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Induction of Suppressor of Cytokine Signaling-1 by *Toxoplasma gondii* Contributes to Immune Evasion in Macrophages by Blocking IFN-γ Signaling

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*Toxoplasma gondii* is an intracellular parasite that survives and multiplies in professional phagocytes such as macrophages. Therefore, *T. gondii* has to cope with the panel of antimicrobial host immune mechanisms, among which IFN-γ plays a crucial role. We report in this study that in vitro infection of murine macrophages with viable, but not with inactivated, parasites results in inhibition of IFN-γ signaling within the infected cells. Thus, infection of RAW264.7 macrophages with *tachyzoites* inhibited IFN-γ-induced STAT-1 tyrosine phosphorylation, mRNA expression of target genes, and secretion of NO. These effects were dependent on direct contact of the host cells with living parasites and were not due to secreted intermediates. In parallel, we report the induction of suppressor of cytokine signaling-1 (SOCS-1), which is a known feedback inhibitor of IFN-γ receptor signaling. SOCS overexpression in macrophages did not affect tachyzoite proliferation per se, yet abolished the inhibitory effects of IFN-γ on parasite replication. The inhibitory effects of *T. gondii* on IFN-γ were diminished in macrophages from SOCS-1<sup>-/-</sup> mice. The results suggest that induction of SOCS proteins within phagocytes due to infection with *T. gondii* contributes to the parasite’s immune evasion strategies.

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3 Abbreviations used in this paper: iNOS, inducible NO synthase; CIS, cytokine-inducible Src homology 2 domain-containing protein; LXA₄, lipoxin A₄; MIG, monokine induced by IFN-γ; IGIP, interferon-inducible GTPase 1; MOL, multiplicity of infection; SOCS, suppressor of cytokine signaling.
SOCS-1 knockout mice were generated from heterozygous intercrosses (mixed 129 x BL6 background) and have been described in detail previously (20). Bone marrow-derived macrophages were prepared from newborn wild-type littermates or SOCS-1 knockout mice as previously described (21). Briefly, bone marrow cells were seeded overnight in RPMI 1640 supplemented with 10% FCS, antibiotics, and 20% L929-conditioned medium as a source of M-CSF. Nonadherent cells were further propagated for 7–9 days, with a boost of fresh medium on day 4. Finally, adherent cells were scraped and used for the experiments. In the same way, macrophages were obtained from IL-10-deficient mice (22). RAW macrophages stably overexpressing SOCS proteins were established by cotransfection of SOCS expression plasmids and a neomycin resistance cassette, as described by us previously (23, 24). BV-2 cells and bone marrow-derived dendritic cells were used as previously described (25).

Infection with T. gondii

Tachyzoites from the T. gondii strain BK were prepared by coculture with RAW264.7 macrophages. Freshly hatched tachyzoites were used for infection experiments at different parasite to host ratios (multiplicity of infection (MOI)). Where indicated, tachyzoites were killed by repeated freeze/thaw cycles or were labeled with CSFE. To this, 1 x 10^8 tachyzoites were incubated in 5 ml of 2 µM CSFE at 37°C for 15 min, followed by PBS for 30 min.

Determination of NO secretion

Cells (1.5 x 10^5) were infected with tachyzoites in 96-well plates as stated in the respective experiment and were treated with recombinant murine IFN-γ (Tebu). Supernatants were harvested after 26 h of stimulation and analyzed. NO accumulation was measured photometrically (550 nm) by mixing equal parts of supernatant and Griess reagent (1/1 mixture of 1 g% sulfanilamide/5% H3PO4 and 0.1% naphthyl-ethylenediamine dihydrochloride).

Cell viability

Cell viability was assessed by determination of MTT turnover.

Quantitative RT-PCR

Cells (1 x 10^6) were stimulated either in 24-well plates or in Transwells (0.4 µm). Total RNA was isolated using a HighPure RNA kit (Roche), which included DNase I digestion. Total RNA (1 µg) was reverse transcribed with a cDNA synthesis kit (MBI Fermentas). Then, cDNA was diluted 1/4 and used as a template in the quantitative PCR-mix according to the manufacturer’s standard protocol (Eurogentec: ABI PRISM 7700; Applied Biosystems). The primer sequences have been previously described (26) and are available on request. Quantifications were made using either fluorogenic probes (FAM/TAMRA) or by means of SYBR Green. The specificity of RT-PCR was controlled by no template and no reverse transcriptase controls. PCR efficiencies for all reactions were determined and were similar (0.96–1.00). Threshold values were normalized to the expression of β-actin. Quantitative PCR results are expressed either as n-fold induction to nonstimulated cells or as relative expression (1/2(Ct target gene – Ct actin)).

Western blot

Cells (2 x 10^5) were infected with tachyzoites in medium containing 0.5% FCS before IFN-γ stimulation. Cells were lysed for 30 min on ice in 250 µl of lysis buffer (50 mM Tris-HCl (pH 7.4); 1% Igepal; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 µg/ml each of aprotinin, leupeptin, and pepstatin 1; 1 mM Na3VO4; and 1 mM sodium fluoride). Lysates were cleared by centrifugation at 4°C for 10 min at 11,000 x g. Equal amounts of lysates were fractionated by 10% SDS-PAGE and electrophoresed to polyvinylidene difluoride membranes. Membranes were stained as indicated, and proteins were detected using an ECL system (Amersham Biosciences). Phosphotyroisine-specific STAT1 Ab was purchased from Cell Signaling Technology; STAT1, STAT3, and actin Abs were obtained from Santa Cruz Biotechnology.

Confocal microscopy

RAW264.7 macrophages (3 x 10^5) were grown on chamber slides in medium containing 0.5% FCS overnight. Then cells were infected with CSFE-labeled tachyzoites and subsequently stimulated with IFN-γ. Cells were fixed in 4% paraformaldehyde/PBS for 20 min and permeabilized in −20°C methanol for 1 h. Cells were incubated with pY-STAT1 (1/50) at 4°C overnight, stained with tetramethylrhodamine isothiocyanate-second-
Tachyzoites resulted in an inhibition of early signal transduction of IFN-γ, as measured by loss of tyrosine phosphorylation of STAT1 (Fig. 2a). Moreover, confocal microscopy with labeled parasites indicated that the inhibitory effects were only observed in infected cells, not in bystander cells, excluding a possible role of paracrine factors (data not shown). This was verified in Transwell...
experiments (Fig. 2c). Only cells that were in direct contact with the parasites and thus could become infected became refractory to IFN-γ, as measured by inhibition of MIG. In contrast, cells on the opposite chamber of the Transwell were not affected in IFN-γ responsiveness by parasite infection.

Beside the inhibition of STAT1 phosphorylation, levels of STAT1 were decreased especially at higher parasite to host cell ratios (Fig. 2a). This, again, could not be accounted for by cell loss, because the loading control actin as well as STAT3 were unaffected. STAT1 activation was also examined using an ELISA-based assay that quantitatively measured nuclear STAT1 accumulation (Fig. 2b). It was observed that T. gondii infection inhibited activation and subsequent nuclear translocation of STAT1, confirming the above findings. However, we also noted that inhibition of IFN-γ-mediated NO production over time was more effectively inhibited than nuclear translocation of STAT1 when comparing two different MOIs (Fig. 2b).

The Transwell experiment (Fig. 2c) already excluded a role of secreted factors for the inhibitory effects of T. gondii infection; however, we furthermore analyzed whether the inhibitory cytokine IL-10 might play a role. We observed that the inhibition of IFN-γ induced NO by T. gondii was equal in IL-10+/− and wild-type macrophages (Fig. 2d).

SOCS proteins have been reported to be natural inhibitors of cytokine receptor signaling acting proximal in the signaling cascade. Therefore, we analyzed the expression of various SOCS family members upon T. gondii infection. We observed that viable, but not killed, parasites were able to increase mRNA expression of SOCS-1 and CIS. Only weak induction of SOCS-3 was observed (Fig. 3, a–c). Expression of SOCS-1 and CIS increased with higher parasite to host ratios. The characteristics were the same as those observed for the IFN-γ inhibitory effects. Thus, only freshly prepared and highly infectious parasites were able to induce SOCS, and these effects were dependent on the applied MOI. Furthermore, the expression of SOCS-1, which plays a crucial role for inhibition of IFN-γ, started to increase 4 h after infection (Fig. 3d), thus paralleling the kinetics of IFN-γ inhibition (Fig. 1e). Transwell experiments demonstrated that SOCS-1 expression was a direct effect of T. gondii infection and was not mediated via paracrine factors (Fig. 3e) as was the infection-associated inhibition of IFN-γ-induced effects (Fig. 2c).

Next, we analyzed RAW264.7 macrophages stably overexpressing various SOCS family members. Cells were infected at a low MOI, and under these conditions, IFN-γ was able to increase NO in infected cells (Fig. 4a; control and mock). This went along with a complete inhibition of T. gondii growth (measured specifically by [3H]uracil incorporation), establishing that the antiparasitic effects of IFN-γ were functional in our assay (Fig. 4b; control and mock). Overexpression of SOCS-1, however, ablated IFN-γ-mediated NO secretion and led to an undisturbed proliferation of parasites in infected cells (Fig. 4). Intermediate effects were observed for CIS overexpression, whereas SOCS-2 overexpression, which has no reported role in IFN-γ signaling, did not show any alterations.

Using macrophages generated from bone-marrow of newborn SOCS-1−/− mice, we could also confirm a role of SOCS-1 in T. gondii-mediated IFN-γ inhibition (Fig. 5). As a readout, we used the IFN-γ-induced expression of MIG. In SOCS-1−/− cells, we noted that IFN-γ increased the expression of MIG to higher levels compared with wild-type cells, consistent with a role of SOCS-1 as an intrinsic negative feedback inhibitor. However, although
infection of wild-type macrophages with *T. gondii* decreased IFN-γ/H9253-induced expression of MIG to 17% of uninfected control values, infected macrophages from SOCS-1/H11002 mice showed only an inhibition to 44% (Fig. 5). Similar results were obtained for iNOS induction. In wild-type macrophages, an inhibition to 76% was observed upon infection. Under the chosen conditions, inhibition was weaker than that in RAW264.7 macrophages. However, macrophages from SOCS-1/H11002 mice did not show any inhibition upon infection (data not shown), confirming the results with MIG.

**Discussion**

We report in this study that *T. gondii* is able to use the host SOCS system for its own purposes, namely, to inhibit IFN-γ signaling. Thereby parasites can evade the antiparasitic effects of IFN-γ in macrophages. We found that viable and infectious parasites from the BK strain, but not killed parasites, are able to inhibit IFN-γ/H9253 signaling and IFN-γ/H9253-induced NO, which are critical mediators of host defense in murine macrophages (2, 3, 29). Although the importance of NO during in vivo infection seems to be dependent on the stage of infection, the mouse strain, and the route of infection (30, 31), it is evident that NO is essential for restricting the growth of parasites. Thus, interfering with the NO-inducing IFN-γ system is a promising point for parasites to evade or retard immune defenses. We found that IFN-γ-inducible genes iNOS, MIG, and RT-PCR after infection with 10:1 tachyzoites for different time periods. Displayed is the relative expression of SOCS-1 normalized to β-actin (mean of triplicate determinations ± SD). e. RAW264.7 macrophages were incubated in 0.4-μm Transwells. Cells were infected 10:1 with *T. gondii* in the lower compartment and subsequently analyzed for SOCS-1 expression after 5 and 20 h (one of two experiments; mean of triplicate determinations ± SD).
IIIGP1] were inhibited, confirming and extending earlier findings of different groups (8, 11, 12, 32), which showed modulation of iNOS activity by various T. gondii strains. We consistently observed that the inhibitory potential varied between different experiments and was dependent on the capacity to infect host cells. Thus, the parasite to host ratio to obtain similar inhibition might be different for various strains, as also proposed by others (11).

Extending the findings on macrophages, we observed that infection with viable T. gondii also led to an impairment of IFN-γ signaling in dendritic cells. Because dendritic cells are a major source for IL-12, which has been reported to be crucial for IFN-γ synthesis and resistance to acute toxoplasmosis (33), it will be interesting to determine whether dendritic cells either activated by toxoplasma lysate or infected by viable parasites behave differentially in terms of induction of primary T cell responses.

Concerning the mode of IFN-γ inhibition, we observed that the signaling pathway was disrupted far proximally at the stage of tyrosine phosphorylation of STAT1. This contrasts with the findings of Luder et al. (10), who did not observe inhibition of phosphorylation. However, they used much higher doses of IFN-γ and a lower parasite to host ratio. Indeed, we also observed that the inhibition can be overcome partially by increasing the IFN-γ dose (data not shown). Our results parallel data obtained in a model of African trypanosomiasis in which trypanosome infection led to an inhibition of IFN-γ signaling via a decrease in STAT1 phosphorylation (34). Moreover, our data indicate that IFN-γ inhibition is not merely due to a decrease in phosphorylated STAT1, but that STAT1 itself becomes degraded (Fig. 2). Effects of T. gondii infection on overall cell viability within the observation period were excluded carefully. Both findings are nevertheless consistent with the proposed mode of action of SOCS proteins. Indeed, SOCS-1 has been reported to inhibit JAKs, and this is achieved via the Src homology 2 and KIR domains (17). Yet, SOCS proteins have another functional domain, the C-terminal SOCS box (35). This domain interacts with elongin B/C, which is part of an E3 ligase, thus mediating ubiquitination and degradation of bound target molecules (36). Accordingly, SOCS-1 would prevent phosphorylation of STAT1 and also initiate degradation. In vivo deletion of the SOCS box alone resulted in a phenotype resembling that of the complete SOCS-1 knockout with milder characteristics, substantiating a role of the SOCS box in gene function (37).

To establish a productive infection in macrophages, T. gondii has to manipulate two branches of macrophage activation. First, the direct recognition of parasites that results in macrophage activation has to be avoided. TLRs are crucial for the initiation of this task. Indeed, molecules prepared from T. gondii can be recognized in an MyD88-dependent manner (38, 39), which is important for downstream TLR signaling. Recently, a T. gondii profilin-like protein has been defined as a new ligand for TLR-11 (40). Thus, it is evident that macrophages are able to sense infectious danger by parasites, and this, in general, results in activation of the NF-κB pathway and subsequent IL-12 and TNF-α secretion. However, it has been observed that this pathway is inhibited by infection with viable T. gondii (7, 8). MAPKs, which are another signaling system of TLRs, were also inhibited by T. gondii infection (5). As was the case for our observations, these effects were dependent on a direct contact of parasite with host cells, thus excluding paracrine factors. We also show that the inhibitory potential is directly related to the process of infection (Transwell experiments, live vs killed parasites) and is not mediated by secreted factors, including IL-10. Recently, it has been shown that T. gondii inhibits the NF-κB pathway in a STAT3-dependent manner (9). STAT3 is the crucial transmitter of the inhibitory cytokine IL-10, yet the inhibitory effects were IL-10 independent. Thus, T. gondii seems to hijack the STAT3-dependent endogenous anti-inflammatory pathway to inhibit an initial branch of macrophage activation. It is unlikely that the SOCS proteins play a role in the inhibition of NF-κB. Although initial reports argued for an inhibitory role of SOCS in TLR signaling (41, 42), our own work strongly argues against this interpretation (21, 23). Thus, the regulatory actions of SOCS proteins remain restricted to cytokine receptor signaling.

Beside the direct activation by contact with parasites, macrophages are efficiently boosted by the effects of IFN-γ. Moreover, IFN-γ and STAT1 are indispensable for defense of T. gondii infections (3, 43). Targeting IFN-γ is thus a promising strategy for parasites. A similar mechanism has been proposed for murine leishmaniasis. In this study it was found that L. donovani induces SOCS-3, thereby suppressing activation of human macrophages (44). Similar to our results, the induction of SOCS was a result of direct host-pathogen contact. Moreover, it was reported that SOCS-1 deficiency results in decreased numbers of infected macrophages in an in vitro infection with Leishmania major and LPS/IFN-γ stimulation (18). Also, infection with Listeria monocytogenes modulated IFN-γ signaling via induction of SOCS-3 (45).

At present, it is only possible to speculate by which means T. gondii and apparently other parasites induce SOCS expression. We and others (24, 26, 46) have shown that TLR stimulation results in the induction of SOCS proteins. However, we could not find SOCS mRNA upon addition of killed T. gondii parasites, although T. gondii proteins can be recognized via TLRs (40). Furthermore, it would be difficult to induce SOCS in a TLR-dependent manner while simultaneously inhibiting the main NF-κB pathway.

Regarding the induction of SOCS, an interesting link might develop that involves lipoxin (LXA₄), an anti-inflammatory eicosanoid mediator. Lack of LXA₄ in 5-lipoxygenase-deficient mice resulted in enhanced mortality with increased IL-12 levels during T. gondii infection (47). Moreover, evidence was given that T. gondii might hijack the endogenous LXA₄ system, because parasitic extracts showed 15-lipoxygenase activity (48). Moreover, it has been found that LXs induce SOCS proteins, which contribute to their anti-inflammatory actions (49). Although LXs are soluble mediators, and we excluded paracrine factors being responsible for the observed effects, it might be speculated that sufficient concentrations are only achieved in infected cells, or the mediators might act directly within infected cells.

Lack of immune activation will result in detrimental effects due to unlimited parasite replication, although, in contrast, overshooting immune responses will result in immunopathology. Indeed, it has been shown that T. gondii infection in susceptible mice can lead to tissue destruction in small intestine and liver (50, 51), and
this was mediated by IFN-γ and NO. To establish a chronic infection, which is a hallmark of toxoplasmosis, dampening acute inflammation to avoid excessive host damage might be a more promising strategy. In this respect, SOCS could also function in avoiding acute toxicity. Indeed, it was found that when SOCS-1+1 mice were challenged with L. major, they developed larger lesions despite having a similar parasitic load as wild-type mice (52). It will be interesting to analyze whether the different susceptibilities of mouse strains toward model parasites might also have a correlate in the ability to induce SOCS proteins.

In summary, T. gondii has developed means to inhibit both direct activation of macrophages by microbial recognition and indirect activation via immuno-activated cytokines, especially IFN-γ. For the latter, in this study we have identified a possible mechanism that involves the manipulation of the host SOCS system. Inducing endogenous SOCS by the parasite results in inhibition of IFN-γ signaling at the level of signal transduction and rescues T. gondii from the otherwise detrimental antiparasitic effects of this cytokine.

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Disclosures

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