Aberrant IL-4 production by SOCS3-over-expressing T cells during infection with *Leishmania major* exacerbates disease manifestations

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Received 28 April 2010, accepted 17 December 2010

Abstract

Suppressor of cytokine signaling (SOCS) 3 is a major negative feedback regulator of signal transducer and activator of transcription 3-activating cytokines. Studies using T-cell-specific SOCS3-deficient mice indicate that the absence of SOCS3 in T cells results in exacerbation of disease progression after infection by *Leishmania major* due to skewing of the Th3 cell phenotype accompanied by hyper-production of IL-10 and transforming growth factor β (TGF-β). Here we show that transgenic mice over-expressing the SOCS3 gene in T cells (Lck-SOCS3 Tg mice) are also susceptible to infection by *L. major*. Forced expression of SOCS3 in T cells did not affect the production of the anti-inflammatory cytokines IL-10 and TGF-β or that of the protective Th1 type cytokine IFN-γ, which is required for parasite clearance. CD4⁺ T cells isolated from infected-Lck-SOCS3 Tg mice produced much higher levels of IL-4 when they were re-stimulated with *L. major* antigen in vitro. Exacerbation of disease progression in Lck-SOCS3 Tg mice was completely reversed by administration of a neutralizing antibody against IL-4. These data suggest that tight regulation of SOCS3 expression in Th cells is crucial for disease control during infection by *L. major*.

Keywords: regulatory T cell, Th1, Th2, cytokine

Introduction

The murine model of infection with the protozoan parasite *Leishmania major* has provided the first evidence of the relevance of the balance between Tₜ₁/Tₜ₂ cells in the regulation of disease outcome in vivo. Disease outcome is strongly correlated with the early patterns of Tₜ₁ subset differentiation such as the IL-12 driven IFN-γ-dominated Tₜ₁ response that promotes parasite clearance, and an IL-4-driven- and IL-4-dominated-Tₜ₂ response that causes the development of progressive lesions (1, 2). The genetic predisposition to infection by *L. major* in mice has been studied intensively. In most inbred mouse strains (including C57BL/6), preferential differentiation towards Tₜ₁ cells upon infection by *L. major* leads to resolution of cutaneous infection via IFN-γ-mediated activation of macrophages, whereas in some strains (e.g. BALB/c) the preferential production of IL-4 and IL-10 from Tₜ₂ cells accompanied by reduced production of IFN-γ cannot efficiently activate macrophage leishmanicidal function (3). Disease outcome can be reversed by treatment of resistant strains with anti-IL-12 or anti-IFN-γ antibody or by treatment of susceptible strains with anti-IL-4 antibody (4–6). Furthermore, recent studies on the differentiation of Tₜ₁ cells regulated by cytokine signals have revealed new Tₜ₁ subsets; IL-17-producing Tₜ₁ cells driven by transforming growth factor β (TGF-β) and IL-6 (7, 8), and immunosuppressive inducible regulatory T (iTreg) cells driven by TGF-β (9). Treg cells modulate effector immune responses (10), and the balance of Tₜ₁/Tₜ₂/
SOCS3 modulates infection by *L. major* through IL-4

T<sub>h</sub>17/Treg is believed to be important for the direction of immune responses. Therefore, the early differentiation of T<sub>h</sub> cells determined by the cytokine milieu is crucial for disease outcome.

In physiological as well as pathological conditions, cytokine signals are tightly regulated by many factors, including a cytoplasmic protein family termed “suppressor of cytokine signaling (SOCS)” (11). Cytokines (including ILs and IFNs) activate the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway. This leads to the expression of various target genes including SOCS. Consequently, SOCS protein inhibits its JAK–STAT activity, thereby forming a negative-feedback loop. Among the eight SOCS family proteins, SOCS3 in particular inhibits IL-6/gp130-mediated activation of STAT3 (12). We have previously shown that deletion of the SOCS3 gene in T-cell compartments results in susceptibility to *L. major* infection, and the development of progressive lesions due to preferential production of IL-10 and TGF-β by SOCS3-deficient CD<sup>4</sup> T cells (13). Loss of SOCS3 in T<sub>h</sub> cells leads to excessive activation of STAT3 that enhances IL-10 and TGF-β promoter activities. It has been demonstrated that IL-10 and TGF-β exert powerful deactivating effects on infected cells. For instance, production of reactive nitrogen intermediates by IFN-γ-activated macrophages is suppressed by IL-4, IL-10 and TGF-β (14). Therefore, SOCS3 in T<sub>h</sub> cells is a crucial factor for protective immunity against *L. major* infection.

We examined the functional significance of SOCS3 expression in T<sub>h</sub> cells for eliminating the intracellular pathogen *L. major*. Contrary to expectations, constitutive expression of SOCS3 in T<sub>h</sub> cells in transgenic mice carrying the SOCS3 gene driven by the Lck-E<sub>μ</sub>-promoter (Lck-SOCS3 Tg mice) results in exacerbation of disease progression rather than reinforced protective immunity. Lck-SOCS3-Tg mice infected with *L. major* exhibited neither an increased number of Treg populations nor enhanced production of anti-inflammatory cytokines such as IL-10 and TGF-β. However, IL-4 production by CD<sup>4</sup> T cells from infected Lck-SOCS3 Tg mice was significantly elevated, and the administration of a neutralizing antibody against IL-4 ameliorated lesions, suggesting a skewing of the immune response toward a T<sub>h</sub>2 phenotype in Lck-SOCS3 Tg mice during the course of infection. Taken together, temporal regulation of SOCS3 expression in T<sub>h</sub> cells is critical for disease control of *L. major* infection.

**Methods**

**Mice**

Transgenic mice carrying the Myc-tagged wild-type SOCS3 gene driven by the Lck-E<sub>μ</sub>-promoter (Lck-SOCS3 Tg mice) have been described (15). Lck-SOCS3 Tg mice were backcrossed on a C57BL/6 genetic background more than 12 times. Mice were kept in specific pathogen-free facilities. Experiments using mice were approved by and carried out according to the guidelines of the Animal Ethics Committee of Kyushu University (Fukuoka, Japan).

*L. major* infection in mice

*Leishmania major* (MHOM/SU/73-5-ASKH) were passed in *vivo* and grown *in vitro* in medium 199 with 10% heat-inactivated fetal bovine serum containing 2 mM glutamine, 10 mM HEPES and gentamicin (100 µl ml<sup>−1</sup>). Infection of *L. major* was carried out as described previously (13). Briefly, 1 x 10<sup>7</sup> stationary-phase promastigotes were subcutaneously injected into the right footpad of mice. To neutralize IL-4 *in vivo*, mice were intra-peritoneally injected with 2 mg of anti-IL-4 (clone: 11B11) 5 h before and 24 h after infection. Footpad swelling was monitored every 2 weeks with a vernier caliper. Parasite burdens in the footpads were quantified by homogenizing tissue in 10 ml of the complete medium 199 as described previously (13).

**Cytokine assay**

Four weeks after infection by *L. major*, CD<sup>4</sup> T cells were isolated from the right popliteal lymph nodes by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) sorting using anti-CD4 antibody (clone: GK1.5) according to the manufacturer’s protocol. Cells (5 x 10<sup>5</sup> per 200 µl per well) were stimulated with or without *L. major* antigens (equivalent to 5 x 10<sup>5</sup> promastigotes) in the presence of irradiated (30 Gy) T-cell-depleted splenocytes for 70 h. Culture supernatants were collected and analyzed for IL-4, IFN-γ, IL-10 and immunoglobulins by ELISA. ELISA kits were purchased from companies as follows: IFN-γ, IL-4 and IL-10 (eBioscience, San Diego, CA, USA); TGF-β (Promega, Madison, WI, USA); and mouse immunoglobulin isotype (Bethyl Laboratories Incorporated, Montgomery, TX, USA); these were used according to the manufacturers’ instructions.

**Flow cytometric analyses**

Anti-CD4-FITC, anti-CD4-allophycocyanin (APC), anti-CD8α-FITC, anti-CD25-PE, and anti-Foxp3-APC antibodies were from eBioscience, and anti-B220-PerCP-Cy5.5 antibody was from BD Bioscience (Franklin Lakes, NJ, USA). For intracellular Foxp3 staining, popliteal lymph node cells were isolated, washed and stained with anti-CD4-FITC and anti-CD25-PE antibodies for 30 min. Cells were washed and fixed in 1 x Fix/Perm solution (eBioscience) for 2 h on ice. Cells were then washed and suspended in permeabilization buffer (eBioscience), stained with APC-conjugated anti-mouse Foxp3 (clone: FJK-16s), and analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Bioscience). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

**Immunohistochemistry**

Tissue samples were isolated and fixed in 10% buffered formalin and embedded in paraffin. Sections were cut, dehydrated and microwaved in 10 mM citrate buffer (pH 6.0) twice, for 5 min each time. Sections were incubated with anti-inducible nitric oxide synthase (anti-iNOS) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:100 dilution). VECTASTAIN ABC kits (Vector Laboratories, Burlingame, CA, USA) and Histofine MOUSESTAIN kit (Nichirei Biosciences, Tokyo, Japan) were used for detection. Sections were counterstained with hematoxylin. The iNOS<sup>+</sup> area was measured in five regions for each condition using ImageJ software.
**Results**

**Exacerbation of disease progression in Lck-SOCS3 Tg mice infected with *L. major***

We have previously demonstrated that T-cell-specific SOCS3 deficiency leads to susceptibility to infection with *L. major* owing to preferential differentiation of immunosuppressive T<sub>H</sub>3 cells that produce large amounts of TGF-β and IL-10. We therefore investigated whether the constitutive expression of SOCS3 in T<sub>H</sub> cells reverses disease outcome.

Wild-type (WT) and Lck-SOCS3 Tg mice (C57BL/6 background) were subcutaneously infected with *L. major*, and lesion size was monitored (Fig. 1A and B). Footpad swelling was exacerbated significantly more in Lck-SOCS3 Tg mice than in WT mice throughout the course of infection. In Lck-SOCS3 Tg mice, lesion size remained large until 8 weeks after infection, whereas in WT mice it gradually reduced to the basal levels (Fig. 1B). We then examined the number of parasites remaining in the footpads. Four weeks after infection, the numbers of parasites in the lesions were greater in Lck-SOCS3 Tg mice than in WT mice (Fig. 1C). These results indicated that the constitutive expression of SOCS3 in T<sub>H</sub> cells exacerbates cutaneous leishmaniasis.

The number of Treg cells does not account for exacerbation of disease progression in Lck-SOCS3 Tg mice

To reveal the molecular and cellular mechanisms underlying the progression of lesions in Lck-SOCS3 Tg mice, we initially examined the lymphocyte population in the popliteal lymph nodes. In uninfected mice, there was no significant difference in lymphocyte counts in the popliteal lymph nodes between Lck-SOCS3 Tg mice and WT mice (Fig. 2A left panel). The number of lymphocytes gradually increased after infection by *L. major*. In Lck-SOCS3 Tg mice, the lymphocyte number was slightly lower than in WT mice; this difference reached statistical significance 4 weeks after infection and became more prominent at 8 weeks after infection (Fig. 2A, right panel). The incidence of B cells among the total population of popliteal lymph node cells was greatly increased at 4 weeks after *L. major* infection, but this increase occurred in both WT and Lck-SOCS3 Tg mice to a comparable degree (WT: 75.7 ± 1.9% versus Tg: 78.9 ± 1.7%) (Fig. 2B, upper panels). In uninfected mice, a slight reduction in CD<sup>8+</sup> T cells was observed in Lck-SOCS3 Tg mice, but there was no significant difference between the two groups of mice in CD<sup>4+</sup> T-cell counts in the popliteal lymph nodes (Fig. 2B, left panel). The ratio of CD<sup>8+</sup> T cells to CD<sup>4+</sup> T cells in the T-cell compartment was significantly reduced in infected Lck-SOCS3 Tg mice (WT: 42.1 ± 1.7% versus Tg: 33.4 ± 2.0%; Fig. 2B, middle panels). Given that the total lymphocyte number in infected Lck-SOCS3 Tg mice was reduced, the cellularity of CD<sup>8+</sup> T cells was drastically reduced in Lck-SOCS3 Tg mice (Fig. 2C, right panel). The Lck promoter induces gene expression not only in peripheral T cells but also in the thymus. The phenotypes of thymocytes in uninfected mice, including percentages and actual numbers of double-negative (DN), double-positive (DP), single-positive (SP) and CD44/CD25 populations, are shown in Fig. 2(D and E). The total number of lymphocytes in the thymus was not significantly different between WT and Lck-SOCS3 Tg mice, although the numbers of CD8SP T cells were lower in Lck-SOCS3 Tg mice than in WT mice, while the numbers of CD4<sup>+</sup> T cells were comparable between the two groups (Fig. 2E). The population of DN1 was lower in Lck-SOCS3 Tg mice than in WT mice (Fig. 2D and E, lower panels). These results indicated that the reduction in CD8<sup>+</sup> T cells in Lck-SOCS3 Tg mice was an intrinsic defect of CD8<sup>+</sup> T-cell development or proliferation that occurred regardless of infection status.

Next, to analyze the frequency of Treg cells in the popliteal lymph nodes, we carried out intracellular staining for Foxp3 (a master transcription factor of Treg cells). The proportion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in the popliteal lymph nodes of uninfected mice was identical between the two groups (WT: 10.1 ± 1.2% versus Tg: 11.1 ± 0.5%), and the frequency did not change at 4 weeks after infection (WT: 11.1 ± 1.3% versus Tg: 10.8 ± 1.5%; Fig. 2B, lower panels). This indicated that Treg cells were not involved in the exacerbated disease outcome observed in Lck-SOCS3 Tg mice.
Exacerbation of disease progression in Lck-SOCS3 Tg mice is not due to impaired production of IFN-γ or enhanced production of anti-inflammatory cytokines

We examined cytokine production by CD4^+ T cells derived from the popliteal lymph nodes of infected mice. IFN-γ production by CD4^+ T cells that were re-stimulated in vitro and the amount of IFN-γ in the serum were comparable between WT and Lck-SOCS3 Tg mice (Fig. 3A and B). Production of anti-inflammatory cytokines such as IL-10 and TGF-β was also comparable between the two groups of mice (Fig. 3A).
Two groups of mice were comparable, however (Fig. 3D), and cytokine levels in the culture supernatant were determined by ELISA. in vitro SOCS3 Tg mice was significantly greater than production by with L. major infection. CD4+ lymph nodes were isolated from WT and Lck-SOCS3 Tg mice 4 weeks after infection. CD4+ T cells were re-stimulated with L. major antigen in vitro in the presence of naive antigen-presenting cells for 70 h. Cytokine levels in the culture supernatant were determined by ELISA. (C and D) Levels of total Ig (C) and IgG1 (D) in the serum from infected mice were measured by ELISA.

Results suggest that neither impaired production of IFN-γ nor enhanced production of anti-inflammatory cytokines accounts for the progression of lesions in Lck-SOCS3 Tg mice infected with L. major. Interestingly, IL-4 production by T cells from Lck-SOCS3 Tg mice was significantly greater than production by T cells from WT mice (Fig. 3A). Moreover, total IgG1 and IgE levels in the serum were significantly elevated in Lck-SOCS3 Tg mice compared with levels in WT mice regardless of infection status (Fig. 3C). The levels of antigen-specific IgG1 in the two groups of mice were comparable, however (Fig. 3D), and antigen-specific IgG2a and antigen-specific IgE were below detection levels (data not shown). Thus, the increased levels of total IgG1 and IgE may be a secondary effect of aberrant IL-4 production from T cells and may not be implicated in the exacerbation of L. major infection in Lck-SOCS3 Tg mice.

Neutralization of IL-4 reverses delayed clearance of parasites and increased footpad swelling in Lck-SOCS3 Tg mice

To investigate whether the enhanced production of IL-4 observed in Lck-SOCS3 Tg mice is responsible for the exacerbation of L. major infection, we neutralized IL-4 using a specific antibody. It is well known that neutralization of initially produced IL-4 in BALB/c mice during infection with L. major inhibits T helpers 2 cell differentiation (16). We therefore used BALB/c mice as a positive control to assess the effect of IL-4 neutralization. The increase in footpad swelling observed in Lck-SOCS3 Tg mice was completely reversed to the level seen in WT mice by administration of neutralizing antibody against IL-4 (Fig. 4A and B). The number of parasites in the lesions in Lck-SOCS3 Tg mice was also drastically reduced to the level seen in WT mice (Fig. 4C). The reduction in total lymphocytes and B cells in the popliteal lymph nodes of infected Lck-SOCS3 Tg mice was recovered by treatment with anti-IL-4 mAb (Fig. 4D and E). These results suggest that the enhanced levels of IL-4 initially produced by T cells in response to infection is responsible for the exacerbation of disease progression in Lck-SOCS3 Tg mice.

T helper 1 effector cells produce high levels of IFN-γ, leading to the expression of iNOS and production of leishmanicidal nitric oxide in macrophages. Despite the normal level of IFN-γ production, eradication of L. major was dampened in Lck-SOCS3 Tg mice. We therefore compared iNOS induction in the lesions of infected mice from an immunohistochemical perspective. iNOS induction in infected tissues of Lck-SOCS3 Tg mice was much weaker than that in the tissues of WT mice, which was completely reversed by the administration of anti-IL-4 mAb (Fig. 4F and G). These results suggest that aberrant production of IL-4 by the mutant T cells suppresses iNOS induction, which is required for the elimination of intracellular pathogens.

Discussion

Cytokines and their signaling pathways define the fate and function of T helper cells, which eventually determines disease outcome. In particular, the balance between T helper 1 and T helper 2 cells is crucial for disease control in the L. major infection model (1, 2). IFN-γ and IL-4 have been identified as the counter-regulatory T helper 1 and T helper 2 cytokines, respectively, that promote resistance and susceptibility to infection with L. major (4, 6, 16). The anti-inflammatory cytokines IL-10 and TGF-β, and the immunosuppressive T helper-17 cell subset known as Treg cells, limit the magnitude of effector responses, which contributes to the prevention of collateral damage to tissues but which may result in failure to eliminate intracellular pathogens (17–20). We have previously shown that the absence of SOCS3 in T cells results in exacerbation of L. major infection due to enhanced production of IL-10 and TGF-β caused by hyper-activation of STAT3 signaling (13).

In the present study, we examined the constitutive expression of SOCS3 in T helper cells during leishmaniasis. It has been shown that Lck-SOCS3 Tg mice show significantly enhanced airway responsiveness, suggesting that SOCS3 expression in CD4+ T cells promotes T helper 2-dependent responses such as allergic reaction (15). Here we found that excessive expression of SOCS3 in T cells also promoted the disease progression of leishmaniasis due to a dominant IL-4 response. Moreover, we demonstrated that aberrant IL-4 production during the early phase of infection is responsible for the exacerbation of disease progression in Lck-SOCS3 Tg mice.
We found that profound expression of IL-4 was induced in Lck-SOCS3 Tg mice after infection with *L. major*. CD4+ T cells from infected transgenic mice also produced a substantial amount of IFN-γ comparable with that produced by WT CD4+ T cells (Fig. 3). The percentages of IFN-γ-positive T cells were comparable between the two groups of mice regardless of anti-IL-4 treatment (data not shown). Serum levels of IFN-γ in infected Lck-SOCS3 Tg mice were almost identical with those in WT mice. Moreover, the expression levels of T-bet and GATA3 in T cells were not significantly

Fig. 4. Suppression of footpad swelling of *Leishmania major*-infected Lck-SOCS3 Tg mice by neutralization of initial IL-4. Mice received intraperitoneal injections of anti-mouse IL-4 antibody (+IL-4 antibody; 2 mg per mouse) 5 h before and 24 h after infection with *L. major*. BALB/c mice were used as a positive control. (A) The size of footpad lesions was monitored weekly. (B) Photographs show lesion development in WT and Lck-SOCS3 Tg mice at 6 weeks after infection. The numbers of parasites in the footpads (C) and draining lymph node cells (D) were counted at 6 weeks after infection. (E) The lymph node cells from infected mice were analyzed by flow cytometry. (F) iNOS expression in the footpad lesions was analyzed by immunohistochemistry. Six weeks after infection, the footpad lesions were stained with anti-iNOS antibody and photographed. iNOS+ areas are shown by arrows. Scale bar represents 100 μm. The iNOS+ area was measured in five regions under each condition as described in Methods. Data are mean ± SD (n = 6). Horizontal bars in (C) and (D) indicate the mean value of each group. ***P < 0.001, **P < 0.01, *P < 0.05.
different between the two groups of infected mice (data not shown). These results indicated that excessive production of IL-4 did not suppress the Th1 response in Lck-SOCS3 Tg mice. Similar phenomena have been reported in which constitutive expression of IL-4 or IL-10 in resistant strains failed to control infection by L. major despite generating a relatively strong Th1 response (19, 24). The mechanism by which an IFN-γ-producing Th1 response is established under Th2-dominated conditions in vivo should be resolved. Nevertheless, the Th2 response dominates disease outcome, probably because of the deactivating effects of Th2 cytokines on infected macrophages.

The mechanism by which IL-4 production is enhanced in Th1 cells in Lck-SOCS3 Tg mice is largely unknown. It has been shown that the high expression of SOCS3 in Th1 cells leads to skewing toward Th2 differentiation, probably due in part to the inhibition of IL-12-mediated STAT4 activation by SOCS3 (15). SOCS3 binds to IL-12Rβ2 and inhibits STAT4 activation, which in turn suppresses further induction of IL-12Rβ2 and T1 development (15, 25). Selective loss of IL-12 signaling due to the down-regulation of IL-12Rβ2 expression in the susceptible BALB/c strain has also been reported (26). Defective IL-12 signaling therefore probably promotes an IL-4-dominant Th2 response in Lck-SOCS3 Tg mice. However, the pathological relevance of the expression of IL-12Rβ2 in L. major infection is controversial because IL-12Rβ2 transgenic BALB/c mice are susceptible to infection by L. major despite normal activation of STAT4 in CD4+ T cells upon IL-12 stimulation (27). Furthermore, the induction of IL-12 in response to L. major infection is delayed in comparison with the induction of IL-4 and IL-13, even in resistant mice (28, 29). Therefore, under pathological conditions, IL-12 may have a limited role in inhibition of the early burst of IL-4 in response to L. major infection.

Another possible effect of Th2-type polarization in Lck-SOCS3 Tg mice during infection with L. major is impaired signaling of IL-27 (an IL-12-related cytokine induced earlier than IL-12 in response to L. major infection). IL-27 activates STAT1 and STAT3 through its receptor, which consists of WSX-1 and gp130 subunits, resulting in positive and negative regulations of immune responses (30). It is thought that IL-27 not only promotes Th1,2 response but also suppresses IL-4 production in Th1,2 cells after infection with L. major (25, 31). Indeed, WSX-1-deficient C57BL/6 mice are highly susceptible to L. major infection accompanied with increased levels of IL-4 (32). Our preliminary data indicated that activation of STAT1 and STAT3 in response to IL-27 was severely impaired in CD4+ T cells from Lck-SOCS3 Tg mice (data not shown). Therefore, constitutive expression of SOCS3 in CD4+ T cells may inhibit the IL-27 signal, leading to the increase in IL-4 in Lck-SOCS3 Tg mice. Further investigation is necessary to clarify the mechanism underlying the enhanced production of IL-4 in Lck-SOCS3 Tg mice.

We found that CD8+ T cells were significantly reduced in infected Lck-SOCS3 Tg mice, but the number of CD8+ T cells in the thymus was already decreased in uninfected mutant mice. Thus, forced expression of SOCS3 in T cells resulted in a reduction in peripheral CD8+ T cells, although the mechanism behind this was uncertain. It has been reported that CD8+ T cells do not play a critical role in the early phase of L. major infection (33). Thus, the reduction in CD8+ T cells observed in Lck-SOCS3 Tg mice may not be implicated in the exacerbation of disease progression. Indeed, the reduction in CD8+ T cells was not reversed by the administration of anti-IL-4 mAb, although the disease was ameliorated by the treatment.

In the present study, we revealed that SOCS3 expression in Th1 cells is functionally important for eliminating L. major. The constitutive expression as well as the absent expression of SOCS3 in Th1 cells results in exacerbation of disease progression with a different mode of action. Therefore, expression levels of SOCS3 must be regulated strictly and temporally during L. major infection. Taken together, the available evidence shows that temporal regulation of SOCS3 expression in Th1 cells is critical for the control of L. major infection.

**Funding**

Special Grants-in-Aid for Scientific Research on Priority Areas (19041058 to T.K.), for Scientific Research on Innovative Areas (22117516 to T.K.), for Scientific Research (20590492 to T.K.), for Young Scientists (22790472 to M.N.) and Special Coordination Fund for Promoting Science and Technology Improvement of research environment for young researchers Carrier development program for young investigators in “Cell and Metabolism Research” from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), the Nakatomi Foundation, the Takeda Science Foundation, The Kato Memorial Trust Foundation for Nanbyo Research, the Suzuken Memorial Foundation, the Japan Intractable Disease Research Foundation, the Naito Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, Astellas Foundation for Research on Metabolic Disorders, the Yakult Bioscience Research Foundation, and the Princess Takamatsu Cancer Research Fund.

**Acknowledgements**

We thank T. Yoshioka-Nakaji, M. Ohtsu, and N. Kinoshita for technical assistance and Y. Nishi for manuscript preparation.

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