

2003-04-01

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<http://hdl.handle.net/10026.1/20394>

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10.4049/jimmunol.170.7.3679

The Journal of Immunology

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### The Possible Role of TGF- $\beta$ -Induced Suppressors of Cytokine Signaling Expression in Osteoclast/Macrophage Lineage Commitment In Vitro<sup>1</sup> ✓

Simon W. Fox; ... et. al

*J Immunol* (2003) 170 (7): 3679–3687.

<https://doi.org/10.4049/jimmunol.170.7.3679>

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# The Possible Role of TGF- $\beta$ -Induced Suppressors of Cytokine Signaling Expression in Osteoclast/Macrophage Lineage Commitment In Vitro<sup>1</sup>

Simon W. Fox,<sup>2\*</sup> S. Jaharul Haque,<sup>†</sup> Alison C. Lovibond,<sup>\*</sup> and Timothy J. Chambers<sup>\*</sup>

Osteoclast formation is dependent on the ability of TGF- $\beta$  to enable receptor activator of NF- $\kappa$ B ligand (RANKL)-induced commitment of hemopoietic precursors to the osteoclastic lineage. The mechanism by which TGF- $\beta$  enables formation is unknown. One possibility is that TGF- $\beta$  opposes Janus kinase (JAK)/STAT signals generated by inhibitory cytokines such as IFN- $\beta$ . The JAK/STAT pathway is activated by cytokines that induce resistance to osteoclast formation, such as IFN- $\gamma$  and M-CSF, and the effect of these is opposed by TGF- $\beta$ . Recently, a group of STAT-induced factors, termed suppressors of cytokine signaling (SOCS), has been identified that inhibit JAK/STAT signals. Therefore, we tested the ability of TGF- $\beta$  to induce SOCS expression in osteoclast precursors and examined the effect of SOCS expression on osteoclast/macrophage lineage commitment. We found that while SOCS mRNA is undetectable in macrophages, osteoclasts express SOCS-3, and TGF- $\beta$  up-regulates this expression. Furthermore, TGF- $\beta$  rapidly induces sustained SOCS-3 expression in macrophage/osteoclast precursors. To determine whether SOCS-3 plays a role in osteoclast differentiation we expressed SOCS-3 in precursors using a retroviral system. We found that osteoclast differentiation was significantly enhanced in SOCS-3-infected precursors, and SOCS-3 expression enables formation in the presence of anti-TGF- $\beta$  Ab. On the other hand, antisense knockdown of SOCS-3 strongly suppressed osteoclast formation and significantly blunted the response to TGF- $\beta$ . Moreover, like TGF- $\beta$ , SOCS-3 expression opposed the inhibitory effect of IFN- $\beta$ . These data suggest that TGF- $\beta$ -induced expression of SOCS-3 may represent a mechanism by which TGF- $\beta$  suppresses inhibitory cytokine signaling, priming precursors for a role in bone resorption. *The Journal of Immunology*, 2003, 170: 3679–3687.

The osteoclast is the cell that resorbs bone. Overactivity by this cell is responsible for the excessive bone loss seen in osteoporosis and other common clinical conditions. It has become clear that receptor activator of NF- $\kappa$ B ligand (RANKL),<sup>3</sup> an osteoblast-expressed member of the TNF superfamily, induces osteoclast differentiation and activity in cells of the mononuclear phagocyte lineage (1, 2). However, while RANKL plays a central role in osteoclast formation, only a proportion of mononuclear phagocyte precursors form osteoclasts when incubated with RANKL in vitro (3, 4). In addition, in semisolid cultures, osteoclasts that develop in colonies are always mixed with macrophages (5), suggesting that RANKL alone might not be sufficient to ensure that precursors become osteoclasts at sites of resorption.

Responses to stimuli from the TNF superfamily are characteristically associated with critical inputs from other factors (6). One such factor that influences response to TNFs is TGF- $\beta$ , which is

the most abundant cytokine found in bone matrix. Interestingly, TGF- $\beta$ , like other members of the TGF- $\beta$  superfamily (7, 8), significantly augments RANKL-induced osteoclast formation (4, 9). Furthermore, RANKL-induced osteoclast formation is abolished by recombinant soluble TGF- $\beta$  type II receptors or anti-TGF- $\beta$  Abs, suggesting that osteoclasts induced by RANKL without the addition of exogenous TGF- $\beta$  are dependent on TGF- $\beta$  present in the culture medium or produced by the precursors themselves (4).

It appears that the primary role of TGF- $\beta$  in osteoclast formation is to maintain and enhance the responsiveness of osteoclast precursors to RANKL (4). Thus, TGF- $\beta$  prevents the increased resistance of mononuclear phagocyte precursors to RANKL-induced osteoclast formation that occurs when precursors are incubated in M-CSF alone (3–5). This is consistent with a model in which connective tissue matrix or bone cells release TGF- $\beta$ , which maintains RANKL responsiveness in precursors during migration to areas of prospective resorption, where they are activated to form osteoclasts by RANKL-expressing osteoblasts (1). However, the mechanism by which TGF- $\beta$  maintains the responsiveness of osteoclast precursors to RANKL is at present unknown. One possibility is that cells perceive the culture environment as essentially inflammatory (10–12), and that TGF- $\beta$ , which has anti-inflammatory actions on the mononuclear phagocyte system, opposes these macrophage-activating influences. Consistent with this, several cytokines produced by macrophages in vitro have been shown to inhibit osteoclast formation (13). In particular, it has been shown that RANKL induces IFN- $\beta$  expression, which inhibits osteoclast formation and has been proposed to act as an autocrine regulator of osteoclast formation (14). In addition, we have shown that IFN- $\gamma$  directly inhibits osteoclast formation by priming precursors for a role as cytotoxic macrophages, activating NO production while strongly suppressing osteoclast formation (15, 16). TGF- $\beta$  opposes both actions of IFN- $\gamma$ .

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Received for publication September 12, 2002. Accepted for publication January 31, 2003.

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<sup>1</sup> This work was supported by the Wellcome Trust, the Arthritis Research Campaign, and National Institutes of Health Grant ROIGM60533.

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<sup>3</sup> Abbreviations used in this paper: RANKL, receptor activator of NF- $\kappa$ B ligand; CIS, cytokine-inducible gene; EMEM, MEM with Earle's salts; JAK, Janus kinase; MNC, multinuclear cell; ODN, oligonucleotide; SMAD, S mothers against decapentaplegic; SOCS, suppressor of cytokine signaling; SSPE, sodium chloride sodium phosphate EDTA; TAK1, TGF- $\beta$ -activated kinase 1; TRAP, tartrate-resistant acid phosphatase.

IFN- $\gamma$  and IFN- $\beta$ , like other factors that can suppress osteoclast formation, such as M-CSF and GM-CSF (17, 18), signal through the Janus kinase (JAK)/STAT pathway (19, 20). Furthermore, the anti-osteoclastic action of several of these cytokines is absent in mice lacking STATs (14, 20, 21). Thus, JAK/STAT activation may represent a central mechanism by which inhibitory cytokines suppress osteoclast formation, and TGF- $\beta$  might therefore act to block JAK/STAT signals to enable formation.

Recently, a group of cytokine-inducible factors (suppressors of cytokine signaling (SOCS)) has been discovered that forms part of the negative feedback mechanisms that inhibit the JAK/STAT pathway after cytokine stimulation (22–26). Most, if not all, members of the cytokine superfamily rapidly induce transcriptional activation of one or more SOCS, which further block JAK/STAT activity and thus prevent overstimulation (24). The prototype of this family, SOCS-1, was identified by its ability to inhibit macrophage differentiation of M1 cells in response to IL-6 (22). Database searches have since identified at least an additional 14 members of this family, all of which contain a highly conserved 40-aa domain at the C terminus termed the SOCS box (27). Cytokines such as IL-3, IL-4, IL-13, GM-CSF, IFN- $\beta$ , and IFN- $\gamma$  induce SOCS expression in numerous cells, including bone marrow cells (22), via a STAT-dependent transcriptional mechanism.

Intriguingly, SOCS expression has been shown to inhibit the activity of several factors that act via JAK/STAT signaling. In view of this, we elected to determine whether TGF- $\beta$  induces SOCS expression in noncommitted precursors, and whether such SOCS signals could direct precursors to the osteoclastic lineage rather than to alternative cytotoxic and inflammatory pathways.

## Materials and Methods

### Media and reagents

Nonadherent, M-CSF-dependent bone marrow cells were incubated in MEM with Earle's salts (EMEM) supplemented with 10% FBS, 2 mmol/liter glutamine, 100 IU/ml benzylpenicillin, and 100  $\mu$ g/ml streptomycin (all from Imperial Laboratories, Andover, U.K.). The Phoenix retroviral packaging cell line (28) was incubated in DMEM supplemented with 10% FBS and 2 mmol/liter glutamine, 100 IU/ml benzylpenicillin, and 100  $\mu$ g/ml streptomycin. Incubations were performed at 37°C in 5% CO<sub>2</sub>, and cultures fed every 2–3 days. Recombinant human TGF- $\beta$ 1, pan-specific TGF- $\beta$  Ab, and recombinant murine IFN- $\beta$  were obtained from R&D Systems (Minneapolis, MN). Recombinant human M-CSF was provided by Chiron (Emeryville, CA), and soluble recombinant human RANKL was obtained from Insight Biotechnology (Wembley, Middlesex, U.K.). Slices of bovine cortical bone were prepared as previously described (29). Bone slices were cut with a low speed diamond saw, cleaned by ultrasonication in water, washed briefly in acetone, immersed in ethanol for 10 min, and stored dry at room temperature.

### Isolation of stroma-depleted, nonadherent M-CSF-dependent bone marrow precursors

Male MF-1 mice (4–6 wk old) were killed by cervical dislocation. Femora were removed and dissected free of adherent soft tissue. The bone ends were cut, and the marrow was flushed out with medium 199 (Imperial) by injecting medium at one end of the bone with a 21-gauge needle. The bone marrow cells were washed twice, resuspended in EMEM, and incubated for 24 h in M-CSF (5 ng/ml) at a density of  $3 \times 10^5$ /ml in a 75-cm<sup>2</sup> flask. After 24 h, nonadherent cells were harvested, washed, and incubated as described below.

### Northern analysis of SOCS expression

Total RNA was prepared from M-CSF-dependent bone marrow precursors incubated ( $3 \times 10^4$ /ml) for 5 days in 75-cm<sup>2</sup> tissue culture flasks in EMEM containing M-CSF (30 ng/ml) with combinations of TGF- $\beta$ 1 (0.1 ng/ml) and RANKL (30 ng/ml), according to an established method (30). For Northern blot analysis, 15  $\mu$ g of total RNA was separated on a 1.2% agarose-formaldehyde gel, transferred to a Hybond-N membrane (Amersham International, Little Chalfont, U.K.), and hybridized with specific <sup>32</sup>P-labeled cDNA probes for murine SOCS-1 (724 bp), SOCS-2 (679 bp),

SOCS-3 (760 bp), cytokine-inducible gene (CIS; 740 bp), and  $\beta$ -actin (760 bp) prepared by the random primer method (Amersham International). Specific murine SOCS probes were prepared by excising cDNA inserts from expression vectors (31), using appropriate digestion enzymes. After hybridization the membrane was washed (2 $\times$  SSPE and 0.1% SDS, twice for 15 min each time; 1 $\times$  SSPE and 0.1% SDS, 30 min; 0.5 $\times$  sodium chloride sodium phosphate EDTA (SSPE) and 0.1% SDS, twice for 15 min each time; all at 42°C) and autoradiographed using Hyperfilm (Amersham International).

### Quantitative RT-PCR

Nonadherent, M-CSF-dependent bone marrow cells ( $3 \times 10^4$ /ml) were incubated for 2 days in 75-cm<sup>2</sup> tissue culture flasks in EMEM containing M-CSF (10 ng/ml). Cultures were then treated with TGF- $\beta$ 1 (0.1 ng/ml) and/or RANKL (30 ng/ml) for 1–24 h, and total RNA was extracted. For assessment of RNA expression, 2  $\mu$ g of total RNA was reverse transcribed for 1 h at 42°C using 100 pmol of random hexamers (Amersham International) and 600 U of Moloney murine leukemia virus (Life Technologies, Paisley, U.K.) in a 50- $\mu$ l reaction. This was then diluted to a final volume of 100  $\mu$ l.

Real-time PCR was conducted with the I-Cycler (Bio-Rad, Hemel Hempstead, U.K.) using the DNA-binding dye SYBR Green for the detection of PCR products. Two microliters of either external plasmid standards or cDNA (equal to 40 ng of total RNA) was added to a final reaction volume of 25  $\mu$ l containing 200  $\mu$ M dNTPs, 200  $\mu$ M primers, 0.25 U of AmpErase UNG (PE Applied Biosystems, Warrington, U.K.), 2.5  $\mu$ l of 10 $\times$  SYBR Green PCR buffer, 3 mM MgCl<sub>2</sub>, and 0.625 U of platinum *Taq* polymerase (Universal PCR Master mix; PE Applied Biosystems). This was then made up to 100  $\mu$ l, and 2  $\mu$ l of this was used as a template for 25- $\mu$ l PCR reactions. Primers used for PCR were as follows: SOCS-3 sense, 5'-GAACCTACGCATCCAGTGTGAG-3'; SOCS-3 antisense, 5'-CGTTGACAGTCTCCGACAAAG-3';  $\beta$ -actin sense, 5'-GTCATCAC TATTGGCAACGAG-3'; and  $\beta$ -actin antisense, 5'-CCTGTGCAATG CCTGGTACAT-3', which yield products of 322 and 197 bp, respectively.

For the generation of standard curves, the corresponding cDNA were cloned into pGEM4Z (Promega, Southampton, U.K.). The concentration of DNA plasmid stock was determined by the OD at 260 nm. Copy number for each plasmid was calculated on the spectrophotometric reading. The linear range of the assay was determined by the amplification of log serial dilutions of plasmids from 500 to  $5 \times 10^6$ . The progress of the PCR amplification was monitored by real-time fluorescence emitted from SYBR Green during the extension time. The cycles were 95°C for 3 min, followed by 40 cycles of 95°C for 20 s, 59°C for 20 s, and 72°C for 20 s. At the end of each PCR run, a melt curve analysis was performed to show the absence of nonspecific bands. The sizes of products were confirmed by agarose gel electrophoresis.

For each sample, SOCS-3 mRNA levels were expressed as relative copy number normalized against  $\beta$ -actin mRNA. This was achieved by constructing a standard curve for each PCR run from serial dilutions of purified plasmid DNA with specified amplicon. The mRNA copy number was calculated for each sample from the standard curves by the instrument's software. Samples were analyzed in triplicate. For each sample SOCS-3 copy number relative to 10<sup>6</sup>  $\beta$ -actin copies in the same sample was calculated. Results were then expressed as a percentage of the M-CSF-treated control value (SOCS-3 copy number per 10<sup>6</sup>  $\beta$ -actin of M-CSF control: A,  $7.55 \times 10^2$ ; B,  $1.51 \times 10^3$ ; C,  $6.42 \times 10^2$ ). Results are the mean of two separate experiments, each consisting of three samples  $\pm$  SEM.

### SOCS-3-expressing retroviral vector

SOCS-3 was transduced using a retroviral vector, pBabe puro, which expresses cDNA inserts constitutively under the control of a retroviral enhancer-promoter (32). The coding region of mouse SOCS-3 (760 bp) was PCR amplified from pEF-FLAG-1/mSOCS-3 (31) with a 5' primer containing a *Bam*HI site and a Kozak sequence and with a 3' primer containing a *Sal*I site, and cloned into pBabe puro. The resulting plasmid pBabe-SOCS-3 was sequenced (Cambridge Biolabs, Cambridge, U.K.) to confirm the SOCS-3 sequence. The pBabe-SOCS-3 and pBabe-empty (control) vectors were then transfected into the Phoenix retroviral packaging cell line (28) using FuGENE-6 (Roche, Indianapolis, IN). After 48 h, stably transfected cells were selected by replacing growth medium with fresh medium containing puromycin (2.5  $\mu$ g/ml). After a further 48-h period, cells were washed twice, and fresh puromycin containing medium was added. Stably transfected clones were picked 4–7 days later and grown to confluence in 25-cm<sup>2</sup> flasks. Clones producing high viral titers were identified by infecting NIH-3T3 cells with viral supernatant for 16 h. After incubation in fresh medium for an additional 2 days, stably infected colonies were selected with puromycin (2.5  $\mu$ g/ml) for 2 days, and viral titer was calculated: puro



CFU/ml = number of puro-resistant colonies/virus volume. Supernatants of the retrovirus producing cells showed a similar viral titer against NIH-3T3 cells (pBabe-SOCS-3  $4.9 \times 10^6$  CFU/ml; pBabe-empty  $4.1 \times 10^6$  CFU/ml).

#### Infection of M-CSF-dependent precursors with control and SOCS-3-expressing retroviruses

Nonadherent, M-CSF-dependent bone marrow cells ( $10^6$  cells/ml) were added to the wells of 96-well plates containing Thermanox coverslips (Nunc, Naperville, IL) or bone slices and cultured in M-CSF (10 ng/ml) for 48 h. Medium was then removed and replaced with supernatant of pBabe-SOCS-3 or pBabe-empty virus-producing Phoenix cells together with 8  $\mu$ g/ml polybrene and M-CSF (10 ng/ml). The cells were then centrifuged at 800 rpm to increase infection efficiency and were incubated for 16 h at 37°C. Viral supernatant was removed and replaced with EMEM containing M-CSF (30 ng/ml) and RANKL (30 ng/ml). After incubation for an additional 2 days, stably infected cells were selected by addition of puromycin (2.5  $\mu$ g/ml) for 3 days. Control cultures to which virus had not been added showed no viable cells. Infection rates for both retroviruses were similar (pBabe-SOCS-3, 55–70%; pBabe-empty, 60–70%). Puromycin was then removed, and coverslips and bone slices were assessed 3–7 days later for osteoclast formation and bone resorption as described below.

#### SOCS-3 antisense studies

The effect of SOCS-3 knockdown on osteoclast formation was examined using a phosphorothioate antisense oligonucleotide (ODN) (5'-TCA CTC TGC AGC GAA AAG-3') specifically targeting nt 320–337 of mouse SOCS-3 mRNA manufactured by Biognostik (Göttingen, Germany; Biognostik is the owner of the intellectual property rights of the antisense sequence). The control ODN is the reverse complement of the target sequence and contains no cross-homology to other sequences on the GenBank database. M-CSF-dependent mononuclear cells were incubated with M-CSF (30 ng/ml), RANKL (30 ng/ml), and FuGENE6 (0.003%, v/v) with or without TGF- $\beta$ 1 (0.1 ng/ml) in the presence of antisense SOCS-3 ODN (2  $\mu$ M) or control ODN (2  $\mu$ M) for 6 days. Cells were fixed and stained for tartrate resistant acid phosphatase (TRAP).

#### Assessment of the effect of TGF- $\beta$ on inhibition of osteoclast formation by IFN- $\beta$

Nonadherent, M-CSF-dependent bone marrow cells were incubated in 96-well-plates ( $10^5$  cells/ml) for 6 days with M-CSF (30 ng/ml) and RANKL (30 ng/ml) with or without IFN- $\beta$  (5 U/ml) and TGF- $\beta$ 1 (0.1 ng/ml). Cultures were fixed and assessed for osteoclast formation.

#### TRAP cytochemistry

Osteoclast formation on coverslips and bone slices was evaluated by quantification of TRAP-positive multinuclear cells (MNC) containing three or more nuclei. After incubation, cells on coverslips or bone slices were washed, fixed in 10% formalin for 10 min, permeabilized in acetone for 10 min, washed, and stained for acid phosphatase in the presence of 0.05 mol/liter sodium tartrate, using naphthol AS-BI phosphate as a substrate (33). Cells were counterstained with hematoxylin.

#### Assessment of bone resorption

Resorption was quantified on bone slices after TRAP staining. For this, cells were removed from the bone surface by immersion in 10% NaOCl (BDH, Poole, U.K.) for 10 min, followed by washing in water. After drying, bone slices were mounted on glass slides and sputter-coated with gold. Resorption was quantified by reflected light microscopy using an eyepiece graticule.

#### Statistical analysis

Differences between groups were assessed using ANOVA (StatView version 5.01; Abacus Concepts, Berkeley, CA). A difference of  $p < 0.05$  was considered significant.

## Results

### Osteoclasts express SOCS-3 mRNA

To assess the pattern of SOCS expression in macrophages and osteoclasts we performed Northern analysis on total RNA extracted from bone marrow precursors incubated in the presence of M-CSF with or without RANKL and TGF- $\beta$  for 5 days. While the expression of SOCS1, -2, or -3 or CIS mRNA was not detectable in M-CSF-treated bone marrow macrophages, SOCS-3 mRNA ex-

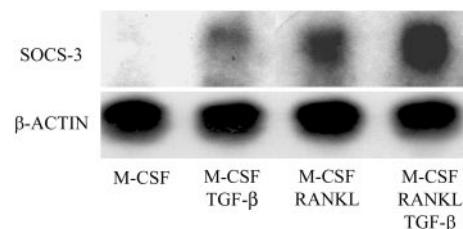
pression was detected in RANKL-induced osteoclasts as previously reported (34) and was further up-regulated in osteoclasts formed in the presence of TGF- $\beta$  (Fig. 1). Furthermore, mononuclear phagocyte precursors incubated with TGF- $\beta$  for 5 days also expressed SOCS-3 mRNA (Fig. 1). The expression of SOCS-1, SOCS-2, or CIS mRNA was not detected in osteoclasts incubated in RANKL or TGF- $\beta$  (data not shown).

To determine the kinetics and magnitude of TGF- $\beta$ -induced SOCS-3 expression in mononuclear-phagocyte precursors we further examined TGF- $\beta$ -induced SOCS-3 expression in noncommitted precursors using quantitative RT-PCR. TGF- $\beta$  induced a >3-fold increase in SOCS-3 mRNA expression in noncommitted precursors within 1 h (Fig. 2). Consistent with the sustained expression of SOCS-3 mRNA in precursors incubated in TGF- $\beta$  for 5 days, SOCS-3 mRNA expression remained 4-fold greater than that of controls 24 h after the addition of TGF- $\beta$ . Thus, TGF- $\beta$  induces a rapid and sustained elevation in SOCS-3 mRNA expression in noncommitted precursors and mature osteoclasts. RANKL itself induced an approximate doubling of mRNA levels of SOCS-3 (Fig. 2). In cultures to which both RANKL and TGF- $\beta$  were added, there was a sustained increase in the level of SOCS-3 mRNA, to  $\sim 7$  times control levels (Fig. 2).

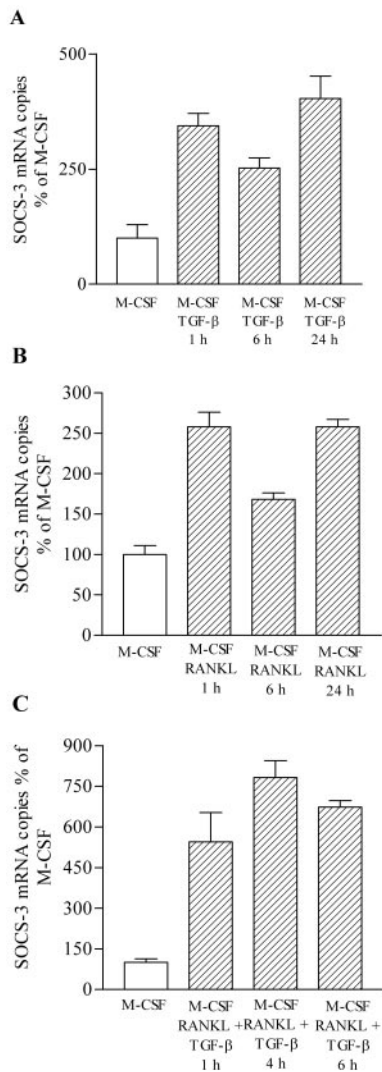
### Retroviral-mediated SOCS-3 expression enhances osteoclast differentiation, but not activation

SOCS-3 is a STAT-induced negative feedback regulator of the JAK/STAT signaling pathway. Interestingly, previous studies suggest that SOCS-3 expression may mediate antagonistic interactions between inflammatory signaling pathways. SOCS-3 expression inhibits the anti-viral effect of IFN- $\gamma$ , and the suppression of IFN- $\gamma$  signaling in response to IL-10 is also attributed to SOCS-3 expression (35, 36).

The rapid and sustained expression of SOCS-3 mRNA in response to TGF- $\beta$  raises the possibility that SOCS-3 expression may form part of the mechanism by which TGF- $\beta$  determines the fate of noncommitted precursors, preventing inhibitory JAK/STAT signals and thereby facilitating osteoclast formation. Therefore, to examine the role of SOCS-3 in lineage switching in mononuclear phagocyte precursors we used a pBabe retroviral vector system to express SOCS-3 in noncommitted precursors. Similar to previous findings, when nonadherent, M-CSF-dependent mononuclear phagocyte precursors infected with empty control virus (pBabe-empty) were incubated with RANKL, strongly TRAP-positive MNCs formed within 8 days (Fig. 3).



**FIGURE 1.** Effect of TGF- $\beta$  on SOCS-3 mRNA expression. Total RNA was prepared from M-CSF-dependent bone marrow precursors incubated ( $3 \times 10^4$ /ml) for 5 days with M-CSF (30 ng/ml) and combinations of TGF- $\beta$ 1 (0.1 ng/ml) and RANKL (30 ng/ml). For Northern blot analysis, 15  $\mu$ g of total RNA was separated on a 1.2% agarose-formaldehyde gel, transferred to a Hybond-N membrane, and hybridized with specific  $^{32}$ P-labeled cDNA probes for murine SOCS-3 or  $\beta$ -actin prepared by the random primer method. After hybridization, the membrane was washed ( $2 \times$  SSPE and 0.1% SDS, twice for 15 min each time;  $1 \times$  SSPE and 0.1% SDS, 30 min;  $0.5 \times$  SSPE and 0.1% SDS, twice for 15 min each time; all at 42°C) and autoradiographed using Hyperfilm.



**FIGURE 2.** Quantitative RT-PCR analysis of TGF- $\beta$ -induced SOCS-3 mRNA expression in mononuclear precursors. Nonadherent, M-CSF-dependent bone marrow cells ( $3 \times 10^4$ /ml) were incubated for 2 days in M-CSF (10 ng/ml). Cultures were then treated with TGF- $\beta_1$  (0.1 ng/ml; A), RANKL (30 ng/ml; B), or TGF- $\beta_1$  (0.1 ng/ml; C) and RANKL (30 ng/ml) for 1–24 h, and total RNA was extracted. For assessment of RNA expression, 2  $\mu$ g of total RNA was reverse transcribed for 1 h at 42°C. Real-time PCR was conducted with the I-Cycler using the DNA-binding dye SYBR Green and specific primers for murine SOCS-3 or  $\beta$ -actin for the detection of PCR products. For the generation of standard curves, the corresponding cDNA were cloned into pGEM4Z (SOCS-3 pGEM4Z or  $\beta$ -actin pGEM4Z). The progress of the PCR amplification was monitored by real-time fluorescence emitted from SYBR Green during the extension time. The cycles were 95°C for 3 min, followed by 40 cycles of 95°C for 20 s, 59°C for 20 s, and 72°C for 20 s. For each sample, SOCS-3 mRNA levels were expressed as the relative copy number normalized against  $\beta$ -actin mRNA. This was achieved by constructing a standard curve for each PCR run from serial dilutions of purified plasmid DNA with specified amplicon. The mRNA copy number was calculated for each sample from the standard curves by the instrument's software. Samples were analyzed in triplicate. For each sample SOCS-3 copy number relative to  $10^6$   $\beta$ -actin copies in the same sample was calculated. Results were then expressed as a percentage of the M-CSF-treated control (SOCS-3 copy number per  $10^6$   $\beta$ -actin of M-CSF control: A,  $7.55 \times 10^2$ ; B,  $1.51 \times 10^3$ ; C,  $6.42 \times 10^2$ ). Results are the mean of two separate experiments each consisting of three samples  $\pm$  SEM.

Interestingly, SOCS-3 expression caused a substantial increase in the number of TRAP-positive MNCs that formed in the presence of RANKL compared with cultures infected with pBabe-

empty at all time points examined (Fig. 3). This increase in MNC number was not attributable to differences in infection rate or total cell number, as cell numbers in SOCS-3- and pBabe-empty-infected cultures did not differ at any time point examined (Table I). Elevated SOCS-3 mRNA levels were confirmed in pBabe-SOCS-3-infected precursors by real-time PCR (pBabe SOCS-3-infected cells,  $4.92 \times 10^5$  SOCS-3 mRNA copies/ $1 \times 10^6$   $\beta$ -actin; TGF- $\beta$ -treated pBabe-control-infected cells,  $2.60 \times 10^3$  SOCS-3 copies/ $1 \times 10^6$   $\beta$ -actin).

Similarly, SOCS-3-infected precursors incubated on bone slices showed a significant, but smaller, increase in TRAP-positive MNC cell numbers compared with controls (Fig. 4). This was accompanied by an increase in the extent of bone resorption in SOCS-3-infected cultures. The increase in the number of MNC by SOCS-3 correlates with the increase in bone resorption. This is consistent with the action of TGF- $\beta$  to increase the number of TRAP-positive MNCs formed by RANKL, but to have no effect on the resorption of each osteoclast so formed (4). The relatively minor increase in TRAP-positive MNCs induced by SOCS-3 virus on bone compared with plastic is also consistent with the only minor enhancement of osteoclast differentiation achieved by TGF- $\beta$  in precursors on bone slices compared with precursors on plastic (4), which was attributable to the presence of TGF- $\beta$  in bone matrix.

RANKL-induced osteoclast formation is abolished by anti-TGF- $\beta$  Abs, suggesting that osteoclasts induced by RANKL without the addition of exogenous TGF- $\beta$  are dependent on TGF- $\beta$  present in the culture medium or produced by the precursors themselves (4). Therefore, to further determine the possible role of SOCS-3 expression in osteoclast formation we examined the ability of SOCS-3 expression to restore osteoclast differentiation in the presence of pan-specific TGF- $\beta$  Ab. Similar to previous studies, RANKL-induced osteoclast formation was significantly suppressed in control pBabe-empty-infected cells incubated with pan-specific TGF- $\beta$  Ab (Fig. 5) (4). On the other hand, in SOCS-3-infected precursors RANKL-induced osteoclast formation was not significantly suppressed by pan-specific TGF- $\beta$  Ab (Fig. 5). This indicates that SOCS-3 enables RANKL-induced osteoclast differentiation in the absence of TGF- $\beta$ . Similarly, addition of TGF- $\beta$  to control pBabe-empty-infected precursors significantly enhanced osteoclast differentiation, whereas it had no significant effect on osteoclast formation in SOCS-3-infected precursors (Fig. 5).

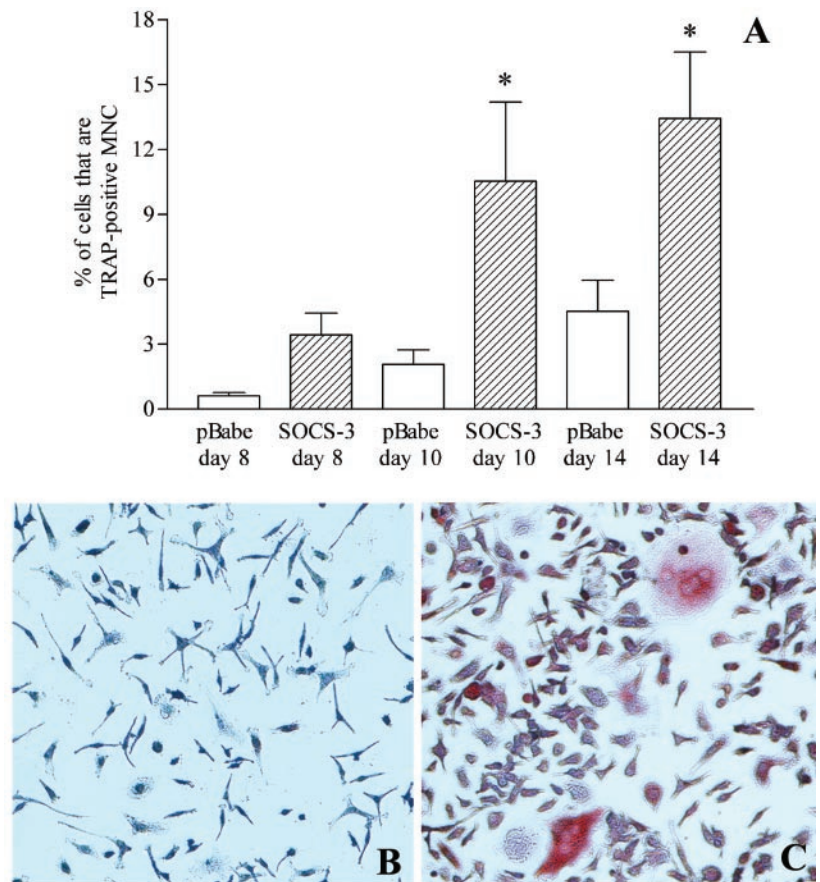
#### *SOCS-3 antisense ODN suppress RANKL-induced osteoclast formation and the enhancing effect of TGF- $\beta$*

The ability of SOCS-3 retroviruses to enhance RANKL-induced osteoclast formation and suppress the inhibitory effect of TGF- $\beta$  Abs strongly suggests that SOCS-3 has a role in the mechanism by which TGF- $\beta$  enables osteoclast formation. To further test the role of SOCS-3 in the enhancement of osteoclast formation by TGF- $\beta$  we examined the effect of knocking down SOCS-3 using a specific SOCS-3 antisense oligonucleotide. RANKL-induced osteoclast formation was markedly suppressed in cultures incubated with SOCS-3 antisense ODN compared with cultures incubated with control ODN (Fig. 6). In addition, SOCS-3 antisense ODN strongly reduced the enhancing effect of exogenously administered TGF- $\beta$  (Fig. 6) compared with control cultures.

#### *TGF- $\beta$ suppresses the antiosteoclastic action of IFN- $\beta$*

The culture environment potentially contains inflammatory stimuli that may act to prime precursors for roles alternative to bone resorption. One such factor is IFN- $\beta$ , which has recently been shown to function as an autocrine regulator of osteoclast differentiation, limiting RANKL-induced osteoclast formation. In the studies by

**FIGURE 3.** SOCS-3 expression enhances RANKL-induced osteoclast formation. Nonadherent M-CSF-dependent bone marrow cells ( $10^6$ /ml) were incubated on plastic coverslips in 96-well plates with M-CSF (10 ng/ml) for 2 days. Cells were then infected with pBabe-SOCS-3 or pBabe-empty (control) retroviruses. Cultures were incubated with M-CSF (30 ng/ml) and RANKL (30 ng/ml) for 8–14 days. Infected cells were selected with puromycin ( $2.5 \mu\text{g}/\text{ml}$ ) between days 2 and 5. Coverslips were fixed and stained for TRAP, and total cell number and the number of TRAP-positive MNC were counted (A). Values are the mean  $\pm$  SEM of three separate experiments ( $n = 24$ ). \*,  $p < 0.05$  vs control cultures. B and C, Photomicrographs of cultures after incubation as described above with pBabe (empty retrovirus; B) and SOCS-3-expressing retrovirus (C).



Takayangi et al. (14), RANKL was shown to induce IFN- $\beta$  expression in osteoclast precursors, which, in turn, suppressed subsequent RANKL-induced osteoclast formation. However, while TGF- $\beta$  has been shown to antagonize inflammatory stimuli (16), it is not known whether TGF- $\beta$  prevents the inhibitory effect of IFN- $\beta$  on osteoclast formation. Therefore, we assessed the ability of TGF- $\beta$  to override the suppressive action of IFN- $\beta$  on osteoclast formation. As expected, addition of IFN- $\beta$  to bipotential osteoclast precursors significantly inhibited RANKL-induced TRAP-positive MNC formation (Fig. 7). Interestingly, osteoclast formation in cultures treated with both TGF- $\beta$  and IFN- $\beta$  was significantly greater than that of controls (Fig. 7), indicating that TGF- $\beta$  prevents the suppressive action of IFN- $\beta$ .

#### SOCS-3 expression enables osteoclast formation in the presence of IFN- $\beta$

IFN- $\beta$ , upon binding to its receptor, activates JAK1 and Tyk2, which, in turn, phosphorylates STAT1 and STAT2, which form part of the IFN-stimulated gene factor-3 signaling complex that is responsible for controlling the transcription of target genes (19, 37). One regulatory factor activated by IFN- $\beta$  is SOCS-3 (38), which inhibits signaling by binding to the kinase activation loop of receptor-associated JAKs, which, in turn, prevents further STAT

activation and STAT-dependent transcription (25, 39). Therefore, to examine whether SOCS-3 expression could account for the suppressive effect of TGF- $\beta$  on IFN- $\beta$  signaling we examined the effect of IFN- $\beta$  on osteoclast formation in precursors expressing pBabe-SOCS-3 retrovirus. Similar to the results described above, SOCS-3 significantly increased RANKL-induced osteoclast differentiation compared with that in control cultures (Fig. 8). Moreover, while IFN- $\beta$  abolished TRAP-positive MNC production in control cultures, it had no significant effect on RANKL-induced osteoclast formation in cultures of SOCS-3-expressing precursors (Fig. 8). Thus, SOCS-3 expression opposes the inhibitory effect of IFN- $\beta$  on osteoclast formation, maintaining the ability of precursors to form osteoclasts. These data raise the possibility that TGF- $\beta$ -induced SOCS-3 expression in bipotential precursors prevents inhibitory JAK/STAT signals, thus acting to prime precursors for a role in bone resorption rather than other macrophage functions.

#### Discussion

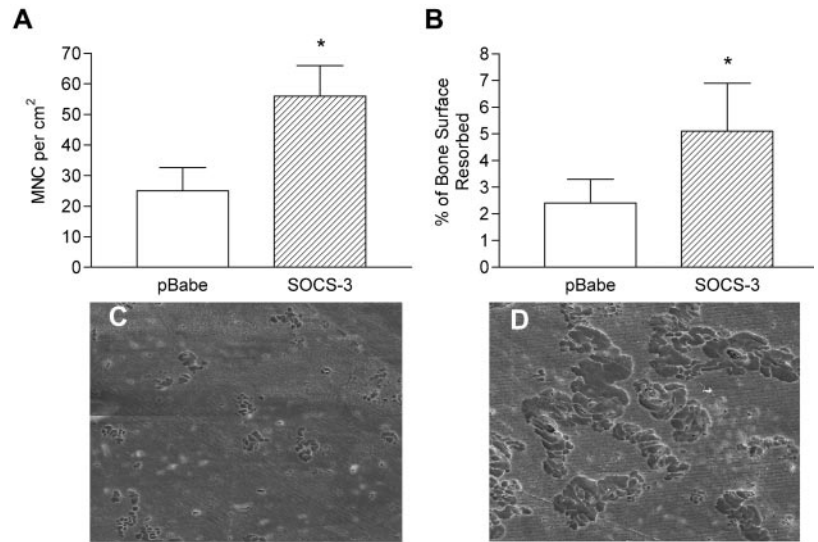
TGF- $\beta$  has a crucial role in determining lineage switching in immature mononuclear phagocytes, acting to maintain and enhance the responsiveness of osteoclast precursors to RANKL (4, 9). In the absence of exogenous TGF- $\beta$ , only a proportion of precursors form osteoclasts. This may be due to the presence of cytokines

Table I. Total cell numbers in pBabe-infected and SOCS 3-infected precursors incubated with M-CSF and RANKL

Group	Total Cell Number Day 8 (mean $\pm$ SEM)	Total Cell Number Day 10 (mean $\pm$ SEM)	Total Cell Number Day 14 (mean $\pm$ SEM)
pBabe-infected	16538 $\pm$ 1238	18731 $\pm$ 1756	17432 $\pm$ 3690
SOCS 3-infected	14327 $\pm$ 2470	16212 $\pm$ 3215	16969 $\pm$ 1960



**FIGURE 4.** Effect of SOCS-3 expression on osteoclast differentiation and activation on bone. Nonadherent, M-CSF-dependent bone marrow cells were incubated on slices of bovine cortical bone with M-CSF (10 ng/ml) for 2 days. Cells were then infected with pBabe-SOCS-3 or pBabe-empty (control) retroviruses. Cultures were incubated with M-CSF (30 ng/ml) and RANKL (30 ng/ml) for 8 days. **A**, Bone slices were fixed, and the number of TRAP-positive MNC was quantified. **B**, The percentage of bone surface resorbed. Following assessment of TRAP-positive MNC the bone slices were immersed in NaOCl and sputter-coated with gold, and the extent of resorption was quantified. Values are expressed as the mean  $\pm$  SEM of three separate experiments ( $n = 24$ ). \*,  $p < 0.05$  vs control. **C** and **D**, Photomicrograph of bone surface after removal of cells after incubation of nonadherent bone marrow cells with M-CSF (30 ng/ml) plus RANKL (30 ng/ml) as described above and infected with either pBabe-empty (**C**) or pBabe-SOCS-3 (**D**).

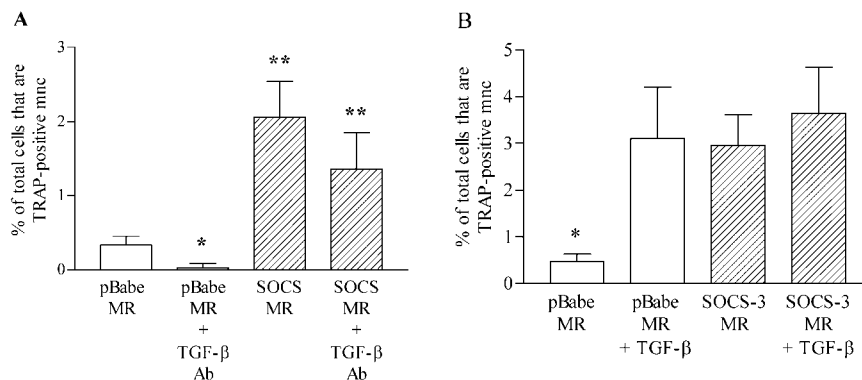


such as IFN- $\beta$  in the tissue culture environment, which direct the differentiation of precursors toward inflammatory and cytocidal macrophagic pathways rather than the osteoclastic lineage (14). IFN- $\beta$ , like many other factors that suppress osteoclast formation (13, 17, 18), signal through the JAK/STAT pathway (19, 20). JAK/STAT activation may therefore represent a central mechanism by which inhibitory cytokines suppress osteoclast formation.

Following cytokine activation, JAK/STAT signaling is modulated by the expression of a number of STAT-induced SOCS isoforms. SOCS expression mediates the antagonistic action of several factors on STAT activation. In the present study we demonstrate that TGF- $\beta$  rapidly induces sustained expression of SOCS-3 mRNA in bipotential osteoclast-macrophage precursors. Like TGF- $\beta$ , SOCS-3 expression in mononuclear precursors significantly augmented RANKL-induced osteoclast differentiation and prevented the suppressive action of IFN- $\beta$  on osteoclast formation. Furthermore, SOCS-3 antisense oligonucleotides suppressed RANKL-induced osteoclast formation and the enhancing effect of exogenously administered TGF- $\beta$ . These data suggest that TGF- $\beta$  may act to facilitate osteoclast formation through the in-

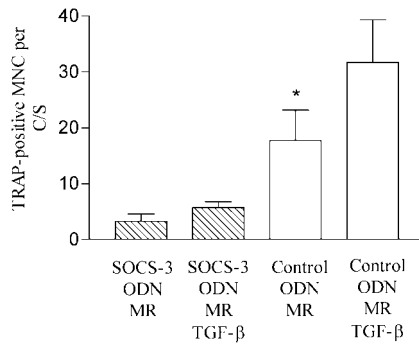
duction of SOCS-3, thereby inhibiting JAK/STAT signaling and facilitating the differentiation of noncommitted precursors to the osteoclastic lineage rather than to alternative, inflammatory macrophage pathways.

Macrophage fate is determined by a relatively short incubation in cytokine, as IFN- $\beta$  rapidly activates transcription of target genes in noncommitted precursors (40). Thus, a candidate for mediating the suppressive action of TGF- $\beta$  would have to be up-regulated and prevent macrophage differentiation within this initial period. SOCS rapidly block the phosphorylation of STATs by inhibiting the kinase activity of JAKs. The mechanism by which SOCS-3 inhibits signaling differs from that of other SOCS isoforms. Unlike SOCS-1, SOCS-3 does not directly inhibit JAK2 activity, the ability of SOCS-3 to inhibit growth hormone-induced JAK2 activation increases significantly in the presence of the growth hormone receptor (41), indicating that SOCS-3 indirectly inhibits STAT phosphorylation through an association with the cytokine receptor (42). SOCS-3 may also suppress signaling by increasing STAT degradation. SOCS box-containing proteins recruit activated signaling proteins to the proteasome (43) through an



**FIGURE 5.** SOCS-3 expression opposes the inhibitory effect of pan-specific TGF- $\beta$  Abs. Nonadherent, M-CSF-dependent bone marrow cells were incubated in M-CSF (10 ng/ml) for 2 days. Cells were then infected with pBabe-SOCS-3 or pBabe-empty retroviruses. **A**, Cultures were incubated with M-CSF (30 ng/ml) and RANKL (100 ng/ml) with or without pan-specific TGF- $\beta$  Ab (20  $\mu$ g/ml) for 6 days. Coverslips were fixed, and the total cell number and the number of TRAP-positive MNC cells were counted. Values are the mean  $\pm$  SEM of two separate experiments ( $n = 16$ ). \*,  $p < 0.05$  vs all other groups; \*\*,  $p < 0.05$  vs pBabe MR. There was no significant difference in the number of TRAP-positive MNC cells formed in SOCS-3-infected cells incubated in M-CSF and RANKL vs SOCS-3-infected cells incubated with M-CSF, RANKL, and pan-specific TGF- $\beta$  Ab. **B**, Cultures were incubated with M-CSF (30 ng/ml) and RANKL (100 ng/ml) with or without TGF- $\beta$  (0.1 ng/ml) for 6 days. Coverslips were fixed, and the total cell number and the number of TRAP-positive MNC cells were counted. Values are the mean  $\pm$  SEM of two separate experiments ( $n = 14$ ). \*,  $p < 0.05$  vs all other groups. There was no significant difference in the number of TRAP-positive MNC cells formed in SOCS-3-infected cells incubated with or without TGF- $\beta$ .

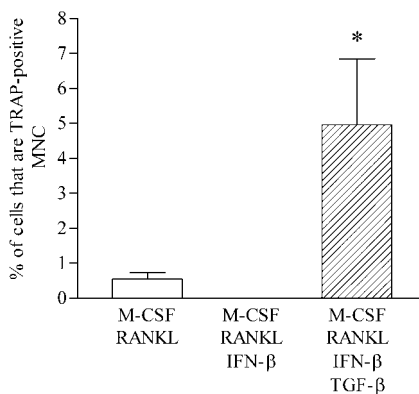




**FIGURE 6.** Antisense SOCS-3 oligonucleotides suppress osteoclast formation and blunt the enhancing effect of TGF- $\beta$ . Nonadherent, M-CSF-dependent bone marrow precursors were incubated for 6 days with combinations of M-CSF (30 ng/ml), RANKL (30 ng/ml), TGF- $\beta$ 1 (0.1 ng/ml), and either antisense SOCS-3 ODN (2  $\mu$ M) or control ODN (2  $\mu$ M). Cells were fixed, and the number of TRAP-positive MNC was quantified. Values are expressed as the mean  $\pm$  SEM of two separate experiments ( $n = 12$ ). There was no significant difference between antisense SOCS-3 ODN cultures incubated with or without TGF- $\beta$ . \*, Significantly different vs all other groups ( $p < 0.05$ ).

association of the SOCS box with elongins B and C (43, 44), facilitating the ubiquitination and subsequent degradation of the signaling protein. Therefore, in view of the rapid nature by which SOCS-3 inhibits STAT activation, it seems likely that induction of SOCS-3 mRNA within 1 h would be sufficient to suppress the differentiation of precursors toward other macrophagic lineages.

The precise stage of maturation when lineage switching in mononuclear phagocyte precursors occurs is not known. It is likely that it is determined within the first few days of culture, since bone marrow cells become increasingly resistant to osteoclast induction as the culture period progresses (3–5). TGF- $\beta$  prevents this resistance from developing; the proportion of cells in bone marrow cultures that form osteoclasts is the same in cultures preincubated in TGF- $\beta$  and those in which RANKL is present from the start (4). The ability of TGF- $\beta$  to maintain precursors in a RANKL-responsive state for several days may be explained by its ability to maintain a level of SOCS-3 expression sufficient to continuously suppress signals from proinflammatory stimuli generated by macrophages in vitro and by the culture environment, which is



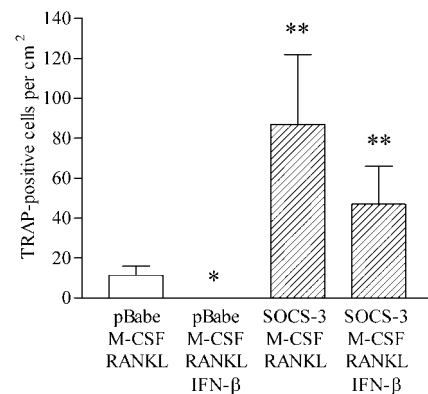
**FIGURE 7.** TGF- $\beta$  opposes the inhibitory effect of IFN- $\beta$  on osteoclast formation. Nonadherent, M-CSF-dependent bone marrow precursors were incubated for 6 days with M-CSF (30 ng/ml) and RANKL (30 ng/ml) with or without IFN- $\beta$  (5 U/ml) and/or TGF- $\beta$ 1 (0.1 ng/ml). Cells were fixed, and the number of TRAP-positive MNC was quantified. Values are expressed as the mean  $\pm$  SEM of two separate experiments ( $n = 16$ ). \*,  $p < 0.01$  vs control.

itself proinflammatory and capable of macrophage activation (10–12).

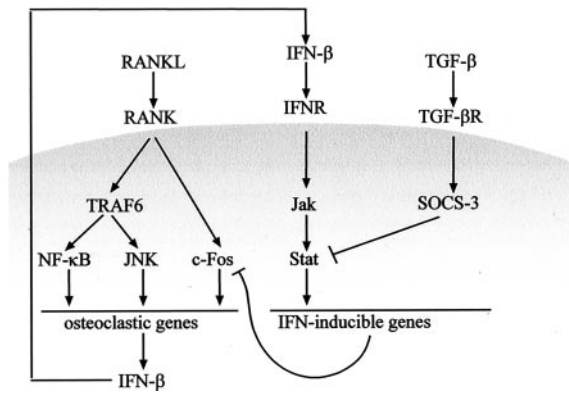
SOCS-3 may inhibit signaling by other osteoclast inhibitory factors in addition to IFN- $\beta$ . Several studies have implicated SOCS-3 in the suppression of IFN- $\gamma$  signaling. SOCS-3 expression inhibits the antiviral effect of IFN- $\gamma$  in macrophages, and the suppression of IFN- $\gamma$  signaling in response to IL-10 is also attributable to an up-regulation of SOCS-3 expression (35, 36, 38). The attenuation of IFN- $\gamma$  signaling by SOCS-3 in these studies is attributed to a reduction in STAT1 phosphorylation (35). Thus, other cytokines that phosphorylate STAT1 may also be inhibited by TGF- $\beta$ -induced SOCS-3 expression. In addition, SOCS-3 has been shown to prevent the phosphorylation of STAT3 (45), which is activated in the IL-10 and IL-4 signaling cascades, both of which have been shown to suppress osteoclast differentiation (21, 46, 47).

The ability of TGF- $\beta$  to induce SOCS-3 expression within 1 h suggests that this is mediated by a direct effect of TGF- $\beta$ -activated intracellular signals on SOCS-3 transcription, since this is too short a time for the transcription and expression of an intermediary factor. Binding of TGF- $\beta$  to its receptor complex induces the phosphorylation of S mothers against decapentaplegic (SMADs) (48), which bind to DNA and associate with numerous transcription factors. TGF- $\beta$  also activates mitogen-activated protein kinases (49, 50) through its ability to stimulate TAK1 (TGF- $\beta$ -activated kinase) (51, 52). The relative importance of mitogen-activated protein kinase and SMAD signaling pathways in TGF- $\beta$ -induced SOCS-3 expression and osteoclast formation is not known.

The ability of SOCS-3 expression to enhance osteoclast formation and of antisense SOCS-3 to significantly blunt the enhancing effect of TGF- $\beta$  does not preclude the involvement of additional mechanisms by which TGF- $\beta$  enables osteoclast formation. It is possible that TGF- $\beta$  may induce the expression of additional, novel SOCS isoforms to switch off JAK/STAT signaling by other inhibitory factors. Alternatively, other mechanisms may be involved in the suppression of inhibitory signals. TGF- $\beta$  has been shown to modulate the signaling response of other cytokines by competing for the transcriptional coactivator CRE binding protein/p300. In fibroblasts the antagonistic regulation of type I collagen



**FIGURE 8.** SOCS-3 expression enables the formation of osteoclasts in the presence of IFN- $\beta$ . Nonadherent, M-CSF-dependent bone marrow cells were incubated in M-CSF (10 ng/ml) for 2 days. Cells were then infected with pBabe-SOCS-3 or pBabe-empty retroviruses. Cultures were incubated with M-CSF (30 ng/ml) and RANKL (30 ng/ml) with or without IFN- $\beta$  (5 U/ml) for 6 days. Coverslips were fixed, and the total cell number and the number of TRAP-positive MNC were counted. Values are the mean  $\pm$  SEM of two separate experiments ( $n = 16$ ). \*,  $p < 0.05$  vs all other groups; \*\*,  $p < 0.05$  vs pBabe M-CSF and RANKL. There was no significant difference in the number of TRAP-positive MNC formed in SOCS-3-infected cells incubated with or without IFN- $\beta$ .



**FIGURE 9.** TGF- $\beta$  facilitates osteoclast formation by preventing the inhibitory effect of IFN- $\beta$  on the RANKL signaling cascade. TGF- $\beta$  induces SOCS-3 expression in noncommitted mononuclear phagocyte precursors, which, in turn, inhibits subsequent signaling through the JAK/STAT pathway. It has been shown that RANKL induces the production of IFN- $\beta$  in precursors, and that IFN- $\beta$  suppresses osteoclastic differentiation via JAK-STAT-mediated suppression of Fos (12).

expression by TGF- $\beta$  and IFN- $\gamma$  is attributed to a competition between activated SMAD and STAT complexes for limited amounts of CRE binding protein/p300 (53). On the other hand, TGF- $\beta$  may act to potentiate RANKL-induced signaling cascade through its ability to activate TAK1. Recently, TAK1 has been suggested to form part of a transcriptional complex that has a central role in the RANKL signaling pathway (54). RANKL stimulates the formation of a complex containing RANK, TNFR-associated factor 6, TAK1 binding protein 2, and TAK1, leading to the activation of NF- $\kappa$ B (55, 56). Therefore, TGF- $\beta$  may facilitate the formation of this complex through the activation of TAK1 and the subsequent increase in NF- $\kappa$ B activity, which is a key component in the signaling cascade leading to osteoclast formation.

Whatever the roles of alternative mechanisms, this study provides a possible mechanistic basis for the regulation of macrophage-osteoclast lineage regulation and also has important implications for the mechanism by which TGF- $\beta$  modulates macrophage activation by inflammatory cytokines. Our data show that TGF- $\beta$  rapidly induces SOCS-3 mRNA expression in immature mononuclear phagocytes. Like TGF- $\beta$ , SOCS-3 expression in precursors significantly enhances RANKL-induced osteoclast formation and opposes the inhibitory effect of IFN- $\beta$  on osteoclast formation. SOCS-3 expression also opposes the inhibitory effect of anti-TGF- $\beta$  Ab on osteoclast differentiation. Furthermore, SOCS-3 antisense oligonucleotide suppresses RANKL-induced osteoclast formation and significantly blunts the enhancing effect of TGF- $\beta$ . This suggests that TGF- $\beta$ -induced SOCS-3 expression in osteoclast precursors may assist the commitment to the osteoclastic lineage by suppressing exogenous and endogenous proinflammatory, antiosteoclastic JAK/STAT signals, thus acting to prime precursors for a role in bone resorption rather than cytotoxic and inflammatory functions (Fig. 9).

Osteoclast differentiation depends on TGF- $\beta$ , and the presence of very large amounts of this cytokine in bone matrix might contribute to an explanation for the regular differentiation of osteoclasts only in bone despite exposure of macrophagic precursors to osteoclast-inductive cytokines in other tissues.

## Acknowledgments

We are grateful to Ray Moss (Medical Imaging Facility, St. George's Hospital Medical School) and Valerie Emmons for assistance in the preparation of text and figures.

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