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Suppressor of Cytokine Signaling 3 Inhibits Antiviral IFN-β Signaling To Enhance HIV-1 Replication in Macrophages

Lisa Nowoslawski Akhtar,* Hongwei Qin,* Michelle T. Muldowney,* Lora L. Yanagisawa,* Olaf Kutsch,† Janice E. Clements,‡ and Etty N. Benveniste*

HIV-1 replication within macrophages of the CNS often results in cognitive and motor impairment, which is known as HIV-associated dementia (HAD) in its most severe form. IFN-β suppresses viral replication within these cells during early CNS infection, but the effect is transient. HIV-1 eventually overcomes this protective innate immune response to resume replication through an unknown mechanism, initiating the progression toward HAD. In this article, we show that Suppressor of Cytokine Signaling (SOCS)3, a molecular inhibitor of IFN signaling, may allow HIV-1 to evade innate immunity within the CNS. We found that SOCS3 is elevated in an in vivo SIV/macaque model of HAD and that the pattern of expression correlates with recurrence of viral replication and onset of CNS disease. In vitro, the HIV-1 regulatory protein transactivator of transcription induces SOCS3 in human and murine macrophages in a NF-κB–dependent manner. SOCS3 expression attenuates the response of macrophages to IFN-β at proximal levels of pathway activation and downstream antiviral gene expression and consequently overcomes the inhibitory effect of IFN-β on HIV-1 replication. These studies indicate that SOCS3 expression, induced by stimuli present in the HIV-1–infected brain, such as transactivator of transcription, inhibits antiviral IFN-β signaling to enhance HIV-1 replication in macrophages. This consequence of SOCS3 expression in vitro, supported by a correlation with increased viral load and onset of CNS disease in vivo, suggests that SOCS3 may allow HIV-1 to evade the protective innate immune response within the CNS, allowing the recurrence of viral replication and, ultimately, promoting progression toward HAD. The Journal of Immunology, 2010, 185: 2393–2404.

Even with the widespread use of highly active antiretroviral therapy, 50% of patients infected with HIV-1 eventually experience neurologic symptoms, including cognitive and motor dysfunction, known as HIV-associated neurocognitive disorder (HAND) (1). Although only a small portion of these patients go on to develop the severe and disabling dementia characteristic of HIV (HIV-associated dementia [HAD]), mild cognitive impairment is common and can be detrimental to a patient’s quality of life (2), opportunity for employment (3), proper medication management (4), and survival (5). HAND is the result of HIV–1–induced neuronal injury and loss, predominantly in the basal ganglia and other subcortical regions of the brain where viral load is the most concentrated (6). Although neurons are not directly infected by HIV-1, viral replication within infiltrating macrophages and microglia, the resident macrophage of the brain, results in the production of viral proteins and activation-induced cytokines that can be damaging, or even toxic, to neurons (7).

Notably, although HIV-1 invades the CNS early following peripheral infection, neurologic symptoms do not occur until later in the course of disease (8). This delay is due to the ability of the innate immune response to effectively suppress viral replication during the early stages of CNS infection (9). IFN-β, a member of the type I or antiviral family of IFNs, is a critical mediator of this response in the brain. In vitro, IFN-β inhibits HIV-1 (10) and SIV (11) replication in macrophages, the cellular initiators of CNS pathogenesis. In vivo, increased IFN-β expression correlates with suppression of CNS SIV replication in an SIV/macaque model of HAD (11), the most faithful model of human HAND available. Unfortunately, although the ability to express IFN-β persists through late stages of HIV-1 (12) and SIV (13) disease, the virus eventually overcomes this antiviral effect through an unknown mechanism, and replication resumes (8). Studies performed in an SIV/macaque model of HAD showed that this resurgence of viral replication correlates with inflammatory dysregulation and progression to CNS disease (14). Therefore, it is critical that we understand the mechanism by which HIV-1 evades IFN-β’s protective antiviral effect to develop more potent therapies against CNS pathogenesis.

IFN-β inhibits viral replication by signaling through the JAK/STAT pathway to induce the expression of multiple antiviral target genes (15). In this pathway, IFN-β stimulates the cell-surface receptor subunits IFNAR1 and IFNAR2 to activate associated JAK family members JAK1 and TYK2. Activated JAKs then phosphorylate cytoplasmic STAT1 and STAT2, allowing them to heterodimerize and subsequently enter the nucleus to induce transcription of IFN-stimulated genes. Collectively, these antiviral genes can inhibit the
replication of a broad range of viral pathogens, including HIV-1, at multiple stages of the viral life cycle. For example, the translation inhibitor PKR prevents reactivation of HIV-1 replication in latently infected cells (16), and ISG20, a 3′-5′ exonuclease that cleaves ssRNA, can delay the replication kinetics of HIV-1 (17). Functions such as these culminate in the terminal antiviral effects of IFN-β signaling.

The JAK/STAT signaling pathway is subject to negative regulation by a family of proteins known as Suppressor of Cytokine Signaling (SOCS). This family contains eight members: cytoplasmic inductible SH2 domain containing protein and SOCS1–SOCS7 (18). Each uses a central SH2 domain to bind specific phosphorylated tyrosine residues within the JAK/STAT signaling receptor complex. They subsequently inhibit pathway function with a variable family member-specific domain in the N terminus or the more conserved C-terminal SOCS box, which targets associated proteins for proteosomal degradation. Classical SOCS upregulation is cytokine dependent, creating a negative feedback loop to prevent excessive cytokine stimulation (18, 19). However, many pathogens are capable of independently inducing SOCS expression to diminish the responsiveness of host cells to protective IFNs. For example, Listeria monocytogenes (20) and Mycobacterium avium (21), macrophage-tropic intracellular pathogens, induce SOCS3 to prevent IFN-γ-mediated host-clearance mechanisms. Toxoplasma gondii (22) achieves the same result through expression of SOCS1. Similarly, influenza A virus induces SOCS3 expression to evade IFN-β–mediated antiviral pathways (23), whereas HSV type I was reported to induce SOCS1 (24) or SOCS3 (25) to evade IFN-γ and IFN-α responses, respectively. SOCS proteins were also reported to be expressed at increased levels during HIV-1 infection (26, 27). However, it is not known whether SOCS proteins are induced by stimuli present in the HIV-1–infected brain or how they may affect protective IFN-β signaling.

One stimulus of particular interest in the HIV-1–infected brain is the multipotent HIV-1 protein transactivator of transcription (Tat) (28). Classically, Tat acts as a regulatory protein in the nucleus of HIV-1–infected cells, recruiting essential components of the transcriptional complex to the viral promoter to enhance HIV-1 transcription. However, Tat has a multitude of additional functions that contribute to HAND pathogenesis. Tat can be secreted to act on uninfected cells (29) extracellularly through interactions with various cell surface receptors (30–32) or intracellularly following endocytosis (33). Acting distally, Tat can directly induce neuronal apoptosis (34), promote the expression of cytokines and chemokines (35–38), induce expression of the HIV-1 chemokine coreceptors CXCR4 and CCR5 (39), and inhibit the expression of important immune molecules, such as MHC class I (40) and class II (41). By extension, additional HAND-promoting effects are likely. However, Tat has not been shown to affect critical IFN-β signaling within the HIV-1–infected brain.

In this study, we investigated whether SOCS proteins play a role in HIV-1 immune evasion in the CNS, thereby contributing to the development of HAND. We show that SOCS3 is expressed in an SIV/macaque model of HAD in a pattern that correlates with recurrence of viral replication and onset of CNS disease. SOCS3 is induced by HIV-1 Tat in macrophages and results in the inhibition of IFN-β signaling, ultimately preventing the ability of IFN-β to suppress HIV-1 replication. Collectively, these studies suggest that Tat-induced SOCS3 expression may promote progression to HAND by allowing HIV-1 to evade the innate immune response within the CNS.
under the control of the LysM promoter, to generate mice in which the conditional SOCS3 allele is excised specifically in macrophages and neutrophils (SOCS3Δ/Δ). BMDMs were collected as described above.

**HIV-1 infection and flow-cytometric analysis**

THP-GFP or THP-GFP-S3 cells were incubated in the presence of 1 ng/ml PMA for 72 h to induce macrophage differentiation, followed by pretreatment with 0–30 U/ml IFN-β for 24 h. Medium was removed, and the cells were infected with the molecular HIV-1 clone SG3 or the patient isolate CUCY. Three hours postviral inoculation, RPMI 1640/10% FBS containing the respective concentrations of IFN-β was readded, and cells were incubated for an additional 24–72 h prior to collection. The percentage of GFP-expressing cells was measured using a Guava EasyCyte flow cytometer.

**Statistical analysis**

Data were analyzed using GraphPad Prism software. Appropriate statistical tests were chosen for each experiment and are indicated in the respective figure legend.

**Results**

SOCS3 expression correlates with viral load and CNS disease in an SIV/macaque model of HAD

SOCS proteins are upregulated by a number of pathogens to evade protective host IFN responses. Specifically, SOCS1 and SOCS3 were shown to prevent IFN-mediated inhibition of viral replication (23, 24, 52). To determine whether either of these molecules is induced in the brain during progression to HAND, we examined SOCS1 and SOCS3 mRNA levels in an SIV/macacaque model of HAD. This model closely recapitulates the most severe form of human HAND (HAD), although in an accelerated fashion that consistently results in disease (11, 14, 50). Animals display an increase in CNS viral load shortly after infection, which rapidly diminishes ∼10 d postinfection in response to an increase in IFN-β expression. Recurrence of viral replication within the brain occurs by 42–84 d postinfection and correlates with the onset of disease. We collected mRNA from the basal ganglia of uninfected animals, or animals at a range of time points along the course of infection, and analyzed SOCS1 and SOCS3 expression by quantitative real-time PCR. SOCS3 mRNA expression was increased in SIV-infected animals in a pattern that correlated with increases in CNS viral load and onset of disease (Fig. 1A). Collectively, seven of nine animals with confirmed CNS disease showed increased SOCS3 expression compared with uninfected controls, whereas animals without disease showed consistently low SOCS3 expression (Fig. 1B). Additionally, the six most severely affected animals (Mo, S) showed the greatest increases in SOCS3 expression. SOCS1 mRNA expression was also detected in SIV-infected animals, but it did not correlate with increases in viral load or CNS disease (data not shown). These data indicate that SOCS1 and SOCS3 mRNA are expressed within the brain during progression to HAD. Because SOCS3 expression correlated with recurrence of viral replication and onset of CNS disease, we concluded that it contributed more significantly to HAD progression and focused subsequent mechanistic studies on this protein.

HIV-1 Tat induces SOCS3 expression in macrophages and microglia

SOCS proteins are expressed in cell types throughout the CNS in a family member- and stimulus-specific manner (19, 42, 53, 54). We hypothesized that SOCS3 must be expressed in macrophages, the most important source of viral replication within the CNS, to have a direct impact on viral replication. To determine whether macrophages could express SOCS3 in response to stimuli present in the HAD brain, we treated cells with HIV-1 Tat, a protein with multiple HAND-promoting effects. Primary macaque PBDMs were treated with HIV-1 Tat1–72aa for 0–24 h. A 10-nM concentration, close to the low physiologic concentration of Tat found in the sera

![FIGURE 1. SOCS3 expression correlates with viral load and CNS disease in an SIV/macaque model of HAD. A, RNA was collected from the basal ganglia of SIV-infected macaques at the times indicated p.i. and analyzed by quantitative RT-PCR with primers specific for SOCS3. Individual samples were normalized to 18S and displayed as fold increase above the noninfected control (C) with the greatest levels of SOCS3 ± SE. CNS viral load is indicated as no infection (0), <100,000 copies (↓), 100,000–1 million copies (↓), 1 million–10 million copies (↑), or >10 million copies (.). B. Infected animals were grouped by disease severity. The number of animals with increased SOCS3 levels (defined as ≥3-fold increase above control levels) and average fold induction of SOCS3 expression for each group were determined. Mi, mild; Mo, moderate; p.i., postinfection; S, severe.](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Disease Severity</th>
<th>Animals with Increased SOCS3 Levels</th>
<th>Average SOCS3 Fold Induction</th>
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<tr>
<td>No Disease</td>
<td>0/11</td>
<td>1.48</td>
</tr>
<tr>
<td>Mild (Mi)</td>
<td>1/3</td>
<td>1.70</td>
</tr>
<tr>
<td>Moderate (Mo)</td>
<td>1/1</td>
<td>6.13</td>
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<tr>
<td>Severe (S)</td>
<td>5/6</td>
<td>28.61</td>
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of HIV-1–infected individuals (55), was sufficient to produce robust SOCS3 expression between 2 and 8 h, at the level of mRNA (Fig. 2A) and protein (Fig. 2B). To assess whether Tat treatment of macaque macrophages in vitro also recapitulated the family member specificity seen in our in vivo model, we directly compared SOCS1 and SOCS3 expression by quantitative real-time PCR. Only negligible levels of SOCS1 mRNA expression were detected in comparison with SOCS3 (Supplemental Fig. 1), indicating that the ability of Tat to induce SOCS expression is relatively specific for SOCS3 in vitro. Notably, SOCS3 mRNA was not elevated in primary macaque PBDMs following a 4-h treatment with SIV alone (Supplemental Fig. 1), suggesting that the early stages of virus infection itself are not sufficient to induce SOCS3 expression. To determine whether human macrophages act similarly, we exposed primary human PBDMs to HIV-1 Tat1–72aa. Increased SOCS3 mRNA (Fig. 2C) and protein (Fig. 2D) levels were detected following Tat exposure, with maximal expression occurring 4 h after treatment. These data indicate that SOCS3 expression is induced by HIV-1 Tat, a stimulus present within the HAD brain, in human and macaque macrophages.

We then examined whether HIV-1 Tat could enhance SOCS3 expression in murine cells, which are a useful experimental tool for transfection and gene-deletion studies. Although Tat is unable to enhance HIV-1 transcription in murine cells (56), it was shown to successfully regulate host gene expression (57). RAW264.7 murine macrophages, chosen for their ability to induce SOCS3 in response to other stimuli (42), expressed SOCS3 in response to treatment with HIV-1 Tat1–72aa between 1 and 8 h at the mRNA (Fig. 2E) and protein (Fig. 2F) levels. Notably, treatment with a mutant form of Tat lacking critical core and basic regions (HIV-1 Tat31–61aa) did not induce a comparable increase in SOCS3 expression (data not shown). These results indicate that murine macrophages respond specifically to functional Tat by inducing SOCS3 expression, similarly to macrophages of other species. We then examined a range of primary murine CNS-relevant cells for their responsiveness to Tat treatment. Primary murine BMDMs (Fig. 2G) and primary murine microglia (Fig. 2H) displayed an increase in SOCS3 mRNA for the duration of HIV-1 Tat1–72aa exposure. In contrast, neither primary murine astrocytes (Fig. 2I) nor primary murine cortical neurons (Fig. 2J) expressed SOCS3 mRNA in response to HIV-1 Tat1–72aa treatment. Both of these cell types were capable of expressing SOCS3 in response to oncostatin M (Fig. 2I) or IFN-γ (Fig. 2J), respectively, and were shown to respond functionally to Tat (36, 37). These data indicate that HIV-1 Tat-induced SOCS3 expression is restricted to cells of macrophage lineage within the CNS.

HIV-1 Tat-induced SOCS3 expression is transcriptionally regulated

SOCS3 expression can be regulated at the level of transcription or mRNA stabilization (58, 59). To determine how HIV-1 Tat regulates SOCS3 expression in macrophages, we assessed activation of the SOCS3 promoter (Fig. 3). RAW264.7 macrophages, chosen for their ease of transfection, were transiently transfected with a murine SOCS3 promoter-driven luciferase reporter construct (Fig. 3A) (42). Cells were then treated with HIV-1 Tat1–72aa for
24 h, which was determined to be the optimal assay point. Tat induced a 17-fold increase in luciferase levels compared with untreated cells (Fig. 3B), a value consistent with Tat-induced increases in SOCS3 mRNA (Fig. 2E). These data suggest that HIV-1 Tat regulates SOCS3 expression in macrophages at the level of transcription.

**HIV-1 Tat signals through the NF-κB pathway to induce SOCS3 expression**

Different signaling pathways contribute to SOCS3 expression in a stimulus- and cell type-specific manner (19–21, 23, 42). Of these, the NF-κB pathway is frequently used by HIV-1 Tat to transcriptionally regulate gene targets (36, 38). To determine whether Tat signals through this pathway to induce SOCS3 expression in macrophages, we first examined the kinetics of Tat-induced NF-κB pathway activation. RAW264.7 macrophages were exposed to HIV-1 Tat1–72aa for 0–120 min and analyzed for markers of NF-κB activation (Fig. 4A). Phosphorylation of NF-κB p65 (serine 276) was detected at 30 min and remained elevated through 120 min. In addition, total levels of IκBa, a protein that sequesters NF-κB prior to activation, diminished noticeably between 15 and 45 min. HIV-1 Tat1–72aa treatment of primary murine BMDMs showed...
a similar result, inducing phosphorylation of NF-κB p65 (serine 276) between 30 and 45 min (Fig. 4A).

We used two methods of pathway inhibition to determine whether the NF-κB pathway was necessary for HIV-1 Tat-induced SOCS3 expression. First, RAW264.7 macrophages were assayed for Tat-induced SOCS3 promoter activity in the absence or presence of an IKK-β–dominant negative construct. This construct prevents the phosphorylation and subsequent degradation of IkB, resulting in inhibition of NF-κB activation (60). Although HIV-1 Tat_{1–72aa} stimulation resulted in a nearly 6-fold increase in luciferase expression, inclusion of the IKK-β–dominant negative construct reduced this expression by ~65% (Fig. 4B). Next, RAW264.7 macrophages, primary murine BMDMs, or primary human PBDMs were analyzed for endogenous Tat-induced SOCS3 expression following pretreatment with a pharmacologic inhibitor of the NF-κB pathway. Cells were pretreated with 10 μM BAY 11-7085, a concentration determined to prevent phosphorylation of p65, and were then exposed to HIV-1 Tat_{1–72aa} for 1 h. Exposure to BAY 11-7085 inhibited Tat-induced SOCS3 mRNA expression (Fig. 4C). These data indicate that activation of the NF-κB signaling pathway is necessary for HIV-1 Tat-induced SOCS3 expression.

Activation of the MAPK pathway (ERK, JNK, and p38) and the JAK/STAT pathway (STAT1 and STAT3) was also examined. Although all three of the MAPK pathways were activated by HIV-1 Tat_{1–72aa} with early kinetics (Supplemental Fig. 2A), pharmacologic inhibition of these pathways did not prevent Tat-induced SOCS3 expression (Supplemental Fig. 2B, 2C). By contrast, neither STAT1 nor STAT3 activation occurred with early kinetics following Tat treatment (Supplemental Fig. 2D). These data suggest that the MAPK and JAK/STAT pathways are not necessary for HIV-1 Tat-induced SOCS3 expression in macrophages.

HIV-1 Tat-induced SOCS3 expression is not mediated by IL-10 or IFN-β expression

SOCS3 can be induced by a variety of stimuli, two of which are readily induced by HIV-1 Tat: IL-10 (61) and IFN-β (62). To determine whether Tat-induced IL-10 or IFN-β is a required intermediate for SOCS3 expression, the kinetics of their expression were first examined. RAW264.7 macrophages and primary murine BMDMs were exposed to HIV-1 Tat_{1–72aa} for the times indicated (Fig. 5A). IL-10 and IFN-β mRNA expression were induced by Tat treatment, albeit with differential kinetics. To examine whether either of these cytokines was necessary for Tat-induced SOCS3 expression, macrophages deficient for IL-10 (Fig. 5B) or the IFNAR (Fig. 5C) were compared with WT macrophages following HIV-1 Tat_{1–72aa} treatment. Neither deficiency altered HIV-1 Tat-induced SOCS3 mRNA expression, indicating that neither IL-10 nor IFN-β is an intermediate in this process.

HIV-1 Tat inhibits IFN-β–induced STAT activation and antiviral target gene expression

Pathogen-induced SOCS proteins can inhibit signaling downstream of the types I and II IFNRs, thereby diminishing the host immune response (20–25). To determine whether HIV-1 Tat-induced SOCS3 expression is capable of inhibiting IFN signaling, RAW264.7 cells were pretreated with HIV-1 Tat_{1–72aa} for 4 h to induce SOCS3 protein expression and then were exposed to IFN-β or IFN-γ for 15 min (Fig. 6A). Treatment with IFN-β induced tyrosine phosphorylation of STAT1, STAT2, and STAT3 (lane 5), which was abolished by Tat pretreatment (lane 6). This inhibition was somewhat stimulus specific, because IFN-γ–induced STAT1 and STAT3 tyrosine phosphorylation (lane 3) were affected to a much lesser extent by Tat pretreatment (lane 4). Although serine phosphorylation of STAT1 and STAT3 did not occur to a significant extent at this experimental time point, Tat pretreatment was sufficient to inhibit basal serine phosphorylation of STAT3 (lanes 2, 4, 6). Tat-induced inhibition of IFN-β signaling was also observed in primary murine BMDMs (Fig. 6B). IFN-β induced tyrosine phosphorylation of STAT1, STAT2, and STAT3 (lane 3), whereas pretreatment with HIV-1 Tat_{1–72aa} inhibited this activation (lane 4).

This phenomenon was also observed in primary human PBDMs. Human macrophages were pretreated with HIV-1 Tat_{1–72aa} for 4 h

FIGURE 5. HIV-1 Tat-induced SOCS3 expression is not mediated by IL-10 or IFN-β expression.

A. RAW264.7 cells or primary murine BMDMs were treated with 10 nM HIV-1 Tat_{1–72aa} or 100 ng/ml LPS as a positive control, for the times indicated. mRNA was analyzed by RT-PCR with primers specific to IL-10 and IFN-β. The vertical line indicates the merge of separate gels. B. WT or IL-10-deficient (KO) primary murine macrophages were treated with 10 nM HIV-1 Tat_{1–72aa} or 100 ng/ml IFN-γ as a positive control, for the times indicated. mRNA was analyzed by RT-PCR with primers specific to IL-10 and SOCS3. C. WT or IFNAR-deficient primary murine macrophages were treated with 10 nM HIV-1 Tat_{1–72aa} or 100 U/ml IFN-β as a positive control, for the times indicated. mRNA was analyzed by RT-PCR with primers specific to SOCS3. GAPDH was examined as a loading control in all experiments. Data are representative of two independent experiments.
to induce SOCS3 protein expression and then were exposed to IFN-β for 15 min (Fig. 6C). IFN-β induced tyrosine phosphorylation of STAT1 and STAT2 (lane 3) but not STAT3, whereas pretreatment with Tat inhibited this activation (lane 4). Collectively, these data indicate that treatment with HIV-1 Tat, at time points sufficient to induce SOCS3 expression, inhibits IFN-β–induced STAT activation in murine and human macrophages.

We also examined the ability of HIV-1 Tat to inhibit downstream IFN-β–induced antiviral gene expression. RAW264.7 cells were pretreated with HIV-1 Tat1-72aa for 4 h to induce SOCS3 protein expression and then were exposed to IFN-β for 4 h to induce target gene expression. IFN-β treatment induced mRNA expression of ISG20 and PKR (Fig. 6D, lane 3), two important inhibitors of HIV-1 replication, whereas pretreatment with Tat

**FIGURE 6.** HIV-1 Tat inhibits IFN-β–induced STAT activation and antiviral target gene expression. RAW264.7 cells (A) or primary murine BMDMs (B) were pretreated for 4 h with 10 nM HIV-1 Tat1-72aa or vehicle alone and then were exposed to 100 U/ml murine IFN-β or 100 ng/ml murine IFN-γ for 15 min. Protein was subjected to immunoblot analysis with Abs specific to phosphorylated STAT1 (tyrosine 701 or serine 727), phosphorylated STAT2 (tyrosine 689), phosphorylated STAT3, (tyrosine 705 or serine 727), or total levels of these proteins. Blots were then stripped and reprobed for actin as a loading control. Quantification of band density (shown to the right of blots) represents pY-STAT levels normalized to total STAT levels and is displayed as the fold decrease below IFN treatment alone. Data are representative of at least two independent experiments. C, Primary human PBDMs were pretreated for 4 h with 10 nM HIV-1 Tat1-72aa or vehicle alone and then were exposed to 100 U/ml human IFN-β for 15 min. Abs specific to phosphorylated STAT1 (tyrosine 701), phosphorylated STAT2 (tyrosine 689), and total levels of these proteins were used for immunoblot analysis. Blots were then stripped and reprobed for actin as a loading control. Quantification of band density (shown to the right of blots) represents pY-STAT levels normalized to total STAT levels and is displayed as fold decrease below IFN treatment alone. Data are representative of three independent experiments. D, RAW264.7 cells were pretreated for 4 h with 10 nM HIV-1 Tat1-72aa or vehicle alone and were then exposed to 100 U/ml murine IFN-β for 4 h. mRNA was analyzed by RT-PCR with primers specific to murine ISG20 and PKR. GAPDH was examined as a loading control. Data are representative of two independent experiments.
attenuated this expression (lane 4). These data show that the inhibitory effect of HIV-1 Tat on IFN-β signaling results in decreased antiviral target gene expression.

**SOCS3 mediates HIV-1 Tat-induced inhibition of IFN-β signaling**

To determine whether the inhibitory activity of HIV-1 Tat is mediated by SOCS3, we evaluated the effect of SOCS3 deficiency on the ability of Tat to dampen IFN-β signaling. Primary murine BMDMs were obtained from mice with a conditional SOCS3 allele that was present and fully functional (SOCS3Δ/Δ) or excised specifically in macrophages (SOCS3Δ/Δ) (51). SOCS3 mRNA expression was abolished in SOCS3Δ/Δ macrophages in response to HIV-1 Tat1–72aa compared with SOCS3Δ/Δ macrophages (Fig. 7A). Cells were then pretreated with HIV-1 Tat1–72aa for 4 h followed by treatment with IFN-β (Fig. 7B). Although macrophages containing a functional SOCS3 allele exhibited inhibition of IFN-β–induced STAT1 and STAT2 activation in response to Tat (lanes 3, 4), SOCS3 deletion substantially attenuated this inhibition (lanes 7, 8). These data indicate that SOCS3 expression mediates HIV-1 Tat-induced inhibition of IFN-β signaling.

**SOCS3 prevents IFN-β’s ability to suppress HIV-1 replication**

The most important functional consequence of IFN-β in the HIV-1–infected brain is inhibition of viral replication in macrophages. To determine whether the inhibitory effect of SOCS3 observed on upstream IFN-β signaling correlates with an inhibition of downstream antiviral function, we examined the effect of SOCS3 on HIV-1 replication. PMA-differentiated THP-1 cells containing a stably integrated HIV-1 LTR-driven reporter construct (THP-GFP macrophages) were used to perform these experiments. In these cells, GFP expression is induced upon productive HIV-1 infection (Fig. 8A) and, therefore, it can be used to monitor viral replication. THP-GFP macrophages were infected with HIV-1 CUCY (R5/macrophage preference) in the absence or presence of IFN-β and then incubated for 24–72 h (Fig. 8B). A significant decrease in HIV-1 LTR-driven GFP expression was observed in the presence of IFN-β, indicating an inhibitory effect on HIV-1 replication in these cells. To evaluate the effect of SOCS3 on this process, THP-GFP macrophages containing an integrated SOCS3 expression construct (THP-GFP-S3; Fig. 8C) were used, rather than inducing SOCS3 with HIV-1 Tat, to avoid Tat’s broader replication-enhancing effects. In these cells, SOCS3 overexpression was sufficient to substantially diminish the robust STAT1 and STAT2 activation induced by IFN-β in parental THP-GFP macrophages (Fig. 8D, lanes 3 and 4). THP-GFP and THP-GFP-S3 macrophages were then infected with HIV-1 CUCY or HIV-1 SG3 (X4/lymphocyte preference) in the presence of increasing amounts of IFN-β and were incubated for 72 h prior to collection (Fig. 8E). HIV-1 LTR-driven GFP expression generated in response to infection with either HIV-1 strain was suppressed ∼60% by the highest dose of IFN-β tested in THP-GFP macrophages, whereas IFN-β had no inhibitory effect in SOCS3-overexpressing THP-GFP macrophages. These results indicate that SOCS3 expression is sufficient to overcome the inhibitory effect of IFN-β and, thereby, enhance HIV-1 replication in macrophages.

**Discussion**

IFN-β is a key mediator of the host immune response to HIV-1 infection within the CNS. Previous work showed that IFN-β can inhibit HIV-1 replication within macrophages (10), the most important source of productive HIV-1 infection in the brain, and that increases in its expression correlate with early suppression of viral replication in an SIV/macaque model of HAD (11). Unfortunately, although the ability to express IFN-β persists through the late stages of disease, its antiviral effect is only transient (13). HIV-1 eventually overcomes this response to resume replication within the CNS, often leading to the cognitive and motor impairments that characterize HAND. In these studies, we describe a novel mechanism by which HIV-1 can evade IFN-β’s antiviral effects in the CNS. We show that HIV-1 Tat-induced SOCS3 expression in macrophages diminishes IFN-β signaling, ultimately preventing IFN-β’s ability to inhibit HIV-1 replication. These studies suggest that SOCS3 may promote progression toward HAND by allowing HIV-1 to evade protective host immune responses in the CNS.

Although SOCS1 and SOCS3 were shown to prevent IFN-mediated inhibition of viral replication, our studies focused specifically on SOCS3. This decision was based primarily on our analysis of mRNA collected from an SIV/macaque model of HAD, which showed that increases in SOCS3 expression, but not SOCS1, correlated with recurrence of viral replication and onset of CNS disease. This model allowed us to evaluate SOCS expression throughout the course of disease progression from identically infected and harvested brains, providing us with an accurate kinetic analysis of SOCS expression during disease. Such well-controlled data are difficult to obtain from human patients. Progression to HAD among
a population of HIV-1–infected patients is much more variable in humans, as are the conditions under which postmortem brain is collected. However, even with these limitations, available data suggest that SOCS3 levels are increased in the human HAD brain. A microarray study performed on 24 basal ganglia samples by the National NeuroAIDS Tissue Consortium revealed that the signal intensity of a probe set for SOCS3 mRNA (227697_at) was increased significantly in postmortem brains from patients with HAD compared with control patients or HIV-1–infected patients without CNS disease (B. Gelman, personal communication). Although variation between patients is substantial and expected, these data support the conclusions of our study that SOCS3 may contribute to the progression toward HAND.

We determined that the HIV-1 protein Tat is sufficient to induce SOCS3 expression in macrophages; therefore, it may be responsible for the most critical elevations in SOCS3 expression observed in the HAD brain. This attributes yet another HAND-promoting effect to Tat: innate immune evasion within the CNS. Interestingly, induction of SOCS3 also reveals a concealed similarity between Tat and the HIV-1 accessory proteins, which are often responsible for inhibiting innate immunity (63). For example, Vif eliminates IFN-induced APOBEC3G, an antiviral effector capable of inducing virus-crippling hypermutation (64), whereas Vpu antagonizes another IFN-induced antiviral factor tetherin, which prevents viral release from the cell membrane (65). Therefore, although the finding that Tat-induced SOCS3 can provide innate immune evasion within...
the CNS is novel, it is supported by significant commonality with the current understanding of HIV-1 function.

In our studies, HIV-1 Tat directly induced high levels of SOCS3 expression. Because SOCS3 is thought to bind TYK2 (66), which is only present in the type I IFN complex, Tat predictably inhibits type I IFN signaling (IFN-β) to a greater extent than type II IFN signaling (IFN-γ). However, HIV-1 Tat was not entirely specific for SOCS3 expression. Low levels of SOCS1 mRNA were also detected with delayed kinetics, suggesting an indirect relationship to Tat treatment. In addition, Cheng et al. (67) showed that HIV-1 Tat induces SOCS2 expression in human monocytes, which we also detected in macrophages at very low levels. Because our data indicate that direct induction of SOCS3 by HIV-1 Tat is NF-κB dependent, the absence of an NF-κB consensus site within the SOCS1 and SOCS2 promoters may account for their limited, and perhaps indirect, expression. Notably though, several studies reported a role for NF-κB in SOCS3 expression (23, 68), none showed NF-κB p65 binding to the SOCS3 promoter. Regardless of their relative expression levels, it remains possible that small quantities of these other SOCS family members could further augment the ability of Tat to inhibit IFN signaling within the HIV-1–infected brain.

In these studies, we clearly outlined an in vitro mechanism whereby HIV-1 Tat induces SOCS3 expression, thereby preventing the ability of IFN-β to suppress viral replication. Although the correlation between SOCS3 expression and recurrence of viral replication in our in vivo model strongly supports this hypothesis, it does not preclude much greater complexity. First, although Tat is certainly one stimulus in the HIV-1–infected brain capable of inducing SOCS3, others are likely present. Cytokines with the ability to induce SOCS3, such as IL-6 and oncostatin M, are reportedly increased in the CNS during HIV-1 infection (36, 69). Other soluble viral proteins, in addition to Tat, may possess SOCS3-inducing activity. Second, although we tested the ability of SOCS3 to inhibit the most well-known suppressor of HIV-1 replication in macrophages, IFN-β, it may have broader effects. Chemokines that bind the HIV-1 coreceptor CCR5, such as MIP-1α, MIP-1β, and RANTES (70), as well as other factors, including macrophage-derived chemokine (71) and IL-10 (72), were shown to contribute to inhibition of HIV-1 replication in macrophages. Based on the inhibitory role that SOCS3 plays in multiple cytokine- and chemokine-signaling pathways, it is reasonable to expect that at least some of these additional inhibitory mechanisms may be affected by SOCS3 expression. Therefore, although our data strongly suggest that SOCS3 plays an important role in the progression to HAND, its mechanism of induction and inhibitory function in the HIV-1–infected brain may be broader than that defined by this study.

Finally, it is important to note that SOCS3 expression likely showed such a robust correlation with CNS disease progression because anti–HIV-1 immunity within the brain depends so heavily on the innate arm of the immune system. Although HIV-1 infection in the periphery is controlled by the innate and adaptive immune systems working in collaboration, restricted access of adaptive effectors (T cells, NK cells, and Abs) into the CNS shifts the immune burden to innate mechanisms. However, it is reasonable to expect that the SOCS3-mediated innate immune evasion observed in this study is transferable to HIV-1 replication in the periphery. Based on the increased importance that has recently been placed on innate immunity in the control of peripheral HIV-1 infection, specifically as mediated by type I IFN signaling pathways [i.e., APOBEC3G (64), TRIM (73)], it seems likely that the effect of SOCS3 on HIV-1 replication is not restricted to the CNS.

In summary, these studies describe a novel mechanism by which HIV-1, through the use of its regulatory protein Tat, can evade innate immune defenses. They specifically suggest a mechanism for progression toward HAND but also more generally enhance our understanding of the complexities of HIV-1 immune evasion.

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References


