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*J Immunol* 2008; 180:2034-2038; doi: 10.4049/jimmunol.180.4.2034

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Cutting Edge: Innate Immune Response Triggered by Influenza A Virus Is Negatively Regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-Dependent Pathway

Julien Pothilhet, Michel Chignard, and Mustapha Si-Tahar

Influenza A virus (IAV) triggers a contagious respiratory disease that produces considerable lethality. Although this lethality is likely due to an excessive host inflammatory response, the negative feedback mechanisms aimed at regulating such a response are unknown. In this study, we investigated the role of the eight “suppressor of cytokine signaling” (SOCS) regulatory proteins in IAV-triggered cytokine expression in human respiratory epithelial cells. SOCS1 to SOCS7, but not cytokine-inducible Src homology 2-containing protein (CIS), are constitutively expressed in these cells and only SOCS1 and SOCS3 expressions are up-regulated upon IAV challenge. Using distinct approaches affecting the expression and/or the function of the IFNαβ receptor (IFNAR1), the viral sensors TLR3 and retinoic acid-inducible gene I (RIG-I) as well as the mitochondrial antiviral signaling protein (MAVS, a RIG-I signaling intermediate), we demonstrated that SOCS1 and SOCS3 up-regulation requires a TLR3-independent, RIG-I/MAVS/IFNAR1-dependent pathway. Importantly, by using vectors overexpressing SOCS1 and SOCS3 we revealed that while both molecules inhibit antiviral responses, they differentially modulate inflammatory signaling pathways.

Influenza A virus (IAV) is the etiological agent of a contagious acute respiratory disease. This virus is a major public health threat, killing >30,000 people annually in the United States of America alone, sickening millions, and inflicting substantial economic costs (1, 2). There is evidence that IAV infections are up-regulated upon IAV challenge. Using distinct approaches affecting the expression and/or the function of the IFNαβ receptor (IFNAR1), the viral sensors TLR3 and retinoic acid-inducible gene I (RIG-I) as well as the mitochondrial antiviral signaling protein (MAVS, a RIG-I signaling intermediate), we demonstrated that SOCS1 and SOCS3 up-regulation requires a TLR3-independent, RIG-I/MAVS/IFNAR1-dependent pathway. Importantly, by using vectors overexpressing SOCS1 and SOCS3 we revealed that while both molecules inhibit antiviral responses, they differentially modulate inflammatory signaling pathways. The Journal of Immunology, 2008, 180: 2034–2038.

Influenza A/Scotland/20/74 (H3N2) virus was prepared as indicated in Ref 13. Short interfering RNA (siRNA)-mediated gene silencing

siRNA against human RIG-1 (catalog no.M-012511-00), human IFNαβ receptor 1 (IFNAR1; catalog no.M-020209-00), human mitochondrial antiviral...
signaling protein (MAVS) (MAVS-1: 5'-UAUGUGAUCUCGGGAGGAAUUAAGUAUCGUUUGCAAGCAAGAdTdT3' (sense) and 5'-UUGUGAUCUCGGGAGGAAUUAAGUAUCGUUUGCAAGCAAGAdTdT3' (antisense); MAVS-2: 5'-CUGGCUUGAGCUAAAGACGAAAGAdTdT3' (sense) and 5'-UCUGUGCUUCGGGAGGAAUUAAGUAUCGUUUGCAAGCAAGAdTdT3' (antisense)) as well as a control siRNA (catalog no.D-001206-13) were obtained from Dharmacon Research. The human bronchial epithelial BEAS-2B cells were described previously (13). The cells were transiently transfected with siRNA at 100 nM or a mixture of siRNA (encoding a functional form of either SOCS1 (generously provided by Dr. J. Hiscott, McGill University, Montreal, Canada), SOCS3 (20 g/ml; Acris Antibodies), and -actin (diluted 1/10,000; Sigma-Aldrich). Bound Abs were detected using ECL immuno-blotting detection system (Amersham Biosciences).

Transfection of pulmonary epithelial cells and reporter gene assays

Stably transfected pZero-hTLR3 or control BEAS-2B cells were described previously (12). Concerning the reporter gene studies, BEAS-2B cells were transfected using FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions. Cells were transiently cotransfected with 100 ng of an NF-kB reporter plasmid (generously provided by Prof. N. Mukaida, Kanazawa University, Kanazawa, Japan), an IFN- promoter-, or an IRF-3-luciferase reporter plasmid (generously provided by Dr. J. Hiscott, McGill University, Montreal, Canada), 50 ng of prSV-galactosidase to control DNA uptake, and 100 ng of a vector encoding a functional form of both SOCS1 (generously provided by Dr. A. Duschl, University of Salzburg, Salzburg, Austria) or SOCS3 (a generous gift of Dr. K. Ghosal, National Institute of Immunology, New Delhi, India) or the respective control empty vector. After 24 h, cells were infected or not infected (i.e., mock) with IAV (MOI = 1). Then, cells were processed as previously reported (12). Results are expressed as relative luciferase units (RLU) normalized with -galactosidase activity.

Statistical analysis

Statistical differences between SOCS levels in mock-treated and IAV-infected cells or in pZero-hTLR3 relative to control BEAS-2B cells infected with IAV were tested using a one-way ANOVA followed by a Fisher test, with a threshold of p < 0.05.

Results and Discussion

IAV infection induces SOCS1 and SOCS3 up-regulation in human lung epithelial cells.

Increasing evidence suggests that SOCS proteins are important regulators for cytokine signaling (17, 18). However, no information is available concerning the constitutive SOCS expression in lung epithelial cells as well as their regulation following infection with IAV. This prompted us to perform a qRT-PCR analysis of the eight SOCS proteins in unstimulated human bronchial epithelial BEAS-2B cells. Fig. 1A shows that SOCS1–7 are constitutively expressed whereas CIS remains under the detection limit. SOCS5 is the least expressed SOCS. Interestingly, constitutive expression of SOCS is rather heterogeneous, with the highest expressions for SOCS3, SOCS6, and SOCS1 relative to SOCS5.

Because inhibition or enhancement of SOCS expression may play an important role in the regulation of innate immune responses of IAV-infected epithelial cells, we conducted a kinetic study of SOCS levels in mock-treated and IAV-infected BEAS-2B cells (Fig. 1B). Cells were infected with an amount of virus previously shown to potently activate these cells (12, 13). As illustrated in Fig. 1B, among all SOCS expressed in bronchial epithelial cells, only SOCS1 and SOCS3 are significantly up-regulated upon IAV infection (p < 0.0001). Increase in SOCS1 and SOCS3 mRNA expression was confirmed at the protein level (Fig. 1C). CIS expression is not set off after IAV challenge (data not shown). Regardless, it is rather difficult at this stage to compare the expression and regulation profiles of SOCS molecules found here in respiratory epithelial cells to other cell types, although several studies did describe the regulated expression of some SOCS, mainly SOCS1 and SOCS3, in several tissues. For instance, an elevated expression of SOCS3 was observed in fibroblast cells expressing the hepatitis C virus core (20) or in an amnion cell line infected by the herpes simplex virus type 1 (21). Also, the immunosuppressive HIV 1 Nef

![FIGURE 1. SOCS expression levels in resting and IAV-infected human bronchial epithelial cells. A, qRT-PCR analysis of the constitutive expression of SOCS1–7 and CIS in BEAS-2B cells. Results are represented relative to SOCS5, the least expressed SOCS in these cells as well as in cycle threshold (CT) values. ND, Not detected. B, Levels of SOCS mRNA in BEAS-2B cells infected with IAV (MOI = 1) or treated with mock for various times. SOCS expression was normalized with the -actin level and expressed as fold increase relative to mock-treated cells at each corresponding time. Data are means ± SD of quadruplicate qRT-PCR. A representative result of three independent experiments is shown. C, Representative immunoblot showing SOCS1 and SOCS3 protein levels in BEAS-2B cells treated with mock or infected with IAV (MOI = 1) for various periods. To confirm similar gel loading, membranes were probed with an anti-actin Ab.](http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/)
protein induces SOCS1 and SOCS3 in B cells (22). However, our study is the first to our knowledge to give an extensive qualitative and quantitative expression pattern of the eight SOCS in a given cell type, especially in both a resting condition and after IAV challenge.

IAV-induced SOCS1 and SOCS3 up-regulation is TLR3-independent

We recently demonstrated that the host recognition of IAV and the subsequent cytokine responses require at least two major viral sensors, i.e., TLR3 and RIG-I (12, 13). To better understand the specific contribution of TLR3 vs RIG-I-dependent signaling in IAV-induced SOCS1 and SOCS3 up-regulation, we first examined the role of TLR3 using the previously described pZero-hTLR3 BEAS-2B cells that constitutively express a dominant negative, nonfunctional form of TLR3 (12). Interestingly, the kinetics of the SOCS1 and SOCS3 levels were statistically similar in both control and pZero-hTLR3 BEAS-2B cells infected with IAV (p > 0.05; Fig. 2A), meaning that IAV induces SOCS1 and SOCS3 up-regulation in a TLR3-independent pathway.

A RIG-I/MAVS- and IFNAR1-dependent pathway mediates SOCS1 and SOCS3 up-regulation in IAV-infected bronchial epithelial cells.

The foregoing findings led us to further evaluate the role of RIG-I-dependent signaling in SOCS expression. To inhibit RIG-I signaling pathways, we knocked down RIG-I and its signaling intermediate MAVS by using specific siRNA. Fig. 2B (left and central panels) indicates that the silencing condition of RIG-I and MAVS signaling was effective as assessed by qRT-PCR. Using these reagents, we noticeably reveal that SOCS1 and SOCS3 up-regulation by IAV is RIG-I and MAVS dependent (Fig. 2C).

Many molecules can regulate SOCS expression, including hormones, colony-stimulating factors, and cytokines (17, 18). Among all mediators released by IAV-infected lung epithelial cells (12, 13), we focused on the central antiviral component type I IFN because it has a major impact on IAV pathogenesis and host immune response (23). We hypothesized that RIG-I-mediated expression of SOCS1 and SOCS3 could be secondary to a type I IFN positive feedback loop. To verify this assumption, we knocked down IFNAR1 expression by using specific siRNA. Using qRT-PCR, we first confirmed the effectiveness of our silencing reagents by showing that relative to control siRNA, IFNAR1 siRNA considerably inhibited IFNAR1 expression in both mock-treated and IAV-infected cells (Fig. 2B, right panel). Importantly, IAV-induced SOCS1 and SOCS3 up-regulation was prevented by IFNAR1 silencing (Fig. 2C).

Altogether, our data reliably demonstrate that RIG-I/MAVS/IFNAR1-dependent antiviral signaling is a pivot of SOCS1 and SOCS3 up-regulation in IAV-infected bronchial epithelial cells. It is noteworthy that this regulatory pathway is likely a broad molecular mechanism that can occur in cells infected by other viral pathogens, e.g., Sendai virus, respiratory syncytial virus, vesicular stomatitis virus, Newcastle disease virus, and hepatitis C virus, all of which trigger a RIG-I-dependent type I IFN secretion (24–26).

SOCS1 and SOCS3 are critical negative feedback regulators of IAV-triggered innate immune response

As Fig. 1 shows that SOCS1 and SOCS3 are among the three most constitutively expressed SOCS in bronchial epithelial cells and are the only members of this family that are up-regulated by IAV, we speculated that SOCS1 and SOCS3 might play an active role in the modulation of IAV-induced innate immune response. We examined whether SOCS1 and SOCS3 could alter IRF-3- and/or NF-κB-dependent signaling by using specific luciferase reporter plasmids as well as vectors encoding functional SOCS1 or SOCS3 or the respective control vector. We also used plasmids reporting the activity of IFN-β and IL-8 promoters, two mediators with antiviral and proinflammatory effects, respectively. Fig. 3 indicates that increasing amounts of IAV (MOI = 0.1–1) strongly stimulate the activity of all gene reporters in BEAS-2B cells transfected with a control plasmid. Interestingly, IRF-3-dependent and IFN-β antiviral pathways are significantly reduced (>60%) in IAV-infected cells overexpressing SOCS1 (Fig. 3A). By contrast, NF-κB- and IL-8-dependent proinflammatory pathways are ∼2-fold-higher in SOCS1-transfected cells than in control plasmid-transfected ones (Fig. 3A). These opposite effects of SOCS1 on antiviral...
and proinflammatory responses are in fact consistent not only with the enhancement of type I IFN signaling in Soc1−/− mice infected with the Semliki Forest virus (27) but also with a recent study showing that SOCS1 overexpression in human keratinocytes stimulated with the synthetic dsRNA polyinosinic-polycytidylic acid enhances NF-κB activity, as revealed by the increased phosphorylation of IκBα, an upstream signaling intermediate (28). In striking contrast to the SOCS1 effect, NF-κB- and IL-8 proinflammatory activities are inhibited in cells transfected with SOCS3. Noteworthily, we found that SOCS3 overexpression similarly inhibits IFN-β expression, even though IRF-3-dependent signaling was not significantly reduced (Fig. 3B). The expression of type I IFNs is strictly regulated by the activation of latent transcription factors, including NF-κB and IRF-3 (29). Thus, one could assume that the SOCS1 regulatory effect on IFN-β expression is a result of its activity on IRF-3, whereas the SOCS3 effect on the same gene is a consequence of NF-κB inhibition. The opposite effect of SOCS1 and SOCS3 on NF-κB signaling raises the question of the resultant NF-κB activity in IAV-infected bronchial epithelial cells. In fact, the different kinetics of SOCS1 and SOCS3 are critical regulators of IAV-triggered innate defense (i.e., increasing the beneficial antiviral activity while decreasing the deleterious proinflammatory aspect) that ultimately clear IAV and lead to a favorable clinical outcome.

Acknowledgments
We are grateful to Prof. A. Duschl (University of Salzburg, Salzburg, Austria) and Dr. K. Ghosal (National Institute of Immunology, New Delhi, India) for providing the SOCS1 and SOCS3 expression vectors, respectively.

Disclosures
The authors have no financial conflict of interest.

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