SOCS3 in T and NKT Cells Negatively Regulates Cytokine Production and Ameliorates ConA-Induced Hepatitis

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Suppressor of cytokine signaling 3 (SOCS3), a negative-feedback molecule for cytokine signaling, has been implicated in protection against liver injury. Previous studies have shown that overexpression of SOCS3 in the liver by adenovirus or membrane permeable recombinant protein protected the liver from various injuries. However, it remained uncertain in which type of cells SOCS3 suppresses liver injury. In this study, we demonstrated that forced expression of SOCS3 in T and NKT cells suppressed ConA-induced hepatitis using T and NKT cell-specific SOCS3 transgenic (Lck-SOCS3 Tg) mice. IFN-γ and IL-4 production was reduced in Lck-SOCS3 Tg mice as well as splenocytes treated with ConA. IFN-γ and IL-4 levels were also reduced in Lck-SOCS3 Tg mice administrated with α-galactosylceramide, suggesting that SOCS3 in NKT cells has suppressive function. Sustained expression of SOCS3 in an NKT cell line also resulted in reduced expression of various cytokines and transcription factors. In contrast, T and NKT cell-specific SOCS3 conditional knockout (Lck-SOCS3 cKO) mice were hypersensitive to ConA-mediated hepatitis. Isolated SOCS3-deficient NKT cells produced higher levels of IFN-γ and IL-4. These data indicate that SOCS3 plays a negative regulatory role in NKT cell activation and that forced expression of SOCS3 in NKT cells is effective in preventing hepatitis.


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at least 12 times. T cell-specific SOCS3-disrupted mice (Lck-SOCS3 eKo) mice have been described previously (20, 21). The mice were given a single i.v. injection of ConA (Sigma-Aldrich) at 10–15 μg/g of body weight or α-GalCer (KRN7000) at 100 ng/g of body weight (15, 22). All mice were kept in pathogen-free facilities, and all experiments using these mice were approved by and performed according to the guidelines of the animal ethics committee of Kyushu University, Fukuoka, Japan. The ala- nine transaminase (ALT) activity in serum was measured by SRL.

**Histological analysis**

Livers were isolated and fixed in 4% paraformaldehyde. They were then embedded in paraffin and consecutive 3-μm sections were mounted on slides. The sections were stained with H&E.

**Immunoblot analysis and ELISA**

After ConA injection, the mouse livers were surgically removed and frozen in liquid nitrogen. NKT cells and T cells were lysated after ConA (1 μg/ml) or IL-27 (10 ng/ml) stimulation. Western blotting was performed as described previously (23) using Abs to phosphorylated STAT1 (Tyr701) and phosphorylated STAT3 (Tyr705; both BD Bioscience); SOCS3 (Immuno-Biological Laboratories); STAT1 (E23), STAT3 (C20), and ERK2 (C14), all from Santa Cruz Biotechnology; and Bcl-XL, Caspase-3 and Cleaved caspase-3 (Cell Signaling Technology). The blot was visualized with the appropriate HRP-conjugated secondary Abs using a Chemi-Lumi One L substrate (Nacalai Tesque, Japan) or Immobilon Western Chemiluminescent HRP Substrate (Millipore). Serum IL-2, IL-6, IFN-γ, and TNF-α were measured by an enzyme-linked immunosorbent assay using an ELISA Ready-SET-GO kit (eBioscience) according to the manufacturer's instructions.

**RNA extraction and quantitative RT-PCR**

Total RNA was extracted from mouse livers or 2E10 cells using RNAiso reagent (Takara). First-strand cDNA was synthesized from 1 μg of total RNA with M-MLV reverse transcriptase using random hexamers. The cDNA was used as a template for polymerization according to the manufacture's instructions (Toyobo). Quantitative real-time reverse transcription PCR was performed using an ABI-Prism 7000 (Applied Biosystems) as described (15). The oligonucleotides used for PCR were 5'-AAGTGTGGC-3' and 5'-GATTTGAGGCATTCATG-3' for T-bet, 5'-CTTCCGTTGCG-3' and 5'-CTTCCGTTGTGCCATG-3' (forward) and 5'-GACHCCACTATG-3' (reverse) for IFN-γ, 5'-GCCAAGAACATTCAAGG-3' (forward) and 5'-AGTAGATCTTCCGTTGCTGT-3' (reverse) for IRF-1, 5'-GATCTGTGCACTTCTCCTAT-3' (forward) and 5'-ACTGCGAAAGGTGATTGAC-3' (reverse) for IL-6, 5'-TCCACCACAGTTGCTGACTC-3' (forward) and 5'-TCCACCACAGTTGCTGACTC-3' (reverse) for TNF-α, 5'-AGTAGAAGGGTCGG-3' (forward) and 5'-AGTAGAAGGGTCGG-3' (reverse) for IL-2, and 5'-AGTAGAAGGGTCGG-3' (forward) and 5'-AGTAGAAGGGTCGG-3' (reverse) for IL-12.

**Isolation of splenocytes and hepatic lymphocytes, and analysis with flow-cytometry**

Spleens were removed from the mice under diethyl ether anesthesia, and the splenocytes were isolated. Hepatic lymphocytes were prepared using Percoll (GE Healthcare). The splenocytes and hepatic lymphocytes were incubated with anti-mouse CD16/CD32 mAb (2.4G2) to block the Fc receptor. They were then stained with FITC-conjugated anti-CD3 Ab and allophycocyanin-conjugated NK1.1 Ab (eBiosciences) in PBS containing 0.1% NaN₃ and 2% FBS. The stained cells were counted using a FACSCalibur flow cytometer (BD Biosciences), and the results were analyzed with FlowJo software (Tree Star).

After staining the hepatic lymphocytes, cell sorting was performed by FACS Aria2 (BD Biosciences) to obtain a pure population of CD3− ACTGGCAAAAGGATGGTGAC-3' and 5'-AAATCGGC-3'. The oligonucleotides used for PCR were 5'-AAATCGGC-3' and 5'-AAATCGGC-3' for T-bet, 5'-CTTCCGTTGCG-3' and 5'-CTTCCGTTGTGCCATG-3' (forward) and 5'-GACHCCACTATG-3' (forward) and 5'-AGTAGATCTTCCGTTGCTGT-3' (reverse) for IRF-1, 5'-GATCTGTGCACTTCTCCTAT-3' (forward) and 5'-ACTGCGAAAGGTGATTGAC-3' (reverse) for IFN-γ, 5'-GCCAAGAACATTCAAGG-3' (forward) and 5'-AGTAGATCTTCCGTTGCTGT-3' (reverse) for IL-6, 5'-TCCACCACAGTTGCTGACTC-3' (forward) and 5'-TCCACCACAGTTGCTGACTC-3' (reverse) for TNF-α, 5'-AGTAGAAGGGTCGG-3' (forward) and 5'-AGTAGAAGGGTCGG-3' (reverse) for IL-12, and 5'-AGTAGAAGGGTCGG-3' (forward) and 5'-AGTAGAAGGGTCGG-3' (reverse) for IL-12. The cells were analyzed using RPII 1640 medium with 10% FBS and 0.1% 2-ME. The mouse SOCS3-pGCD2Nsam1/E vector containing an internal ribosome entry site-GFP cassette was transfected into 293GPG-packaging cells as described previously (25). The cells were retrovirally transduced with virus supernatants. Thirty-two hours later, GFP-positive cells were sorted using a FACS Aria cell sorter (BD Biosciences). The cells were stimulated with 5 μg/ml anti-CD3e Ab (145.2C-11).

**Statistical analysis**

The Student’s paired two-tailed t test was used. Values of p < 0.05 were considered significant. All error bars shown in the figures in this article represent the SD.

**Results**

**Lck-SOCS3 Tg mice are resistant to Cona-induced hepatitis**

First, to investigate the role of T/NKT SOCS3 in liver injury, we injected ConA into Lck-SOCS3 Tg mice. As shown in Fig. 1A, 60% of WT control mice died within 9 h of injection with ConA, and only 30% survived after 24 h. In contrast, 100% of Lck-SOCS3 Tg mice survived after 24 h (Fig. 1A). As shown in Fig. 1B, serum ALT levels 12 h after ConA injection were significantly lower in Lck-SOCS3 Tg mice than in WT mice. Then, we compared cytokine levels after ConA injection between WT and Lck-SOCS3 Tg mice in vivo. Serum IFN-γ, TNF-α, and IL-4 levels were down-regulated in Lck-SOCS3 Tg mice (Fig. 1C). In these mice, proinflammatory cytokines such as IFN-γ were particularly strongly suppressed compared with WT mice. In contrast, the serum IL-6 level was not significantly different between WT and Lck-SOCS3 Tg mice (Fig. 1C).

Histology of the liver cells confirmed that Lck-SOCS3 Tg mice appeared to be protected from hepatic injury (Fig. 1D). Consistent with these findings, liver STAT1 activation induced by ConA was much weaker in Lck-SOCS3 Tg mice than in WT mice (Fig. 1E). However, there was no significant difference in the activation of STAT3 between WT and Lck-SOCS3 Tg mice, which may reflect similar serum IL-6 levels. The level of IFN-γ-STAT1 regulated genes, Fas and IRF-1 were up-regulated after ConA administration. The levels of these genes in Lck-SOCS3 Tg mice were lower than in WT mice. Caspase 3 activation has been shown to be associated with apoptosis as a downstream of the Fas-FasL system. The processing of the 32 kDa pro-caspase3 to the 18 kDa active form was suppressed in Lck-SOCS3 Tg mice (Fig. 1E). These findings indicate that Lck-SOCS3 Tg mice were more resistant to ConA-induced hepatitis than WT mice.

**Activation of T and NKT cells in SOCS3 Tg mice treated with ConA**

Then activation of T cells and NKT cells was examined in ConA-treated mice. The number of NKT cells in the liver and spleen decreased after the injection of ConA in both WT and Lck-SOCS3 Tg mice, and there was no significant difference between the two types of mice (Fig. 1, F and G). It has been shown that ConA injection induced FasL expression in NKT cells, which is important for liver damage (5). In contrast to the expression of Fas on liver parenchymal cells (Fig. 1E), the expression level of FasL in NKT cells was not significantly different between WT and Lck-SOCS3 Tg mice (Fig. 1H). The numbers of CD4+ CD69+ T cells (activated helper T cells) were also not significantly different between WT and Lck-SOCS3 Tg mice (Fig. 1I). These data indicate that SOCS3 suppressed cytokine levels without affecting T/NKT cell number and cytotoxic activity.

**Reduced cytokine production from T/NKT cells from Lck-SOCS3 Tg mice**

Both T and NKT cells expressed exogenous SOCS3 in Lck-SOCS3 Tg mice. First, we compared the levels of endogenous and
exogenous SOCS3 in NKT and T cells from the spleen of WT and Tg mice. NKT cells were collected by FACS as CD3+H11001 NK1.1+ cells. Endogenous SOCS3 mRNA was upregulated in T and NKT cells after ConA stimulation. The endogenous SOCS3 was not detectable without stimulation, but rapidly increased after 1 h stimulation (Fig. 2A, left). The levels of SOCS3 mRNA induced by 1 h ConA stimulation were comparable to those induced by IL-6 (Fig. 2A). The endogenous SOCS3 in NKT cells was detectable at low levels before stimulation and the expression level was increased and maintained at high levels after ConA stimulation (Fig. 2A, right). The level of exogenous SOCS3 mRNA in NKT cells from Lck-SOCS3 Tg mice was about a half that of WT NKT cells stimulated with ConA.

Next, naive splenocytes from WT and Lck-SOCS3 Tg mice were treated with ConA in vitro. As shown in Fig. 2B, IFN-γ production in splenocytes from Lck-SOCS3 Tg mice was lower than that from WT mice at 12 and 24 h. IL-4 was undetectable in this condition (data not shown).

To confirm the suppressive function of SOCS3 in T and NKT cells, we compare the STAT1 and SAT3 activation in these cells between WT and SOCS3 Tg mice. NKT cells were expanded with α-GalCer and IL-2. As shown Fig. 2C, the phosphorylation of both STAT1 and STAT3 was severely suppressed in SOCS3 Tg NKT cells compared with WT NKT cells after ConA stimulation. In contrast, STAT1 and STAT3 phosphorylation levels were similar between WT and SOCS3 Tg T cells (Fig. 2D). These data suggest that the effect of SOCS3 was stronger in NKT cells than T cells in response to ConA.

SOCS3 negatively regulates IFN-γ production from NKT cells
Activated T cells and NKT cells play a critical role in ConA-induced hepatitis. We previously showed that IFN-γ production was reduced and IL-4 production was increased in Lck-SOCS3 Tg helper T cells (18); however, the effect of SOCS3 on NKT cells has not been investigated. To activate NKT cells directly, splenocytes were incubated with α-GalCer which binds specifically to Vα14 receptors on NKT cells (26). As shown in Fig. 3A, in vitro IFN-γ and IL-4 production in response to α-GalCer was lower in Lck-SOCS3 Tg splenocytes than in WT splenocytes. When α-Gal-Cer was injected into mice, IFN-γ and ALT levels in Lck-SOCS3 Tg mice were lower than that of WT mice (Fig. 3, B and C).
Data indicate that SOCS3 suppressed both IFN-γ and IL-4 production in NKT cells in vivo.

SOCS3 overexpression suppresses cytokine expression in an NKT cell line

To investigate the molecular basis of the suppression of IFN-γ production by SOCS3, SOCS3 was overexpressed in mouse NKT hybridoma 2E10 cells (Fig. 4A). 2E10 cells have been shown to produce IFN-γ by TCR stimulation with anti-CD3ε Ab (24). The SOCS3 expression levels in 2E10 (SOCS3-2E10) cells were slightly reduced by anti-TCR stimulation. SOCS3 levels in 2E10 cells stably expressing SOCS3 were maintained for first but reduced thereafter (Fig. 4A). This suggests that down-regulation of SOCS3 is connected to activation of NKT cells. As shown in Fig. 4B, anti-CD3ε Ab-mediated IFN-γ production was lower in SOCS3-2E10 cells. IL-2, IL-4, and IL-17 levels were also lower in SOCS3-2E10 cells than in control 2E10 cells. Thus, SOCS3 suppressed cytokine production in 2E10 cells similarly to primary NKT cells. As shown in Fig. 4C, the IFN-γ, T-bet, and IL-4 mRNA levels were down-regulated in SOCS3-2E10 cells compared with Empty-2E10 cells. IL-2 mRNA levels was also suppressed in SOCS3-2E10 cells, which was consistent with ELISA data (Fig. 4B). GATA3 mRNA levels were reduced by anti-CD3 stimulation but there was no significant difference between Empty-2E10 cells. These data suggest that SOCS3 down-regulates cytokine production through suppressing transcription factors.

Lack of SOCS3 in T/NKT cells enhanced ConA-induced hepatitis

Next, we examined the effect of SOCS3 deletion on T cell-mediated and NKT cell-mediated hepatitis using the ConA model. Twelve hours after challenge with ConA, hepatic injury was assessed. As shown in Fig. 5A, the serum ALT level after ConA injection was markedly higher in Lck-SOCS3 cKO mice than in WT mice. Serum IFN-γ and TNF-α levels in Lck-SOCS3 cKO mice were higher than those in WT mice, although IL-4 and IL-6 levels were not significantly different (Fig. 5B). Histological examination revealed more severe apoptosis and necrosis in Lck-SOCS3 cKO mice (Fig. 5C). We confirmed that SOCS3-deficient NKT cells produce higher levels of cytokines than control NKT cells. As shown Fig. 5D, α-GalCer-mediated IFN-γ and IL-4 production in splenocytes from Lck-SOCS3 cKO mice was higher than that from WT mice. These data confirmed that SOCS3 suppressed cytokine production from NKT cells.

Discussion

In this study, we showed that SOCS3 suppresses cytokine production from NKT cells, thereby protecting against liver injury. It has been reported that SOCS3 induced by adenovirus (16) or by membrane permeable recombinant protein (17) can suppress ConA-induced hepatitis. However, it was uncertain in which type of cells SOCS3 suppressed ConA-induced hepatitis. Our preliminary data suggested that macrophage specific SOCS3 conditional knockout
mice (21) were as sensitive as WT mice to ConA-induced hepatitis (M. Hashimoto, unpublished data). The fact that mice lacking the SOCS3 gene in their T and NKT cells were hyperresponsive while those overexpressing SOCS3 in their T and NKT cells were resistant to ConA-induced hepatitis suggested that SOCS3 in T and/or NKT cells likely plays major role in the suppression of hepatitis.

It was also reported that the IL-6/STAT3 pathway inhibited NKT cells via targeting CD4+ T cells and consequently prevented T cell-mediated hepatitis (27). Because the IL-6/STAT3 pathway induces SOCS3, it is possible that STAT3 inhibited NKT cells via induction of SOCS3 in T and NKT cells. Similarly, Uchida et al. (28) reported that IL-18 time-dependently modulates IFN-γ and IL-4 production in α-GalCer-activated NKT cells by up-regulating SOCS3 expression. Being consistent with this idea, SOCS3-deficient NKT cells produced more IFN-γ than WT NKT cells in response to α-GalCer (Fig. 5D). These support our present data showing that SOCS3 suppresses iNKT cell activation.

A previous report showed that forced expression of SOCS3 suppresses IFN-γ production but up-regulates IL-4 production in helper T cells (18). However, in NKT cells, SOCS3 suppressed both IFN-γ and IL-4 production. Because NKT cells play a more important role in ConA-induced hepatitis than T cells, we focused on NKT cells in this study. It was previously reported that TNF-α, IFN-γ, and IL-4 production by NKT cells contributed to the aggravation of ConA-induced hepatitis (3, 29, 30) and our data demonstrated that accelerated IFN-γ and TNF-α productions were observed in Lck-SOCS3 cKO mice, whereas IL-4 production was almost same as that of WT. Furthermore, α-GalCer treated Lck-SOCS3 Tg mice produced lower amount of IFN-γ than WT mice. Although both IFN-γ and IL-4 production by splenocytes from these Lck-SOCS3 Tg mice were reduced in response to α-GalCer in vitro, the reduction of IFN-γ was more evident rather than IL-4. These results indicated that SOCS3 induction or degradation may be a mechanism for appropriate Th1/2 cytokine balance in NKT cells.

However, unexpectedly, after α-GalCer injection into mice, the serum IFN-γ as well as ALT levels were lower in SOCS3 cKO mice than in WT mice (M.N., unpublished data). We think that other T cells such as SOCS3-deficient helper T cells negatively regulated the effect of α-GalCer-induced NKT cell activation in vivo. SOCS3-deficiency in T cells has been shown to produce higher levels of IL-10 and TGF-β (20), which suppress the IFN-γ production. Therefore, SOCS3 deficiency in T and NKT cells may exhibit a complex phenotype in vivo. Further study is necessary for the role of SOCS3 in pathologies which are involved in T and NKT cells.

FIGURE 3. α-GalCer-induced IFN-γ production was suppressed in Lck-SOCS3 Tg mice. **A**, The level of IFN-γ and IL-4 in the culture medium as measured by ELISA (n = 3) 24 h after the splenocytes were stimulated with α-GalCer at the indicated concentrations; *p < 0.05. **B**, The serum IFN-γ level at the indicated times after i.v. injection with α-GalCer (100 ng/g body weight; n = 6); *p < 0.05. **C**, The serum ALT level at indicated times after i.v. injection with α-GalCer (100 ng/g body weight; n = 6); *p < 0.05.

FIGURE 4. SOCS3 overexpression suppressed IFN-γ mRNA expression. 2E10 cells were retrovirally transfected with control GFP or SOCS3-GFP. **A**, The cells were stimulated with anti-CD3 Ab at indicated times. Western blotting of whole cell extracts using the indicated Abs. **B**, The levels of IFN-γ, IL-4, IL-2, and IL-17 after 12 h stimulation in culture medium as measured by ELISA (n = 3); *p < 0.01. **C**, The mRNA levels for IFN-γ, T-bet, IL-2, IL-4, and GATA3 were determined by real-time PCR. Each group was normalized with β-Actin. One representative data from two independent experiments are shown.
We noticed that SOCS3 is expressed at steady-state levels in 2E10 cells, and TCR stimulation reduced the levels. Similarly, Yu et al. (31) reported that Ag stimulation of naive T cells causes down-regulation of SOCS3, followed by a gradual increase in the level of SOCS3 and a corresponding decline in IL-2 secretion. This study yielded similar results using NKT cells. Therefore, SOCS3 plays a role in maintaining T and NKT cells in a quiescent state, and inhibition of SOCS3 by Ag stimulation may be essential to activate resting T and NKT cells.

The molecular mechanism underlying SOCS3 inhibition of cytokine production from NKT cells remained to be clarified. Suppression of T cell activation by SOCS3 has been extensively studied. SOCS3 may regulate T cell function as part of a negative feedback loop involving IL-2 (31). Overexpression of SOCS3 can inhibit IL-2 promoter activation by SOCS3 binding to costimulatory CD28 (19). SOCS3 also negatively affects TCR signaling by interacting with the catalytic subunit of calcineurin (8). In addition, tyrosine-phosphorylated SOCS3 binds to Ras-GAP, thereby activating Ras, which enhances T cell proliferation (32). Thus, SOCS3 regulates T cell activation not only by suppressing cytokine signaling but also by modulating TCR signaling.

Molecular basis for the regulation of cytokine production in NKT cells by SOCS3 has been unclear. We compared downstream targets of TCR using 2E10 and SOCS3-2E10 cells. To our surprise, tyrosine phosphorylation of cellular proteins including STAT1, STAT3, Lck, and c-Cbl was enhanced in SOCS3-2E10 cells compared with control 2E10 cells (M. Nakaya, unpublished data). In contrast, phosphorylation of JNK and IκBα was similar between control and SOCS3-2E10 cells (data not shown). We noticed that the reduction of IFN-γ production in SOCS3-2E10 cells occurred only in the late phase of TCR stimulation (24 h after stimulation). Thus, SOCS3 may not directly affect TCR signaling, but it suppresses secondary TCR signaling responses such as the induction of T-bet. This is consistent with the fact that FasL-induction was not affected by SOCS3 overexpression. Further study is necessary to clarify the mechanism of SOCS3-mediated suppression of cytokine production in NKT cells. We are currently trying to identify the target molecule of SOCS3 downstream of TCR signaling in NKT cells.

In conclusion, SOCS3 modulates NKT cell responses and thereby against liver injury. SOCS3-mediated suppression of IFN-γ and IL-4 production is implicated in this protective effect against hepatitis.

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Disclosures
The authors have no financial conflict of interest.
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