 vs. time zero. Expression of interferon interferon- β (IFN- β) and its inducible genes (*MxA, 2,5-OAS*) in RSV-persistent cells (C, D). Cells were pretreated with siRNA and total cellular RNA was extracted for RT-PCR to detect the target genes. The data are representative of one of three identical results.

during the passage of cells, half of the cells were viral antigen-positive, and the other half were viral antigen-negative. These RSV-persistent cells secreted high levels of cytokines/chemokines, and showed upregulated expression of SOCS1, which helps the virus to impair the innate antiviral response by inhibiting STAT1/2 phosphorylation.

A cloned single RSV-persistent cell could always develop into a differentiated viral-persistent and "virus-free" culture, but the mechanism of this phenomenon is not clear. One possible mechanism may be the imbalance in the abundance of viral particles and the innate immune status between the two cell types; cells with more viral particles and an impaired innate immune are more prone to be RSV-positive, whereas those with fewer viral particles and innate immune activation are more prone to become RSV-negative. In the heterogeneous persistent culture, the survival of RSV-negative cells depends on the coexistence of RSV-positive cells, because the former cannot resist re-infection alone. This result is consistent with Martinez *et al.* 's observation that the RSV-persistent culture contains cells with variable morphology (Martínez et al., 2009), and that apoptosis of persistently infected cells is inhibited through induction of the expression of anti-apoptotic genes and inhibition of pro-apoptotic genes (Herranz et al., 2011).

At the early phase of acute RSV infection, a small amount of IFN- β is secreted and there is transient expres-



Figure 6. (A) STAT1 and STAT2 phosphorylation in RSV-persistent cells. RSV-persistent cells were grown to 70% confluence and pretreated with siRNA against *SOCS1* (siSOCS1) for 24 h. The cellular proteins were harvested for a western blot assay to detect the levels of STAT1, STAT2, pSTAT1, and pSTAT2. Data are normalized to levels of the internal control β -actin and are shown as the relative density compared to untreated cells (designated as 1). (B) Cells were pretreated with siRNA against *SOCS1* or *TLR3* for 24 h. The duplicate supernatants were subjected to ELISA to determine the chemokine concentrations. **p* < 0.05 vs. untreated cells.

sion of SOCS1, which constitutes a feedback regulation mechanism that inhibits the IFN-β-inducible activation of antiviral gene expression (Zhao et al., 2007; Hashimoto et al., 2009). Unlike RSV acute infection, in this study, both abundant IFN-β secretion and SOCS1 overexpression were consecutively found in RSV-persistent cells. SOCS1 is well known as a strong negative regulatory factor that limits the extent of TLR3 signaling by inhibiting type I IFN signal transduction (Baetz et al., 2004). Furthermore, RSV nonstructural proteins could up-regulate SOCS1 expression independently of the TLR3 and RIG-I signaling pathways, and such upregulation of SOCS1 could be responsible for the inhibition of STAT2 activation (Xu et al., 2014). These studies imply that RSV becomes latent or persistent by subverting the innate antiviral response; however, it is not clear why the persistent viral cells survived under virus replication. One possibility is that the cells developed the ability to resist RSV interference of innate immunity, and thus a balance existed between viral replication and cell proliferation. In RSV-persistent cells, the silencing of TLR3 decreased SOCS1 expression, which suggests that TLR3 contributes to the upregulation of SOCS1. However, the overexpression of SOCS1 did not completely inhibit STAT1/2 phosphorylation, and the IFN-β-stimulated genes MxA and 2,5-OAS were still expressed. The incomplete block of the effects of IFN-β via SOCS1 may lead to a balance between viral replication and clearance. The RSV-persistent cells are found in an inflammatory state characterized by the secretion of high amounts of the type II cytokines Mip-1a, IL-8, and Rantes, which are related to the TLR3 pathway (Groskreutz et al., 2006). The

silencing of *TLR3* decreased the secretion of cytokines in persistent cells, which indicates that RSV replication upregulates the expression of cytokines in a TLR3 activation-dependent manner. Furthermore, the overexpression of SOCS1 had no effect on TLR3-induced expression of Mip-1 α , IL-8, and Rantes.

Taken together, our results demonstrate that in a persistent culture, mediated by the activation of TLR3, RSV replication induced the production of cytokines and suppressed innate antiviral signaling by upregulation of SOCS1.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

AUTHOR CONTRIBUTIONS

JZ, PY and YT carried out the experiments. PY and DZ performed the statistical analysis. DZ conceived and supervised the study, and worte the manuscript. All authous read and approved the final manuscript.



REFERENCES

- Baetz A, Frey M, Heeg K, Dalpke AH. 2004. Suppressor of cytokine signaling (SOCS) proteins indirectly regulate toll-like receptor signaling in innate immune cells. J Biol Chem, 279: 54708–54715.
- Bangham CRM, McMichael AJ. 1986. Specific human cytotoxic T cells recognize B cell lines persistently infected with respiratory syncytial virus. Proc Natl Acad Sci USA, 83: 9183–9187.
- Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, Heinrich PC, Häussinger D. 2003. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. Faseb J, 17: 488–490.
- Borg I, Rohde G, Löseke S, Bittscheidt J, Schultze-Werninghaus G, Stephan V, Bufe A. 2003. Evaluation of quantitative real-time PCR for the detection of respiratory syncytial virus in pulmonary diseases. Eur Respir J, 21: 944–951.
- Dalpke A, Heeg K, Bartz H, Baetz A. 2008. Regulation of innate immunity by suppressor of cytokine signaling (SOCS) proteins. Immunobiology, 213: 225–235.
- Falsey AR, Formica MA, Hennessey PA, Criddle MM, Sullender WM, Walsh EE. 2006. Detection of respiratory syncytial virus in adults with chronic obstructive pulmonary disease. Am J Respir Crit Care Med, 173:639–643.
- Groskreutz DJ, Monick MM, Powers LS, Yarovinsky TO, Look DC, Hunninghake GW. 2006. Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells. J Immunol, 176: 1733–1740.
- Hashimoto K, Ishibashi K, Ishioka K, Zhao D, Sato M, Ohara S, Abe Y, Kawasaki Y, Sato Y, Yokota S, Fujii N, Peebles RS Jr, Hosoya M, Suzutani T. 2009. RSV replication is attenuated by counteracting expression of the suppressor of cytokine signaling (SOCS) molecules. Virology, 391:162–170.
- Herranz C, Melero JA, Martínez I. 2011. Reduced innate immune response, apoptosis, and virus release in cells cured of respiratory syncytial virus persistent infection. Virology, 410: 56–63.
- Hobson L, Everard ML. 2008. Persistent of respiratory syncytial virus in human dendritic cells and influence of nitric oxide. Clin Exp Immun, 151: 359–366.
- Jeanette I, Webster M, Jacqueline C. 2013. Poly I:C and respiratory syncytial virus (RSV) inhibit glucocorticoid receptor (GR)-mediated transactivation in lung epithelial, but not monocytic, cell lines. Virus Res,176: 303–306.
- Le Goffic R, Pothlichet J, Vitour D, Fujita T, Meurs E, Chignard M, Si-Tahar M. 2007. Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. J Immunol, 178: 3368–3372.
- Liu P, Jamaluddin M, Li K, Garofalo RP, Casola A, Brasier AR. 2007. Retinoic acid inducible gene-I mediates early anti-viral response and toll like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. J Virol, 81: 1401– 1411.
- Martínez I, Lombardía L, Herranz C, García-Barreno B, Domínguez O, Melero JA. 2009. Cultures of HEp-2 cells persistently infected by human respiratory syncytial virus differ in chemokine expression and resistance to apoptosis as compared to lytic infections of the same cell type. Virology, 388: 31–41.
- Matsuse H, Behera AK, Kumar M, Rabb H, Lockey RF, Mohapatra SS. 2000. Recurrent respiratory syncytial virus infections in allergen-sensitized mice lead to persistent airway inflammation and hyperresponsiveness. J Immunol, 164: 6583–6592.

- Oshansky CM, Krunkosky TM, Barber J, Jones LP, Tripp RA. 2009. Respiratory syncytial virus proteins modulate suppressors of cytokine signaling 1 and 3 and the type I interferon response to infection by a toll-like receptor pathway. Viral Immunol, 22: 147–161.
- Pothlichet J, Chignard M, Si-Tahar M. 2008. Cutting Edge: Innate immune response triggered by influenza A virus is negatively regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-dependent pathway. J Immunol, 80: 2034–2038.
- Prêle CM, Woodward EA, Bisley J, Keith-Magee A, Nicholson SE, Hart PH. 2008. SOCS1 regulates the IFN but not NFκB pathway in TLR-stimulated human monocytes and macrophages. J Immunol, 181: 8018–8026.
- Psarras S, Papadopoulos NG, Johnston SL. 2004. Pathogenesis of respiratory syncytial virus bronchiolitis-related wheezing. Paediatr Respir Rev, (Suppl A): 179–184.
- Riedel F, Oberdieck B, Streckert HJ, Philippou S, Krusat T, Marek W. 1997. Persistence of airway hyperresponsiveness and viral antigen following respiratory syncytial virus bronchiolitis in young guinea-pigs. Eur Respir J, 10: 639–645.
- Schwarze J, O'Donnell DR, Rohwedder A, Openshaw PJ. 2004. Latency and persistence of respiratory syncytial virus despite T cell immunity. Am J Respir Crit Care Med, 169: 801–805.
- Seemungal T, Harper-Owen R, Bhowmik A, Moric I, Sanderson G, Message S, Maccallum P, Meade TW, Jeffries DJ, Johnston SL, Wedzicha JA. 2001. Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. Am J Respir Crit Care Med, 164: 1618–1623.
- Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. 2000. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. Am J Respir Crit. Care Med, 161: 1501–1507.
- Sikkel MB, Quint JK, Mallia P, Wedzicha JA, Johnston SL. 2008. Respiratory syncytial virus persistence in chronic obstructive pulmonary disease. Pediatr Infect Dis J, 27(Suppl): 63–70.
- Stowell NC, Seideman J, Raymond HA, Smalley KA, Lamb RJ, Egenolf DD, Bugelski PJ, Murray LA, Marsters PA, Bunting RA, Flavell RA, Alexopoulou L, San Mateo LR, Griswold DE, Sarisky RT, Mbow ML, Das AM. 2009. Long-term activation of TLR3 by Poly (I: C) induces inflammation and impairs lung function in mice. Respir Res, 10: 43.
- Tripp RA. 2004. The brume surrounding respiratory syncytial virus persistence. Am. J Respir Crit Care Med, 169: 778–779.
- Valdovinos MR, Gomez B. 2003. Establishment of respiratory syncytial virus persistence in cell lines: association with defective interfering particles. Intervirology, 46:190–198.
- Vlotides G, Sörensen AS, Kopp F, Zitzmann K, Cengic N, Brand S, Zachoval R, Auernhammer CJ. 2004. SOCS-1 and SOCS-3 inhibit IFN-alpha-induced expression of the antiviral proteins 2,5-OAS and MxA. Biochem Biophys Res Commun, 320: 1007–1014.
- Xu X, Zheng J, Zheng K, Hou Y, Zhao F, Zhao D. 2014. Respiratory syncytial virus NS1 protein degrades STAT2 by inducing SOCS1 expression. Intervirology, 57: 65–73.
- Yasukawa H, Yajima T, Duplain H, Iwatate M, Kido M, Hoshijima M, Weitzman MD, Nakamura T, Woodard S, Xiong D, Yoshimura A, Chien KR, Knowlton KU. 2003. The suppressor of cytokine signaling-1 (SOCS1) is a novel therapeutic target for enterovirus-induced cardiac injury. J Clin Invest, 111: 469–478.
- Zhao DC, Tang Y, Li L, You SY, Zhang CY. 2007. Respiratory syncytial virus inhibits interferon-α-inducible signaling in macrophage-like U937 cells. J Infect, 54: 393–398.