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Suppressors of Cytokine Signaling-1 and -3 Regulate Osteoclastogenesis in the Presence of Inflammatory Cytokines

Masanobu Ohishi,*† Yumiko Matsumura,* Daisuke Aki,* Ryuichi Mashima,* Koji Taniguchi,* Takashi Kobayashi,* Toshio Kukita,‡ Yukihide Iwamoto,† and Akihiko Yoshimura2*

Bone metabolism and the immune system have a correlative relationship, and both are controlled by various common cytokines, such as IFNs and ILs, produced in the bone microenvironments. The suppressor of cytokine signaling-1 (SOCS1) and SOCS3 are negative regulators of such cytokines. Although SOCSs are shown to be induced during osteoclast differentiation, their physiological roles in osteoclast differentiation and function have not been clarified. Thus, we examined the roles of SOCS1 and SOCS3 in osteoclastogenesis using SOCS1- and SOCS3-deficient mice. IFN-γ-mediated inhibition of osteoclast differentiation from bone marrow-derived monocytes (BMMs) was strongly enhanced in SOCS1-deficient BMMs, but was diminished in SOCS1-overexpressing BMMs. Moreover, LPS-induced osteoclastogenesis and bone destruction in vivo were suppressed in SOCS1+/− mice compared with those in wild-type mice, suggesting that SOCS1 antagonizes the inhibitory effect of IFN-γ on osteoclastogenesis. SOCS3 did not alter the inhibitory effect of IFNs in osteoclastogenesis in both gain and loss of functional assays; however, the suppressive effect of IL-6 on osteoclast differentiation was greater in SOCS3-deficient BMMs than in wild-type BMMs in vitro. In addition, IL-6 significantly prevented LPS-induced bone destruction in SOCS3-deficient mice, although it failed in wild-type mice in vivo. In SOCS3-deficient BMMs, expression levels of TNF-receptor-associated factor-6 and IκB were drastically reduced and receptor activator of the NF-κB ligand-induced IκB phosphorylation was severely impaired in the presence of IL-6. These data suggest that both SOCS1 and SOCS3 regulate osteoclastogenesis by blocking the inhibitory effect of inflammatory cytokines on receptor activator of the NF-κB ligand-mediated osteoclast differentiation signals. Selective suppression of SOCS1 and SOCS3 in osteoclast precursors may be a possible therapeutic strategy for inflammatory bone destruction. The Journal of Immunology, 2005, 174: 3024–3031.

A balance between bone resorption and bone formation by bone-resorbing osteoclasts and bone-forming osteoblasts is critical for the maintenance of bone strength and integrity (1). Hyperactivation and/or accumulation of osteoclasts cause bone destruction in pathological conditions, such as autoimmune arthritis, periodontitis, postmenopausal osteoporosis, Paget’s disease, and bone tumors (2, 3). Enhanced osteoclastic bone resorption causes severe bone damage, leading to progressive joint destruction in autoimmune arthritis, in which receptor activator of the NF-κB ligand (RANKL), 3 (4) also known as TNF-related activation-induced cytokine (5)/osteoclast differentiation factor (6)/osteoprotegerin ligand (7), produced by activated T cells stimulates its cognate receptor, RANK, expressed on osteoclast precursors (8). RANKL, a member of the TNF family, is essential for the differentiation of monocyte/macrophage precursors to osteoclasts (9). Binding of RANKL to its receptor, RANK, results in the recruitment of the TNF-receptor-associated factor (TRAF) family of proteins, including TRAF6, which is an essential adapter molecule for osteoclastogenesis, leading to the activation of NF-κB and JNK (9). In addition, RANKL induces the expression of c-Fos, an essential transcription factor for osteoclastogenesis, by an unknown mechanism (10).

A recent study by Takayanagi et al. (11) demonstrated that IFN-γ secreted from activated T cells counterbalances the action of RANKL, which contributes to maintaining bone homeostasis. Moreover, it was demonstrated that IFN-β inhibits osteoclastogenesis by interfering with the RANKL-induced expression of c-Fos (10). IFN-γ is shown to induce ubiquitination and proteasomal degradation of TRAF6 (11), whereas IFN-β induces the suppression of c-Fos expression by a posttranscriptional control mechanism(s) (10). Therefore, the modulation of RANK signaling by inflammatory cytokines is important for bone remodeling to maintain bone homeostasis in both physiologic and pathologic states. How IFN-γ induces degradation of TRAF6 and how IFN-β induces c-Fos reduction remain to be determined.

IFNs use the JAK/STAT pathways for their signal transduction (12). A group of cytokine-inducible factors, the suppressor of cytokine signaling (SOCS) family, which mediate negative feedback mechanisms of cytokine signaling by inhibiting the JAK/STAT pathway, has recently been discovered (12). Among them, SOCS1 and SOCS3, negative feedback regulators for STAT1 and STAT3 pathways, respectively, inhibit JAK tyrosine kinase activity in

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3 Abbreviations used in this paper: RANKL, receptor activator of NF-κB ligand; BMM, bone marrow-derived monocyte; IRES, internal ribosomal entry site; KO, knockout; MNC, multinuclear cell; RANK, receptor activator of NF-κB; SOCS, suppressor of cytokine signaling; TRAF, TNF receptor-associated factor; TRAP, tartrate-resistant acid phosphatase; WT, wild type.
slightly different manners; SOCS1 directly binds to the activation loop of JAKs through the Src homology 2 domain, whereas SOCS3 directly binds to cytokine receptors, including gp130, a signal transducer of IL-6-related cytokines. The Src homology 2 domain of SOCS3 has been shown to bind to Y757 of gp130, which is also known as a binding site for the protein tyrosine phosphatase 2 (Src homology 2 domain-containing phosphatase-1) (13). These two molecules contain a similar kinase inhibitor region at the N terminus, which is essential for JAK inhibition (13). Analysis of SOCS1- and SOCS3-deficient macrophages indicated that SOCS1 negatively regulates the IFNα/STAT1 (12) as well as TLR (14, 15) signaling pathways, whereas SOCS3 inhibits the IL-6/ gp130 signaling pathways (16–18). Through examination of knockout mice, it has been reported that IL-6 and gp130 are involved in regulation of osteoclast development in pathologic (19) and physiologic states (20). Interestingly, the absence of the Soc3 gene in macrophages resulted in modulation of the character of IL-6 function. For example, in SOCS3-deficient macrophages, IL-6 functions like IL-10 (16) and IFNs (17, 18), both of which are known as negative regulators of osteoclastogenesis (10, 11, 21). However, it is unknown whether IL-6 functions as a negative regulator of osteoclastogenesis, particularly in the absence of SOCS3.

Hayashi et al. (22) reported that bone marrow-derived osteoclast progenitors constitutively express IFN-β, which is further up-regulated by RANKL. IFN-β intrinsically inhibits the differentiation of osteoclasts. In addition, RANKL simultaneously induces the expression of SOCS1 and SOCS3, although their putative roles in osteoclastogenesis have not been confirmed (22). It has also been demonstrated that SOCS3 is induced by TGF-β, which antagonizes the effect of IFN-β (23). These findings suggest that SOCS1 and SOCS3 suppress the intrinsic inhibitory effect of IFN-β on RANK signaling. However, no evidence supporting these proposals has been provided using knockout mice. In the present study we used SOCS1- and SOCS3-deficient mice and demonstrated that SOCS1 and SOCS3 regulate osteoclastogenesis induced by RANKL in a pathological state. These genes are especially important for suppression of the inhibitory effect of exogenous IFNs and IL-6. Therefore, SOCS1 and SOCS3 are potentially critical factors for regulating bone remodeling during inflammation.

Materials and Methods

**Mice**

SOCS3+/−, SOCS1−/+ , IFN-γ−/− SOCS1+/−, IFN-γ−/− SOCS1−/−, SOCS3−/− and LysM-Cre:SOCS3−/− mice have been described (14, 16, 24). All mice were kept in specific pathogen-free conditions in Station for Collaborative Research.

**Confirmation of Soc3 deletion in LysM-Cre:SOCS3−/− bone marrow-derived monocytes (BMMs)**

Floxed Soc3 gene deletion was evaluated by PCR using genomic DNAs obtained from SOCS3−/− and LysM-Cre:SOCS3−/− BMMs. For the detection of the floxed Soc3 allele (Soc3−/−), the following primers were used: primer A, 5′-GGGGG CAGGGGAAGACAGCTGCCTGGGC-3′; primer B, 5′-GGGGG CAGGGGAAGACAGCTGCCTGGGC-3′; and primer C, 5′-AGTCCCGTGT GTCATAAGGTATTGCATCCAC-3′. The band of the Soc3−/− locus (380 bp) was obtained with primers A and B. The band of the Soc3−/− locus (250 bp) was obtained with primers A and C.

We also evaluated Soc3 deletion in LysM-Cre:SOCS3−/− BMMs by RT-PCR. cDNAs were obtained from BMMs cultured for 5 h in the presence of M-CSF (40 ng/ml) and RANKL (50 ng/ml). RT-PCR was performed using following primers: SOCS3 forward, 5′-GGGTTGCGGCAA GAAAGAGG-3′; and SOCS3 reverse, 5′-GGTGGAGCGTCAAGA CCCAGT-3′.

**Retroviral gene transduction**

We constructed a retrovirus vector carrying SOCS1 and SOCS3 cDNA with an internal ribosomal entry site (IRES)-enhanced GFP (pMX-SOCS1/ SOCS3-IRES-EGFP), pMX-IRES-EGFP (empty) was used as a control vector. Retrovirus packaging was performed as previously described (25). Bone marrow cells obtained from 5- to 8-wk-old ddY mice were cultured for 24 h in the presence of M-CSF (R&D Systems; 100 ng/ml). Cells were vigorously washed by pipetting, and adherent cells were incubated in a virus stock medium containing polybrene (Roche) at 4 μg/ml for 4 h. Infected cells were cultured for 2 days in the presence of M-CSF (100 ng/ml) and subjected to in vitro osteoclastogenesis.

**In vitro osteoclastogenesis**

In vitro osteoclastogenesis using BMMs was performed as previously described (26). Bone marrow cells were collected from the femurs and tibia of 5- to 8-wk-old old mice. These cells were suspended in αMEM containing 10% FBS and cultured in 48-well plates (1.5 × 10^5 cells/well) in the presence of M-CSF (100 ng/ml) for 3 days. These cells were then washed vigorously by pipetting. Adherent cells were cultured for another 3 days in the presence of both M-CSF (100 ng/ml) and RANKL (PeproTech; 100 ng/ml) with or without IFN-β (CalBiochem), IFN-γ (PeproTech), or IL-6 (PeproTech). The cells were fixed with formalin, acetone, and acetic acid and stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (Sigma-Aldrich) according to the manufacturer’s protocol. The number of TRAP-positive multinucleated and/or mononuclear cells was determined by counting. In vitro osteoclastogenesis using spleen cells was performed as follows. Spleen cells were collected from 2- to 3-wk-old SOCS1+/− mice or SOCS1−/− mice. These cells were cultured in 96-well plates (1 × 10^5 cells/well) in the presence of M-CSF (25 ng/ml) and RANKL (50 ng/ml) for 4 days. After fixation, the cells were stained for TRAP.

**Quantification of NO release**

BMMs were cultured for 3 days in the presence of M-CSF, RANKL, and IFN-γ as described above, then stimulated with TNF-α (PeproTech; 100 ng/ml) for 24 h. Culture supernatant was collected, and accumulation of nitrite was measured using a nitrate/nitrite assay kit (BioDynamics Laboratory).

**Endotoxin-induced bone resorption**

Mice (8–12 wk old) were administered a local calvarial injection of LPS (Sigma-Aldrich) at 25 mg/kg. Some SOCS3−/− and LysM-Cre:SOCS3−/− mice received an s.c. injection of IL-6 at the same site. After 5 days, the mice were killed, and histological sections of the calvarial bones were prepared as described previously (10). The specimens were stained for TRAP and counterstained with hematoxylin. Bone destruction was evaluated by calculating the percentage of eroded surface over total bone surface (10). The osteoclast number per millimeter of trabecular bone surface was counted as described previously (10).

**Immunohistochemistry**

Immunohistochemical staining for SOCS3 in calvarial sections was performed using anti-SOCS3 Ab (IBL). Briefly, calvarial sections were first treated with proteinase K. After blocking with goat serum for 30 min, the sections were incubated with rabbit anti-SOCS3 Ab (OncogeneScience), anti-phosphotyrosine-STAT1 (Cell Signaling), anti-phosphotyrosine-RelA (NF-κB p65 subunit) in BMMs (Cell Signaling), and anti-IκB Ab (B Cell Signaling) followed by incubation with Alexa 546-labeled anti-rabbit IgG (Molecular Probes) for 30 min. The sample sections were further stained for TRAP as described above.

Immunohistochemical staining for RelA (NF-κB p65 subunit) in BMMs was performed as follows. The cells were fixed with 70% ethanol for 5 min. After fixation, the cells were treated with 0.1% Triton X-100 for 3 min, followed by incubation with rabbit anti-RelA Ab (Santa Cruz Biotechnology). Then they were incubated with Alexa 546-labeled anti-rabbit IgG.

**Western blotting**

Total cells were lysed in lysis buffer A (20 mM HEPES [pH 7.3), 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM DTT, 1 mM sodium vanadate, 1 mM PMSF, and 1% aprotinin). Total cell extracts were resolved by SDS-PAGE, and proteins were detected by immunoblotting using anti-TRAF6 Ab (Santa Cruz Biotechnology), anti-phospho-ResErk-1/2 (Cell Signaling), anti-phospho-P38 (Cell Signaling), anti-phospho-ERK1/2 (Cell Signaling), anti-phospho-STAT1 (Cell Signaling), anti-phospho-p38 (Cell Signaling), and anti-Actin Ab (Cell Signaling).
RT-PCR for osteoclast-specific genes

BMMs infected with retrovirus were cultured for 3 days in the presence of M-CSF (100 ng/ml) and RANKL (100 ng/ml) with or without IFN-γ. cDNA was obtained, and RT-PCR was performed as previously described (22).

Statistical methods

The significance of differences between group means was determined by Student’s t test.

Results

Effects of SOCS1 and SOCS3 overexpression on osteoclast differentiation

To determine the effects of SOCS1 and SOCS3 on osteoclast differentiation, we overexpressed SOCS1 and SOCS3 in BMMs using a pMX retroviral vector system. Comparable levels of retroviral transduction were observed among pMX-empty, -SOCS1, and -SOCS3 (~60%; Fig. 1A). Consistent with previous reports, generation of osteoclasts (TRAP-positive multinuclear cells (MNCs)) from control BMMs (pMX-empty) occurred within 3 days of culture with RANKL, but was strongly suppressed by IFN-γ/H9253 (Fig. 1B–D). Neither SOCS1 nor SOCS3 overexpression caused an increase in the number of RANKL-induced, TRAP-positive MNCs, suggesting that overexpression of SOCS1 or SOCS3 does not affect RANKL-induced osteoclastogenesis under normal conditions (Fig. 1, B–D). We then examined the effects of SOCS1 and SOCS3 on exogenous IFN-γ and IFN-β. As shown in Fig. 1, C and D, SOCS1, but not SOCS3, overexpression conferred partial resistance to both IFN-γ and IFN-β. SOCS1-overexpressing BMMs could differentiate into TRAP-positive MNCs, even in the presence of 5 U/ml IFN-γ or 10 U/ml IFN-β, whereas control and SOCS3-overexpressing BMMs failed to do so (Fig. 1, C and D). Notably, SOCS1-overexpressing BMMs could generate many TRAP-positive mononuclear cells, even in the presence of IFNs, whereas control and SOCS3-overexpressing BMMs could not generate any TRAP-positive cells (Fig. 1B). Resistance to IFNs by SOCS1 overexpression was confirmed by monitoring the expression of osteoclast-specific genes including TRAP, calcitonin receptor, and cathepsin K, as shown in Fig. 1E. These markers were detectable in SOCS1-overexpressing BMMs even in the presence of IFN-γ, suggesting that SOCS1, but not SOCS3, confers resistance to exogenous IFNs on osteoclast progenitors by suppressing IFN signaling. SOCS1 overexpression inhibited IFN-γ-induced STAT1 activation and TRAF6 degradation (data not shown).

Effect of SOCS1 gene deletion on osteoclastogenesis

To confirm the physiological relevance of the effect of SOCS1, we examined RANKL-induced osteoclast differentiation using BMMs from control (IFN-γ/H9253/H11002/SOCS1/H11001/SOCS1/H11001/SOCS1/H11001) and SOCS1-deficient (IFN-γ/H9253/H11002/SOCS1/H11002/SOCS1/H11002/SOCS1/H11002) mice. Similar numbers of TRAP-positive MNCs...
were obtained from wild-type (WT; IFN-γ+/+SOCS1+/+) and IFN-γ-deficient (IFN-γ−/−SOCS1+/+) bone marrow (Fig. 2A). The numbers of osteoclasts differentiated from SOCS1-deficient BMMs were less than those from control BMMs. This indicates that SOCS1 might be an intrinsic regulator of osteoclastogenesis. However, SOCS1 seems not to be indispensable for regulating osteoclastogenesis in vivo under normal conditions, because bone histomorphometric analysis showed no difference in the number of osteoclasts between SOCS1-deficient and control mice (data not shown).

Next we examined the effect of IFN-γ on osteoclast differentiation of SOCS1-deficient BMMs. As shown in Fig. 2B, SOCS1-deficient BMMs were much more sensitive to IFN-γ than control BMMs. Osteoclastogenesis was almost completely inhibited in osteoclasts between SOCS1-deficient and control mice (Fig. 2A). Histomorphometric analysis showed no difference in the number of osteoclasts between SOCS1-deficient and control mice (data not shown).

SOCS1 deficiency suppressed endotoxin-induced bone resorption

To determine the effect of SOCS1 on osteoclastogenesis in vivo, we used an established model of endotoxin-induced bone resorption, in which T cells and IFN-γ play an essential regulatory role (10, 11). We administered a local calvarial injection of LPS to 7- to 8-wk-old Socs1 heterozygous (Socs1+/−) and WT (Socs1+/+) mice. Because Socs1−/− mice show severe inflammation in various organs and die at ~3 wk of age (12, 27, 28), it is not possible to assess LPS-induced bone resorption in vivo. In contrast, IFN-γ−/−SOCS1−/− mice are not adequate for this model, because IFN-γ is not present. Therefore, we compared Socs1−/− and SOCS1−/− mice. Five days after calvarial injection of LPS, we measured the percentage of bone surface covered by osteoclasts (eroded surface) and the osteoclast number per millimeter of trabecular bone surface (osteoclast number). As shown in Fig. 3A, a notable suppression of osteoclast formation and bone destruction induced by LPS was observed in mice with haploinsufficiency of the Socs1 gene. Quantitative analyses evaluated by eroded surface and osteoclast number revealed that the LPS-induced osteoclast development and activation were significantly suppressed in Socs1−/− mice (Fig. 3, B and C). These findings indicate that SOCS1 is an important regulator of bone remodeling in inflammation sites.

Effects of Socs3 gene deletion on osteoclast differentiation

Socs3 expression is also up-regulated by RANKL in BMMs (22). To determine the physiological role of SOCS3 in osteoclastogenesis, we used a conditional knockout (KO) approach, because Socs3-deficient mice die during embryonic development as a result of placental defects (29). To generate a monocytic-specific deletion, a mouse line in which the Cre cDNA was knocked-in at the lysozyme M gene locus (LysM-Cre mice) (24) was crossed with Socs3lox/lox mice. Deletion of the Socs3 gene in BMMs was confirmed by genomic PCR and RT-PCR (Fig. 4, A and B). BMMs from LysM-Cre:Socs3lox/lox mice (Socs3-KO) showed a 250-bp band corresponding to the Cre-mediated deletion of the Socs3 gene, whereas no deletion band was observed in the DNA from Socs3lox/− mice (Socs3-WT; Fig. 4A). In contrast, BMMs from Socs3-WT exhibited a 380-bp band corresponding to the floxed Socs3 gene, whereas the floxed gene could not be amplified from Socs3-KO BMMs (Fig. 4A). Socs3 mRNA was induced by
RANKL treatment within 5 h in BMMs from SOCS3-WT, although it could not be detected in those from SOCS3-KO mice (Fig. 4B). Furthermore, we confirmed that SOCS3 protein was not detected in osteoclasts of SOCS3-KO mice in vivo (Fig. 4C).

The numbers of TRAP-positive MNCs differentiated from SOCS3-deficient BMMs were significantly reduced compared with those from WT BMMs (Fig. 5A). This indicates that SOCS3 may play a role in osteoclast differentiation induced by RANKL, although the mechanism is unclear. We did not see any differences in the inhibitory effect of IFN-β and IFN-γ on osteoclast differentiation between WT and SOCS3-deficient BMMs (Fig. 5, B and C). Thus, SOCS3, unlike SOCS1, does not play a major regulatory role in IFN-γ sensitivity. Other studies as well as ours have shown that IL-6-gp130 signaling is specifically enhanced by the Socs3 gene deletion in macrophages (16–18). This prompted us to examine the effect of IL-6 on osteoclastogenesis. As shown in Fig. 5D, IL-6 did not affect osteoclast differentiation of WT BMMs at a low concentration (0.1 ng/ml), but partially suppressed it at higher concentrations (≥1 ng/ml). In contrast, IL-6 strongly suppressed the osteoclast differentiation of SOCS3-deficient BMMs even at low IL-6 concentrations and completely blocked it at higher concentrations.

To define the molecular mechanism of this phenomenon, we examined the levels of TRAF6, c-Fos, and IκB as well as the phosphorylation of IκB, STAT1, and STAT3 (Fig. 6). IL-6-induced phosphorylation of STAT3 as well as STAT1 was enhanced in SOCS3-KO BMMs (Fig. 6A). In WT BMMs, TRAF6, c-Fos, and IκB levels were not much affected by IL-6 pretreatment, whereas the expression levels of TRAF6 and IκB, but not that of c-Fos, were significantly reduced in the presence of IL-6 in SOCS3-deficient BMMs (Fig. 6B). Moreover, phosphorylation of IκB was diminished by IL-6 pretreatment in SOCS3-deficient BMMs. Consistent with these data, RANKL-induced nuclear translocation of RelA (p65), a subunit of NF-κB, was inhibited by IL-6 in SOCS3-deficient BMMs (Fig. 6C). These data indicate that RANKL signaling was severely suppressed by IL-6 in SOCS3-deficient BMMs, resulting in the suppression of osteoclast differentiation.

IL-6 prevents LPS-induced bone resorption in SOCS3-deficient mice

Next we examined the role of SOCS3 in vivo using LysM-Cre; Socs3fl/fl mice. Unlike osteoclastogenesis in vitro, bone density and mass were not significantly different between SOCS3-KO and control SOCS3-WT mice under normal conditions in vivo. To assess the role of SOCS3 in the regulation of inflammatory bone destruction, we used LPS-induced bone destruction model (Fig. 7). LPS-induced bone destruction was less severe, but not significantly different, in SOCS3-KO mice compared with SOCS3-WT mice. However, when exogenous IL-6 was s.c. administered at the same site of LPS injection, bone destruction in SOCS3-KO mice was strongly suppressed, which was not observed in control

FIGURE 3. LPS-induced bone resorption in SOCS1+/+ and SOCS1−/− mice. A, Histology of calvarial bone of SOCS1+/+ and SOCS1−/− mice injected with saline or LPS (TRAP and hematoxylin staining). Higher magnifications of the encircled area of LPS-injected calvaria are shown in the lowest panels. Osteoclast-resorbing bone surfaces are pointed out by arrows. B, The percentage of erosive surface per bone surface (eroded surface) and the numbers of osteoclasts per millimeter of trabecular bone surface (osteoclast number) were scored. All values are the mean ± SEM from five mice per group. *, p < 0.01, SOCS1−/− vs SOCS1+/+.

FIGURE 4. SOCS3 gene deletion in BMMs from LysM-Cre;Socs3fl/fl mice. A, PCR analysis of genomic DNA from BMMs. The PCR product of the WT SOCS3 allele is 280 bp, and that from the SOCS3fl allele is 380 bp. A band of 250 bp indicates Cre-mediated deletion of the Socs3 gene in LysM-Cre;Socs3fl/fl-derived BMMs. B, mRNA expression levels of SOCS3 in RANKL-stimulated BMMs. BMMs from both SOCS3fl/fl and LysM-Cre;Socs3fl/fl mice were incubated with RANKL (100 ng/ml) for 5 h. SOCS3 mRNA was detected by RT-PCR analysis. C, SOCS3 deletion in osteoclasts in vivo. Specimens of calvarias were immunohistochemically stained for SOCS3 as described in Materials and Methods. Then, the same samples were stained for TRAP for osteoclast detection. SOCS3-KO, LysM-Cre;Socs3fl/fl; SOCS3-WT; SOCS3fl/fl.
SOCS3-WT littermates. These results are consistent with in vitro experiments showing that IL-6 strongly suppressed the osteoclast differentiation of SOCS3-deficient BMMs.

Discussion

In this study we tried to clarify the physiological and pathological roles of SOCS1 and SOCS3 expression in osteoclastogenesis. We have found that neither SOCS1 nor SOCS3 overexpression in BMMs impacts RANKL-induced osteoclastogenesis. Although the number of multinucleated TRAP-positive osteoclasts induced by RANKL was reduced in both SOCS1- and SOCS3-deficient BMMs in vitro, the numbers of osteoclasts were not significantly different between IFN-γ−/−SOCS1+/+ and IFN-γ−/−SOCS1−/− mice in vivo (data not shown). Moreover, the bone density and mass of SOCS3−/− and LysM-Cre:SOCS3−/− mice were almost identical (data not shown). Thus, neither Socs1 nor Socs3 gene disruption impacts on osteoclastogenesis under physiological conditions in vivo. The mechanism of reduced osteoclast differentiation from SOCS1- and SOCS3-deficient BMMs in response to RANKL (Figs. 2A and 5A) remains to be solved.

Importantly, our present data indicate that SOCS1 and SOCS3 play critical roles in osteoclastogenesis in pathological situations both in vitro and in vivo. SOCS1- and SOCS3-deficient BMMs were more sensitive to the suppressive effect of IFN-γ (Fig. 2B) and IL-6 (Fig. 5D), respectively, than the control BMMs in vitro. LPS-mediated calvarial bone destruction and osteoclast induction were suppressed in SOCS1−/− in vivo (Fig. 3). Furthermore, LPS-induced bone destruction was strongly blocked by coadministra-

FIGURE 5. SOCS3-deficient BMMs are hyper-responsive to IL-6, but not to IFNs. A, BMMs derived from both SOCS3-WT and SOCS3-KO mice were cultured for 3 days in the presence of RANKL (100 ng/ml) and M-CSF (100 ng/ml). Cells were fixed and stained for TRAP. The numbers of TRAP-positive MNCs were counted. *, p < 0.05. B–D, BMMs were cultured for 3 days with various concentrations of IFN-γ (B), IFN-γ (C), or IL-6 (D) in the presence of RANKL (100 ng/ml) and M-CSF (100 ng/ml). The number of TRAP-positive MNCs in the cultures without IFNs and IL-6 is valued as 100%.

FIGURE 6. Effects of IL-6 on both STAT and TRAF6-NF-κB activation in SOCS3-KO BMMs. A, Prolonged phosphorylation of STAT1 and STAT3 induced by IL-6 in SOCS3-deficient BMMs. BMMs from SOCS3-WT and SOCS3-KO mice were cultured with M-CSF (100 ng/ml) and RANKL (100 ng/ml) with or without IL-6 (1 ng/ml). Whole cell extracts were immunoblotted with the indicated Abs. B, Suppression of TRAF6 and IκB expression level and RANKL-induced IκB phosphorylation by IL-6 in SOCS3-deficient BMMs. Whole cell extracts from BMMs cultured as described in A were immunoblotted with the indicated Abs. C, Inhibition of NF-κB nuclear translocation by IL-6 in SOCS3-KO BMMs. SOCS3-WT and SOCS3-KO BMMs were stimulated with RANKL with or without IL-6. The cells were immunohistochemically stained with anti-RelA Ab as described in Materials and Methods.
IL-6 (31) contributes to osteoclastogenesis. However, direct administration of inflammatory cytokines, such as IFN-γ (Fig. 2) and IL-6 (Fig. 5), to osteoclast progenitors was found to suppress osteoclast differentiation. Therefore, inflammatory cytokines regulate osteoclastogenesis both positively and negatively. It is likely that the balance between the induction of RANKL and the inhibition of RANK signaling by inflammatory cytokines controls the development of bone destruction in inflammatory diseases. As shown in this study, SOCS1 and SOCS3 are important regulators of RANK signaling in osteoclasts and might contribute to this balance at inflammation sites.

In chronic synovitis of RA, the balance may be skewed in favor of RANKL expression even in the presence of high levels of inflammatory cytokines, including IL-6. Other studies as well as ours have reported that SOCS1 (32) and SOCS3 (33) are highly expressed in arthritic joints. Such high SOCS1 and SOCS3 expression in osteoclast precursors may inhibit inflammatory cytokine signaling, thereby canceling the suppressing effect of these inflammatory cytokines on RANK signaling. There is a possibility that the protection from bone damage in SOCS1- and SOCS3-deficient mice is partly due to the elevated levels of endogenous IFN-γ or IL-6. However, we could not detect significant differences in serum levels of these cytokines after LPS challenge. Therefore, we think that this possibility is unlikely, although we could not exclude high levels of those cytokines in inflammation sites.

In our view, SOCS1 and SOCS3 are not involved in the homeostatic regulation of osteoclasts. This is surprising, because IFN-β, which is rapidly up-regulated by RANKL, is reported to be a part of the negative feedback regulation of osteoclastogenesis (10). The number of osteoclasts was notably greater in the bones of IFN-β receptor-deficient mice than in those of WT mice (10). Because SOCS1 overexpression inhibits IFN-β signaling, and SOCS1 deficiency resulted in low efficiency of osteoclast differentiation, we expected to see a reduction of osteoclasts in SOCS1-deficient mice. However, there was little difference in the number of osteoclasts between SOCS1-deficient mice and controls. Moreover, no difference in the suppressive effect of exogenous IFN-β was seen between SOCS1-deficient BMMs and SOCS1−/+ BMMs (Fig. 2F). The reason for this is not clear at present. The SOCS1 protein level induced by RANKL may not be high enough to block IFN-β signaling. Additional study is necessary to define the role of SOCS1 in the regulation of autocrine IFN-β.

The molecular mechanism of the suppression of RANK signaling by inflammatory cytokines has not been clarified. Takayanagi and his colleagues proposed that IFN-γ induces the ubiquitination and degradation of TRAF6 by a STAT1-dependent mechanism, and that IFN-β suppresses c-Fos induction by an ISGF3-dependent mechanism (10). However, little is known about how STAT1 induces degradation of TRAF6 and IκB expression levels and RANKL-induced IκB phosphorylation by IL-6 in SOCS3-deficient BMMs. This phenomenon is similar to that observed in osteoclast precursors treated with IFN-γ (11). IL-6 may inhibit the TRAF6-NF-κB pathway in the absence of SOCS3 through hyper-STAT1 activation (Fig. 6). This is consistent with an IFN-γ-like effect of IL-6 in the absence of SOCS3 (17, 18). In addition, NF-κB activation may be down-regulated by STAT3, because the anti-inflammatory cytokine IL-10 suppresses the TLR-mediated NF-κB pathway through a STAT3-dependent mechanism (34, 35). IL-10 was shown to be a potent inhibitor of osteoclastogenesis (21). We showed that IL-6 functions like IL-10 in the absence of SOCS3 in macrophages (16). Therefore, we propose that IL-6 functions like IFN-γ and/or IL-10, which negatively regulate osteoclastogenesis, in SOCS3-deficient BMMs. Therefore, IL-6/gp130 signaling probably suppresses RANKL-induced NF-κB activation by multiple mechanisms in the absence of SOCS3. In other words, SOCS3 determines the nature of cytokine function. The molecular mechanisms of suppression of TRAF6-NF-κB activation by STAT1 and STAT3 are still open questions.

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Disclosures
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