Suppressors of cytokine signaling 1 and 3 are upregulated in brain resident cells in response to virus-induced inflammation of the central nervous system via at least two distinctive pathways

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ABSTRACT

Suppressors of cytokine signaling (SOCS) proteins are intracellular proteins that inhibit cytokine signaling in a variety of cell types. A number of viral infections have been associated with SOCS upregulation; however, not much is known about the mechanisms regulating SOCS expression during viral infection. In this study, we used two pathologically distinct intracerebral (i.c.) infection models to characterize temporal and spatial aspects of SOCS expression in the virus-infected central nervous system (CNS), and by employing various knockout mouse models, we sought to identify regulatory mechanisms that may underlie a virus-induced upregulation of SOCS in the CNS. We found that i.c. infection with either lymphocytic choriomeningitis virus (LCMV) or yellow fever virus (YF) results in gradual upregulation of SOCS1/3 mRNA expression peaking at day 7 postinfection (p.i.). In the LCMV model, SOCS mRNA was expressed in brain resident cells, including astrocytes and some neurons, and for SOCS1 in particular this upregulation was almost entirely mediated by gamma interferon (IFN-γ) produced by infiltrating T cells. After infection with YF, we also found SOCS expression to be upregulated in brain resident cells with a peak on day 7 p.i., but in this model, the upregulation was only partially dependent on IFN-γ and T cells, indicating that at least one other mediator was involved in the upregulation of SOCS following YF infection. We conclude that virus-induced inflammation of the CNS is associated with upregulation of SOCS1/3 mRNA expression in brain resident cells and that at least two distinctive pathways can lead to this upregulation.

IMPORTANT

In the present report, we have studied the induction of SOCS1 and SOCS3 expression in the context of virus-induced CNS infection. We found that both a noncytolytic and a cytolytic virus induce marked upregulation of SOCS1 and -3 expression. Notably, the kinetics of the observed upregulation follows that of activity within proinflammatory signaling pathways and, interestingly, type II interferon (IFN), which is also a key inducer of inflammatory mediators, seems to be essential in initiating this counterinflammatory response. Another key observation is that not only cells of the immune system but also CNS resident cells are actively involved in both the pro- and the counterinflammatory immune circuits; thus, for example, astrocytes upregulate both C-X-C motif chemokine 10 (CXCL10) and SOCS when exposed to type II IFN in vivo.

SOCS1 and SOCS3 inhibit signaling from both overlapping and separate subsets of cytokine receptors. Classically, SOCS1 is thought to inhibit interferon (IFN) signaling by interacting either directly with the cytoplasmic domain of the receptor (IFNAR1) or with receptor-associated JAK proteins (IFN-γR), abrogating IFN-induced activation of STATs (7–10). However, SOCS1 has also been shown to inhibit a wider range of signaling pathways, among those the NF-κB pathway, implicating SOCS1 in the regulation of Toll-like receptor (TLR) signaling (11). The classical function of SOCS3 is to inhibit signaling from the interleukin-6 (IL-6) receptor subfamily by interacting directly with the common receptor subunit for this family (gp130) or by inhibiting JAK activity, whereby JAK-mediated phosphorylation of STATs is prevented (12–16). However, SOCS3 also inhibits signaling from receptors that bind lipopolysaccharide, type I and II IFNs, IL-2, and IL-12 and inhibits the NF-κB pathway (17–21).

SOCS1 and -3 have been found to be expressed by immune cells, as well as by cells of the central nervous system (CNS) (2, 21) and therefore appear to have the potential to impact various immune processes within the CNS; indeed, it has been found that

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both IFN-β and IFN-γ have the capacity to induce SOCS1 and -3 expression in astrocytes in vitro (21, 22). However, at the present time, our insight into the regulation of SOCS expression in the inflamed CNS in vivo is limited (4). In mice with experimental autoimmune encephalomyelitis (EAE), the expression of both SOCS1 and -3 is upregulated, and mRNA expression is limited almost entirely to the infiltrating mononuclear cell population, which has led to the hypothesis that SOCS1 and -3 might function to downmodulate the response of the infiltrating immune cells to IFN-γ and possibly also other cytokines (23, 24). Generally, both SOCS1 and SOCS3 gene expression tend to be induced by the same cytokines they regulate, so the activation of STAT transcription factors (especially STAT1 and -3) upregulates SOCS1 and -3 expression (4). Immumomodulating cytokines, such as IL-10, have also been shown to induce the expression of SOCS1 and -3, and this upregulation is thought to be one of the mechanisms by which IL-10 mediates its downstream effects (2, 20).

A number of virus infections result in the upregulation of host SOCS proteins, and it is the general belief that some viruses actually exploit SOCS protein functions to evade the host immune system (6). However, not much is known about the regulatory mechanisms that cause the upregulation of host SOCS expression in vivo following a viral infection.

In the present study, we used two pathologically distinct models of intracerebral (i.c.) infection to address whether SOCS expression is upregulated in the context of virus-induced inflammation in the CNS. Furthermore, by employing various knockout (KO) mouse models, we have sought to investigate which regulatory mechanisms may be involved in mediating a virus induced upregulation of SOCS in the infected CNS. We found that i.c. infection with lymphocytic choriomeningitis virus (LCMV), as well as yellow fever virus (YF), results in a significant upregulation of SOCS1/3 mRNA expression peaking at day 7 postinfection (p.i.). In the LCMV model, we observed that SOCS mRNA was expressed in brain resident cells such as meningeal cells, ependymal cells, astrocytes, and to some extent in neurons, and for SOCS1 in particular this upregulation was predominantly mediated by IFN-γ produced by infiltrating CD8⁺ T cells. Interestingly, we have previously revealed a similar mode of regulation, even within the same cell types, with regard to C-motif chemokine 10 (CXCL10), a chemokine that serves as an important proinflammatory mediator and is known to be involved in the recruitment virus-specific effector CD8⁺ T cells (25, 26). In the YF model, we also found that SOCS expression was upregulated in CNS resident cells and peaked around day 7 p.i., but in this model, the upregulation seemed to depend only partially on IFN-γ production and T cells, indicating that at least one other mediator/signaling pathway was involved in the upregulation of SOCS following YF infection of the CNS.

Thus, we propose that upregulation of SOCS1/3 mRNA expression in brain resident cells represents an integrated part of the tuning of inflammatory responses in the virus-infected CNS and that at least two distinctive pathways may lead to this result.

MATERIALS AND METHODS

Mice. IFN-γR-deficient and IFN-αβR-deficient (IFN-γR⁻/⁻ and IFN-αβR⁻/⁻, respectively) mice on a C57BL/6 background were the progeny of breeder pairs provided by D. Pinschewer and R. Zinkernagel (Universitätsspital, Zürich, Switzerland). C57BL/6 wild-type (WT) mice and matched T-cell-deficient nu/nu mice were purchased from Taconic Farms, while IL-10 deficient (IL-10⁻/⁻) mice were obtained from the Jackson laboratories (Bar Harbor, ME); mice from outside sources were always allowed to acclimatize to the local environment for at least a week before entering into experiments, by which time the animals were between 7 and 9 weeks old.

All animals were housed in a pathogen-free environment, as validated by testing of sentinels for unwanted infections according to the Federation of European Laboratory Animal Science Association standards. Female mice were used in most experiments, but when both genders were included, no gender effect was observed. Experiments were conducted according to national guidelines regarding animal experiments.

Virus infection. Mice were anesthetized and infected i.c. with either a 10⁵ LD₅₀ (≈200 PFU) of LCMV Truba or a 10⁵ PFU of YF-17D, and phosphate-buffered saline (PBS) was used for sham inoculation. LCMV is a noncytolytic virus that causes little if any disease in immunodeficient mice (27, 28). However, i.c. inoculation of LCMV leads to infection of the CNS, and in adult immunocompetent mice the result is a severe CD8⁺ T cell-mediated meningoencephalitis to which the animals succumb around days 8 to 10 p.i. (29, 30). YF-17D is an attenuated vaccine strain derived from YF; the seeding virus was the commercial vaccine obtained from Sanofi-Pasteur. YF is a cytoplastic virus that in humans has a preferential tropism for the liver; however, in i.c.-infected mice the attenuated vaccine strain induces a fatal encephalitis, causing death from paralysis on days 8 to 9 postinfection. This encephalitis is observed also in RAG-deficient mice and therefore, unlike LCMV-induced meningoencephalitis, it is not mediated by CD8⁺ T cells or any other specific immune cell (M. R. Bassi et al., unpublished data).

Bone marrow chimeras. Syngeneic and allogeneic bone marrow chimeras were made using IFN-γR⁻/⁻ and WT mice. Mice were lethally irradiated (9 Gy) in the afternoon and transplanted i.v. with 10⁷ femur cells from allo- or syngeneic donors the following day. After reconstitution for at least 8 weeks, the mice were infected with virus as described above.

Isolation of total RNA for quantitative PCR (qPCR). Brains from mice deeply anesthetized and exsanguinated were immediately removed, snap-frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted from homogenized brains by use of an RNeasy midikit (Qiagen).

Detection of mRNA in the brain by qPCR. One microgram of RNA was reverse transcribed to cDNA by using a RevertAid first-strand cDNA synthesis kit (Fermentas/Thermo Scientific). For the qPCRs, a Brilliant II SYBR green qPCR Mastermix was used according to the manufacturer’s instructions (Stratagene/AH Diagnostics). The qPCR components included Brilliant II qPCR Mastermix, Milli-Q water, reverse-transcribed cDNA, and forward and reverse target gene primers (see Table 1 for the primer sequences). Target gene expression was normalized to expression of the reference gene PBGD (porphobilinogen). An Mx3005P real-time qPCR instrument was used with the following program: initial denaturation at 95°C (10 min), followed by 40 cycles of denaturation at 95°C (30 s), annealing at 60°C (60 s), and extension at 72°C (60 s). Each reaction was run in triplicate plus a no-template control and a no-reverse transcriptase control. The results were analyzed using Mx3005P system software. The relative expression ratio (R) in each sample was calculated using a mathematical model based on the amplification efficiency (31): $R = \frac{\Delta C_P(\text{control-sample})}{\Delta C_P(\text{control-sample})}$. An amplification efficiency (E) of 100% corresponds to a doubling of the PCR product for every cycle. E is calculated from the slope of a standard curve based on 5-fold dilutions for each primer pair used [E = 10⁻¹/slope]. Thus, E_target corresponds to the target gene primers, and E_reference corresponds to the reference gene primers. In the present study, WT brains infected i.c. 7 days before removal with LCMV or YF-17D were used as standard curve templates. ΔC_P (control-sample) refers to the difference in threshold cycle (C_T) between day 7 p.i. (control) and day 3, 5, or 7 p.i. (sample). C_T reflects the number of cycles it takes to reach a point in which the fluorescent signal is above the background fluorescence (31).
In situ hybridization and immunohistochemistry. Brains were removed from lethally anesthetized and exsanguinated mice, frozen in liquid nitrogen, and sectioned in a cryostat into serial 20-μm thick sections, which were mounted on RNase-free Superfrost+ glass slides (Hounisen) and stored at −80°C. mRNA in situ hybridization was performed as described by Clausen et al. (32) using a mixture of two alkaline phosphatase (AP)-labeled DNA oligonucleotide probes (4 to 6 pmol/ml) complementary to murine CXCL10, SOCS1, or SOCS3 mRNA (probe sequences are depicted in Table 2). All probes were designed by using Oligo-design software 6.0 and tested for the ability to recognize other sequences by the NCBI blastn tool, before they were fabricated by DNA Technology A/S. The in situ hybridization signal was developed within 3 days in a Tris-HCl MgCl₂ buffer containing the AP substrates, BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma-Aldrich), and nitroblue tetrazolium (Sigma-Aldrich). The hybridization specificity was documented on parallel sections showing the absence of a hybridization signal when sections were (i) treated with RNase A (Pharmacia Biotech), (ii) hybridized with 100-fold excess of the unlabeled probes, and (iii) incubated with buffer only (Fig. 1 and previously published for CXCL10 by Christensen et al. [26]). Furthermore, hybridization with each of the two probes individually resulted in similar hybridization signals as when hybridized together (data not shown). All control reactions were performed on brain sections from C57BL/6 mice 7 days after infection with LCMV or YF, when the CXCL10, SOCS1, and SOCS3 mRNA expression was maximal (Fig. 1). Furthermore, parallel sections were hybridized for the widely expressed glyceraldehyde-3-phosphate dehydrogenase mRNA to ensure the overall suitability of the tissue for in situ hybridization (data not shown).

When in situ hybridization for CXCL10 or SOCS mRNA was combined with immunohistochemistry for glial fibrillary acidic protein (GFAP), sections were subjected to the standard in situ hybridization, rinsed in 0.5 M Trizma base-buffered saline (pH 7.4) containing 1% Triton (TBS-T), and incubated overnight at 4°C with monoclonal Alexa 488-conjugated mouse anti-GFAP IgG1 (5 μg/ml; catalog no. A21294; Invitrogen) or mouse Alexa 488-conjugated mouse IgG1 isotype control (5 μg/ml; MG120; Caltag Laboratories) in TBS-T and 10% fetal calf serum (FCS). After a final rinse in TBS, sections were counterstained with DAPI (4‘,6’-diamidino-2-phenylindole) nuclear staining. By using Adobe PhotoShop CS6 Extended v13.0 ×32, the bright-field pictures of the in situ hybridization signal were inverted before the red channel was selected and merged with the channels showing the fluorescence signals of DAPI (blue) and Alexa 488 (green). Since the fluorescent signal does not

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′–3′) Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>SOCS1</td>
<td>CACCTCTGTGTTGGCGGC</td>
<td>AAGCCATCTCAGCAGCTGAC</td>
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<td>SOCS3</td>
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<tr>
<td>IL-10</td>
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<td>GTCAAATTACATTAGG</td>
</tr>
<tr>
<td>PBGD</td>
<td>GTGAGTGTTTGTCAGGATC</td>
<td>GGTCATCTTCCTGAGCAT</td>
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![FIG 1](https://jvi.asm.org/)[In situ hybridization control reactions. (A to D) Signal absence after in situ hybridization with AP-labeled probes on RNase-treated brain sections and after hybridization with a 100-fold excess of unlabeled probes on normal brain sections from C57BL/6 mice at day 7 p.i. with LCMV. (E to J) Signal absence after in situ hybridization with AP-labeled probes on RNase-treated tissue and after hybridization with a 100-fold excess of unlabeled probes on normal brain sections from C57BL/6 mice at day 7 p.i. with YF. 3V, third ventricle; cc, corpus callosum; Ctx, cortex; ec, external capsule; Hip, hippocampus. Scale bar, 200 μm.]
shine through a strong AP signal, a yellow color cannot be expected in all cases of colocalization.

**Immunohistochemistry for cleaved caspase-3 (c-Casp3) and cleaved poly(ADP-ribose) polymerase (c-PARP).** Parallel brain sections were fixed in 4% paraformaldehyde for 45 min and rinsed in TBS. After incubation with 10% FCS in TBS, sections were incubated overnight at 4°C with purified rabbit anti-cleaved caspase-3 (Asp175) IgG (1:200; catalog no. 9661S; Cell Signaling Technologies), monoclonal rabbit anti-cleaved-PARP IgG (2 µg/ml; ab32064; Abcam), or rabbit IgG isotype control (Dako) diluted in 10% FCS in TBS. After another rinse in TBS, endogenous peroxidase activity was blocked with 1.9% H2O2 in TBS, and sections were rinsed in TBS before incubated with Envision System HRP-labeled polymer conjugated goat anti-rabbit IgG (ready to use; K4003; Dako). After a final rinse in TBS, the color signal was developed in 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.003% H2O2 in TBS.

**Statistical analysis.** A nonparametric Mann-Whitney U test was used to compare quantitative data (*, P < 0.05; **, P < 0.01). GraphPad Prism version 4 or 6 was used for statistical analysis.

**RESULTS**

SOC1 and -3 mRNA expression is upregulated in response to LCMV-induced inflammation of the CNS. Both SOCS1 and -3 are known to be important negative regulators of proinflammatory cytokine activity, and since it is particularly important in the

![Graph](https://jvi.asm.org/14093)

**FIG 2** Kinetics of expression of key regulators of inflammation in the CNS after i.c. infection with LCMV. WT mice were infected i.c. with LCMV, and on the indicated days we determined the mRNA levels for SOCS1 and SOCS3 (A), IRF7 and USP18 (B), and IFN-γ and IL-10 (C) in the brain by qPCR. Brains from mice inoculated with PBS 3 days earlier served as negative controls. Each point represents one animal.
CNS that inflammation does not lead to excessive tissue damage, we speculated that virus-induced inflammation of the CNS would lead to upregulation of SOCS gene expression as part of an immune dampening response. To test this hypothesis, we initially used the well-established LCMV model of intracerebral (i.c.) infection in which the virus is injected directly into the forebrain. In adult immunocompetent mice this causes a massive influx of virus-specific effector CD8$^{+}$ T cells mediating severe immunopathology and death by paralysis on day 8 to 10 p.i. (33–36). On day 3, 5, and 7 p.i. following inoculation of a lethal dose of virus, we determined the mRNA levels of SOCS1 and -3 in the brain of infected mice by qPCR; mice inoculated with PBS served as baseline controls. As can be seen in Fig. 2A, mRNA expression of both SOCS genes increased quite markedly from day 3 to day 7 p.i., verifying our initial prediction that virus-induced inflammation of the CNS is associated with upregulation of SOCS1 and -3 gene expression in the brains of WT mice.

In order to put the upregulation of SOCS1 and -3 expression in the proper functional context, we proceeded by studying other pro- and anti-inflammatory signaling pathways believed to play a role in virus-induced inflammation. In previous studies, we have clearly demonstrated that type I IFNs represent key mediators in initiating the local inflammatory process in the LCMV-infected CNS. However, since type I IFNs tend to be expressed in too low amounts for detection in whole-brain lysates (26), we focused on the transcriptional levels of SOCS1 and -3 in the brain of infected mice by qPCR; mice inoculated with PBS served as baseline controls. As can be seen in Fig. 2A, mRNA expression of both SOCS genes increased quite markedly from day 3 to day 7 p.i., verifying our initial prediction that virus-induced inflammation of the CNS is associated with upregulation of SOCS1 and -3 gene expression in the brains of WT mice.

Unlike type I IFNs, the expression of type II IFN (IFN-γ) is delayed, and peak expression correlates with maximal T cell influx into the infected brains immediately prior to the death of the host (26). Based on this, we hypothesized that the increase in SOCS expression would correlate with the appearance of IFN-γ. As previously reported (26), IFN-γ mRNA could not be detected until day 7 p.i., at which time mRNA levels increased markedly (Fig. 2C), coinciding with maximal expression of both SOCS1 and -3 mRNA in the inflamed brain. The correlation between IFN-γ and SOCS gene expression suggests that type II IFN or other factors produced by the infiltrating T cells might be the dominating mediator(s) causing the observed increase in SOCS gene expression after LCMV-induced inflammation of the CNS.

Prior studies have revealed IL-10 production by infiltrating T cells after virus-induced inflammation of the CNS (40, 41), and given the role of IL-10 as a positive regulator of SOCS expression (2), the possibility existed that IL-10 might be involved in the induction of SOCS gene expression. However, we did not find a good temporal correlation between IL-10 and SOCS gene expression (Fig. 2C) or to the kinetics of IL-10 expression resembled that of type I IFN-induced genes such as USP18 (Fig. 2B and C). This suggested that IL-10 was not a major regulator of SOCS gene expression after LCMV infection and that IL-10 was induced by early inflammatory events, rather than being produced by infiltrating T cells.

LCMV induces the expression of SOCS1 and -3 in brain resident cells. Having revealed a significant upregulation of SOCS gene expression in the LCMV-infected CNS, it became of interest to determine the spatial distribution of this expression, as well as the cell types induced to express SOCS1 and -3 mRNA in response to LCMV infection. To answer these questions, we performed in situ hybridization...
situ hybridization on brain sections of WT mice to study the localization of SOCS expression after i.c. LCMV injection. Neither SOCS1 nor SOCS3 mRNA was detected on day 3 p.i. (Fig. 3A and B), except near the injection site, whereas both SOCS1 and -3 mRNA were upregulated in multiple cells even far away from the injection site by day 7 p.i. (Fig. 3C and D). As expected, injection of PBS did not result in the upregulation of SOCS1 or -3 (Fig. 3E and F; results are only shown for day 7 p.i.), except near the injection site (data not shown), verifying that SOCS expression was induced by the infection and not by the local trauma caused by the injection. As previously observed for CXCL10 mRNA at day 7 p.i. with LCMV (26), both SOCS1 and -3 mRNA were expressed by cells in the meningeal membrane (Fig. 4A and B), by ependymocytes lining the lateral ventricles and the third ventricle (Fig. 4C and D), by vessel-associated cells (Fig. 4E and F), by cells in periventricular structures, such as the external capsule and the corpus callosum (Fig. 4G and H and Fig. 4I and J), and finally in cells in the deepest layers of neocortex and in hippocampus proper (Fig. 4G and H). Interestingly, the mRNA expression of SOCS1, but not SOCS3, was upregulated in pyramidal neurons especially in CA1 (cornu ammonis 1) of the hippocampus proper and also in neuron-like cells in the deepest layers of neocortex (Fig. 4G and H). Furthermore, combined in situ hybridization for SOCS mRNA and immunostaining for GFAP protein revealed, as previously demonstrated for CXCL10 (26), that at least some of the SOCS1 and -3 mRNA-expressing cells were GFAP+ astrocytes, as shown for the corpus callosum (Fig. 4K to P).

SOCS1 and to some extent SOCS3 expression is dependent on IFN-γ produced by infiltrating T cells. Our initial correlative analyses suggested a possible role for IFN-γ, and not type I IFNs or IL-10, in the induction of SOCS gene expression after LCMV infection. However, to directly assess the role of these cytokines in LCMV-induced upregulation of SOCS expression, we infected IL-10−/−, IFN-αβR−/−, IFN-γR−/−, T cell-deficient (nu/nu) and WT mice with LCMV i.c. and quantified the SOCS1 and -3 mRNA levels on day 7 p.i. As can be seen in Fig. 5, the lack of IL-10 did not impact the virus-induced upregulation of either SOCS1 or SOCS3, whereas the absence of type I and II IFN signaling or T cells significantly reduced the expression of both SOCS genes in the infected CNS. Notably, consistent with published data on the role of type I IFN in the generation of LCMV-specific CD8 T cells (42, 43), additional analysis showed that IFN-γ mRNA levels in the brains of IFN-αβR−/− mice 7 days p.i. were markedly reduced compared to those in WT mice (Fig. 6), indicating that the LCMV-induced T cell response was severely compromised in these animals and that the reduction in SOCS expression might not be a direct result of the lack of type I IFN signaling.
LCMV-induced expression of both SOCS genes was similarly reduced in T cell deficient mice, whereas the absence of IFN-γ seemed to have less impact on SOCS3 expression, suggesting that SOCS1 mRNA expression was primarily regulated through IFN-γ, whereas alternative T cell-dependent pathways might be able to induce the expression of SOCS3. Notably, the importance of type II IFN in regulating LCMV-induced SOCS expression does not reflect substantial differences in baseline expression since the SOCS expression levels in naive IFN-γR−/−, IFN-γR−/−, nude, and WT mice are equivalent (Fig. 7).

The time course of LCMV-induced upregulation of SOCS expression (in particular SOCS1) cannot solely be ascribed to type II IFN, since some SOCS upregulation may be observed even before this cytokine can be detected (cf. Fig. 2). To resolve this conundrum, we performed an additional experiment in which we analyzed SOCS expression in IFN-γR−/− mice on day 5 p.i. Since no type II IFN can be detected at this time point, the reduction of SOCS 1 expression, which we observed in IFN-γR−/− mice compared to WT mice (Fig. 8), could suggest that type I IFNs may also impact SOCS expression but that its role is transient.

The above-mentioned results led us to hypothesize that upon LCMV infection, the massive influx of T cells would activate a negative-feedback mechanism in which resident brain cells responded to the sudden increase in IFN-γ by increasing SOCS expression in an attempt to dampen potentially negative effects of a high IFN-γ level (44). To more directly investigate whether the role for IFN-γR expression in the LCMV-induced upregulation of SOCS1 and -3 mRNA expression could be linked to a requirement for resident brain cells to respond to IFN-γ, we generated syngeneic and allogeneic bone marrow chimeras using WT donor mice and IFN-γR−/− or WT recipient mice. Eight weeks after transplantation, all of the inflammatory cells (T cells and monocytes/macrophages) recruited to the infected brain would be of donor (WT) origin, while the radioresistant cells of the CNS would be of recipient origin (i.e., either IFN-γR−/− or WT). Seven days after virus inoculation, SOCS1 mRNA levels in IFN-γR−/− recipients were found to be substantially reduced compared to both syngeneic chimeras and intact WT mice (Fig. 9A), and given that the fold decrease in IFN-γR−/− recipients compared to WT recipients roughly matches that in intact IFN-γR−/− mice compared to WT mice (cf. Fig. 5), these results strongly suggest that brain resident cells contribute substantially to the expression of SOCS1. SOCS3 mRNA levels showed the same trend, but in this case the differences observed were not statistically significant (Fig. 9B). These observations fit well with the results obtained in intact IFN-γR−/− mice, in which the SOCS3
mRNA levels were also affected to a lesser degree than SOCS1 mRNA levels, and could further point to the existence of alternative regulatory pathways involved in SOCS3 induction after LCMV infection of the CNS.

Yellow fever-induced inflammation of the CNS leads to induction of SOCS1 and -3 gene expression in a pattern similar to that induced by LCMV. Intracerebral infection with LCMV constitutes a well-described but perhaps also somewhat unique model in that the host does not succumb to the virus infection itself but to the immune response toward the virus (34, 35). We were therefore interested in investigating whether the findings in the LCMV model could be extended to other viral infections of the CNS. As an example of an i.c. infection model distinct from LCMV, we utilized an i.c. YF challenge model in which the YF is injected directly into the forebrain, causing encephalitis and death 7 to 10 days p.i. Unlike LCMV, which does not cause any disease in the absence of T cells (34), encephalitis and ultimately death in the YF model does not require the involvement of an adoptive immune response (Bassi et al., unpublished), thus providing a pathologically distinct model of virus-induced CNS inflammation. In agreement with this, cleaved caspase-3, which is a critical executioner of apoptosis being either partially or totally responsible for the proteolytic cleavage of many key proteins (45), is detected in several cells throughout the brains of YF-infected mice, while being mainly present in the meningeal membrane in LCMV-infected mice 7 days p.i. (Fig. 10A to C). In comparison, cleaved caspase-3 was not detected on day 7 after PBS injection (Fig. 10D), except at the injection site (data not shown). Cleaved poly(ADP-ribose) polymerase (PARP) was also present in the CNSs of YF-infected mice at 7 days p.i., especially in cells close to vessels and in neurons in the hippocampal formation, but rarely found in comparable brain sections of LCMV-infected mice at day 7 p.i. (Fig. 10E to G). PARP is one of the main cleavage targets of caspase-3 involved in DNA repair in response to environmental stress (46, 47), and cleavage of PARP serves as a caspase-3 downstream marker of cells undergoing apoptosis (47). Cleaved PARP was not detected in the brain on day 7 after PBS injection (Fig. 10H), except at the injection site (data not shown).

To assess whether the upregulation of SOCS mRNA could also be found in YF-infected mice, WT mice were infected i.c. with YF virus, and on days 3, 5, and 7 p.i. the mRNA levels of SOCS1 and -3 in the brain were determined by qPCR; mice inoculated with PBS served as baseline controls. As can be seen in Fig. 11A, the mRNA levels of both SOCS genes increased quite significantly from day 3 to day 7 p.i., and as observed previously in the LCMV model, SOCS mRNA expression correlated with the appearance of mRNA for IFN-γ (Fig. 11B), which could suggest that the regulation of SOCS mRNA expression in the YF and LCMV models occurred through similar pathways. Interestingly, following YF infection SOCS mRNA expression also correlated with the appearance of IL-10, which displayed significantly different kinetics of expression from those observed after LCMV infection, roughly correlating with the expression of type II IFN and not type I IFNs (Fig. 11B; cf. Fig. 2C). This result not only suggested that IL-10 gene expression was regulated through distinct pathways in the two models but also pointed to the possibility that SOCS gene expression after YF infection might be induced by IL-10.

As observed in the LCMV model, in situ detection of SOCS mRNA day 7 p.i. with YF showed that both SOCS1 and -3 mRNA were upregulated in multiple cells even far away from the injection site (Fig. 12A and B), while neither SOCS1 nor -3 were detectable at day 7 after injection of PBS (Fig. 12C and D), except near the injection site (data not shown). SOCS1 and -3 mRNA were upregulated in cells in the meningeal membrane (Fig. 13A and B) and by ependymocytes lining the lateral ventricles and the third ventricle (Fig. 13C and D). Furthermore, SOCS1 and -3 mRNA levels were also affected to a lesser degree than SOCS1 mRNA levels, and could further point to the existence of alternative regulatory pathways involved in SOCS3 induction after LCMV infection of the CNS.

**FIG 7** Baseline SOCS expression. The levels of mRNA expression for SOCS1 and -3 were determined in the CNSs of naive IFN-αR−/−, IFN-γR−/−, and WT mice. Each point represents one animal.

**FIG 8** SOCS1 expression is reduced on day 5 p.i. in IFN-αR−/− mice. The levels of mRNA expression for SOCS1 and -3 were determined in the CNSs of IFN-αR−/− and WT mice infected i.c. with LCMV 5 days earlier. Each point represents one animal.
were upregulated in vessel-associated cells (Fig. 13E and F) and other cells in the entire brain, including the hippocampus proper (Fig. 13G and H) and the neocortex (Fig. 13I and J). SOCS1 mRNA and to a lesser extent SOCS3 mRNA were upregulated in pyramidal neurons in CA1-3 of the hippocampus proper (Fig. 13G and H) and by large neuron-like cells in neocortex (Fig. 13I), while SOCS3 mRNA preferentially was upregulated by cells with glia-like distribution (Fig. 13J, shown for the neocortex). Com-

FIG 9 SOCS1 upregulation is dependent on the expression of IFN-γR on resident brain cells. Syngeneic and allogeneic bone marrow chimeras were generated by using WT donor mice and lethally irradiated IFN-γR−/− and WT recipient mice. Eight weeks after reconstitution, chimeras were infected i.c. with LCMV virus, and the mRNA levels for SOCS1 (A) and SOCS3 (B) were determined on day 7 p.i. Brains from intact WT mice (Nil = no pretreatment) infected 7 days earlier served as positive controls. Each point represents one animal. The data are pooled from two independent experiments.

FIG 10 YF and to some extent LCMV induce cleavage of caspase-3 and PARP in the brains of WT mice. (A to D) Cleaved caspase-3 (c-Casp3) was detected by immunohistochemistry in cells (arrows) throughout the brains of YF-infected mice (A and B) and in cells (arrows) in the meningeal membranes of LCMV-infected mice day 7 p.i. (C) but undetectable in comparable sections on day 7 after the injection of PBS (D). (E to H) Cleaved PARP (c-PARP) was detected by immunohistochemistry in cells (arrows) throughout the brains of YF-infected mice (E and F) but only rarely detected in LCMV-infected mice 7 days after infection (G) and undetectable in comparable sections on day 7 after injection of PBS (H). CA1 and -3, cornu ammonis 1 and 3; cc, corpus callosum; Ctx, cortex; DG, dentate gyrus; v, vessel; Scale bars: 400 μm (A, C to E, G, and H) and 10 μm (B and F).
combined in situ hybridization for SOCS mRNA and immunostaining for the GFAP protein confirmed that at least some of the SOCS1 and -3 mRNA-expressing cells appeared to be GFAP+/H11001 astrocytes (Fig. 13K to P).

CXCL10 mRNA was also upregulated in multiple cells in the entire brain 7 days after infection with YF (Fig. 14A) but undetectable after injection of PBS (Fig. 14B), except near the injection site (not shown). As previously observed in the LCMV model (26), CXCL10 mRNA was highly expressed by cells in the meningeal membrane (Fig. 14C) and by ependymocytes lining the lateral ventricles and the third ventricle (Fig. 14D). However, unlike the situation in the LCMV-infected CNS, CXCL10 mRNA was also highly expressed by vessel-associated cells (Fig. 14E) and by glia-like cells in the entire brain, including the hippocampus (Fig. 14F) and neocortex (Fig. 14G). Combined in situ hybridization for CXCL10 mRNA and immunostaining for GFAP protein indicated that most of the CXCL10 mRNA-expressing parenchymal cells were GFAP+/H11001 astrocytes (Fig. 14H to J). Expression of both

FIG 11 Intracerebral infection with a flavivirus also causes SOCS1 and SOCS3 mRNA upregulation. WT mice were infected i.c. with YF and, on the indicated days, we determined the mRNA levels for SOCS1 and SOCS3 (A) and IFN-γ and IL-10 (B) in the brain by qPCR. Brains from mice inoculated with PBS 3 days earlier served as negative controls. Each point represents one animal.

FIG 12 YF-induced SOCS1 and SOCS3 mRNA upregulation detected by in situ hybridization in brains from WT mice. (A to D) Both SOCS1 and SOCS3 mRNA were expressed in multiple cells (arrows) throughout the brain on day 7 after infection with YF virus (A and B) but undetectable in comparable sections day 7 after injection of PBS (C and D). 3V, third ventricle; CA1 and -3, cornu ammonis 1 and 3; cc, corpus callosum; Ctx, cortex; Hip, hippocampus; LV, lateral ventricle; v, vessel. Scale bar, 400 μm.
CXCL10 and SOCS mRNA by cells in the meningeal membrane, by ependymocytes, by vessel-associated cells, and by astrocytes suggests that at least some of the cells coexpress CXCL10 and SOCS1 and/or 3 day 7 p.i. with YF.

**YF-induced expression of SOCS1 and -3 is only partially dependent on IFN-γ produced by infiltrating T cells.** To directly assess the role of IL-10 and IFN-γ in the induction of SOCS1 and -3 in the context of YF-induced inflammation of the CNS, we infected IL-10−/−, nu/nu, and IFN-γR−/− mice with YF and assessed the SOCS mRNA level 7 days later. As can be seen in Fig. 15, SOCS1 and -3 expression was not affected by the lack of IL-10 expression but was significantly reduced in both nu/nu and IFN-γR−/− mice compared to WT mice. However, after YF infection, there seemed to be a substantial residual expression of SOCS mRNA in the absence of T cells and IFN-γ expression (cf. Fig. 11 for expression in uninfected mice), suggesting that T cells and IFN-γ were only partially responsible for the induction of SOCS1 and -3 expression associated with this infection.

**DISCUSSION**

A successful immune response—particularly in the CNS—is a balancing act between proinflammatory and anti-inflammatory mediators, and an abundance of cytokines and chemokines interact in deciding the outcome of an infection.

Cytokines are produced by numerous cells and have the potential to impact intracellular processes in both immune cells and parenchymal cells of the infected tissue. If allowed to signal unhindered, cytokines can potentially impact the course of infection negatively and/or induce massive tissue damage. Consequently, feedback mechanisms capable of dampening the intracellular response to cytokines are vital to ensure tissue integrity. The SOCS proteins have emerged as important intracellular inhibitors of cytokine signaling, and SOCS1 and -3 in particular have received increasing attention due to their pivotal roles in regulating both innate and adaptive immune responses, as evident from the fatal effects of a deficiency in either protein (10, 48, 49). SOCS1 and -3 expression may often be observed in the periphery upon viral infection (6); in the CNS, however, only West Nile virus and tick-borne encephalitis virus have previously been found to induce the expression of SOCS, and the involved cell types, as well as the underlying regulation, are completely unknown (50).

In the present study, we investigated the expression and regulation of SOCS1 and -3 in the CNS after virus-induced inflammation by using two pathologically very distinct virus models: LCMV and YF. LCMV is a nonlytic virus that upon injection directly into the brain replicates primarily in the meningeal membranes and induces a massive influx of virus-specific T cells, resulting in severe meningoencephalitis and death 8 to 10 days p.i. (33, 35).
Since the LCMV model of CNS inflammation represents a rather extreme example of virus-induced immunopathology, we decided to include in our studies also a model in which a lytic virus (YF) induces severe encephalitis and death 7 to 10 days p.i. by a direct effect on the brain cells (Bassi et al., unpublished). That the selected viruses cause quite distinct pathologies is supported by our immunohistological analysis. Thus, while the virus-induced lesions induced by LCMV infection are focused around the meninges and the ependymal and choroid plexus, with limited involvement of the neuroparenchyma, YF infection induces widespread apoptosis throughout the brain. In spite of the differences between the two models, we established a general pattern of virus-induced SOCS expression in which SOCS mRNA levels increase markedly from day 3 to day 7 p.i., correlating with the appearance of T cells and IFN-γ/H9253.

Further analyses revealed that IFN-γ produced predominantly by infiltrating T cells was in fact partially responsible for the induction of SOCS1 and -3 gene expression in both models, but that other, as-yet unidentified mediators also play a role; a number of other cytokines have been found to induce SOCS expression (2),

FIG 14 YF-induced CXCL10 mRNA upregulation detected by in situ hybridization of the brains from WT mice. (A and B) CXCL10 mRNA was upregulated by cells (arrows) throughout the brains of WT mice on day 7 after infection with YF (A) but absent in comparable sections on day 7 after the injection of PBS (B). (C to G) Higher-magnification images showing YF-induced CXCL10 mRNA expression in cells (arrows) in the meningeal membrane (C) and in the ependyma (D), in association with vessels (E), and in the hippocampus proper (F) and neocortex (G). (H to J) In situ hybridization for SOCS mRNA and immunohistochemistry GFAP showing CXCL10 mRNA expression by GFAP/H11001 astrocytes; the images were either single stained (H and I) or merged (J). 3V, third ventricle; CA1 and -3, cornu ammonis 1 and 3; cc, corpus callosum; Ctx, cortex; E, ependyma; EC, external capsule; Hip, hippocampus; LV, lateral ventricle; MM, meningeal membrane; v, vessel. Scale bars: 400 μm (A and B), 30 μm (C, D, and G), 100 μm (E), 180 μm (F), and 20 μm (H to J).

FIG 15 Upregulation of SOCS1 and SOCS3 mRNA after i.c. infection with YF virus is only partially dependent on IFN-γ and T cells. The levels of mRNA expression for SOCS1 (A) and SOCS3 (B) were determined in the CNSs of IL-10/H11002, IFN-γR/H11002, nude, and WT mice infected i.c. with YF 7 days earlier. Each point represents one animal.
and several of these may be produced during viral infection, e.g., IL-6 and TNF. Unfortunately, we were not able to detect SOCS1 or -3 proteins in brain sections from virus-infected mice; however, other groups have experienced the same problem (23), and the explanation given is either instability and/or rapid turnover of the these protein (51). Indeed, these qualities may be essential for a proper dynamic regulation of negative regulatory circuits.

Surprisingly, in situ hybridization of brain sections revealed that SOCS1 and -3 mRNA was widely expressed in brain resident cells on day 7 p.i., as opposed to previous studies in EAE mice in which SOCS expression is reserved for the infiltrating inflammatory cell populations (23). Similarly, although virus-induced upregulation of SOCS expression in parenchymal cells of peripheral organs has been observed, increased expression in the inflammatory cells seems to be to the predominant feature (6), e.g., during peripheral infection of mice with a variant of LCMV causing chronic infection (clone 13), marked upregulation of SOCS3 in T cells has previously been described (52). Our qPCR results could also reflect upregulation of SOCS expression in the infiltrating cells, particularly after i.c. YF infection. Nevertheless, the current observations clearly underscore the ability of CNS resident cells to take an active part in regulating virus-induced inflammatory responses of the CNS.

Interestingly, we found that SOCS1 and -3 are expressed in astrocytes, which are also a major source of proinflammatory CXCL10 production following i.c. infection with LCMV (26), and in both cases IFN-γ acts as a positive regulator. Thus, apparently within the same cell population, IFN-γ may induce both proinflammatory and inhibitory signals. This model has some precedence in a study in which the authors showed that overexpression of SOCS1 in pancreatic islet cells in part prevented the development of virus-induced diabetes in a CD8+ cell-mediated RIP-LCMV diabetes model by preventing the induction of CXCL10 and thereby the recruitment of CD8+ T cells (55). That study provides strong support for a functional link between SOCS1 expression and the extent of IFN-γ-induced CXCL10 release, and, together with our data, suggests that further exploration of the immunomodulatory roles of SOCS proteins could lead to advances within the therapeutic fields of both autoimmunity and infectious diseases where excessive inflammation may contribute to the induced tissue damage.

It has previously been speculated that viruses could exploit the functions of host SOCS proteins to hide from the immune response, for example, by inhibiting type 1 IFN-induced signaling (6). However, in neither of our two models of virus-induced CNS inflammation did we see marked SOCS gene expression at early time points when type 1 IFN signaling is expected to be most important. Consequently, we are inclined to conclude that the induction of SOCS1 and -3 more likely represents an attempt to reduce the positive-feedback loop between IFN-γ produced by infiltrating T cells and CXCL10 produced by astrocytes and causing the influx of T cells (25, 26). Obviously, this normally fails to prevent LCMV-induced lethal disease; however, we do not take this to contradict our interpretation. Most likely, the lethal outcome of LCMV infection simply represents a case of too-little, too-late counter-regulation, and perhaps overexpression of SOCS 1 and/or 3 might reduce the mortality. Unfortunately, studying the clinical outcome of infection in the various knockout strains we have used to identify regulatory mechanisms is of no help in evaluating our hypothesis, because the involved genes also affect other critical parameters of the virus-host interaction, such as immune response and/or virus distribution.

Although a negative-feedback loop makes immediate sense in a situation where clinical disease directly results from the immune attack, it is interesting that similar mechanisms seem to be at work during infection with YF, a cytolytic virus. However, depending on the circumstances even following infection with cytolytic viruses, immune pathology may contribute to the severity of disease, and there are well-described examples in the literature where the course of an infection with a cytolytic virus is improved by a certain measure of downregulation of the immune response. For example, in the context of respiratory viruses, such as influenza virus and respiratory syncytial virus, T cell-produced IL-10 has been found to limit excess pulmonary inflammation without negatively affecting viral clearance (54, 55). Similarly, in the case of coronavirus-induced encephalitis it has been found that IL-10 produced by infiltrating cytolytic CD8 T cells diminishes disease severity (41). Consequently, we propose that the function of SOCS1 and -3 proteins in the context of the inflamed CNS is to prevent excess inflammation and, in this manner, protect the brain tissue from severe inflammation-induced tissue damage, e.g., incarceration as a consequence of cerebral edema.

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