The Potential Role of Suppressors of Cytokine Signaling in the Attenuation of Inflammatory Reaction and Alveolar Bone Loss Associated with Apical Periodontitis

Renato Menezes, DDS, MS, PbD,* Thiago Pompermaier Garlet, DDS, MS,† Ana Paula Fávaro Trombone, DPharm, MS, PbD,‡ Carlos Eduardo Repeke, DDS, MS,* Ariadne Letra, DDS, MS, PbD,* José Mauro Granjeiro, MS, PbD,‡§ Ana Paula Campanelli, MS, PbD,* and Gustavo Pompermaier Garlet, DDS, MS, PbD*

Abstract
Inflammatory cytokines contribute to periapical tissue destruction. Their activity is potentially regulated by suppressors of cytokine signaling (SOCS), which down-regulate signal transduction as part of an inhibitory feedback loop. We investigated the expression of the cytokines tumor necrosis factor α (TNF-α), interleukin (IL)-10, and SOCS1, -2, and -3 by real-time polymerase chain reaction in 57 periapical granulomas and 38 healthy periapical tissues. Periapical granulomas exhibited significantly higher SOCS-1, -2, and -3, TNF-α, IL-10, and RANKL messenger RNA levels when compared with healthy controls. Significant positive correlations were found between SOCS1 and IL-10 and between SOCS3 and IL-10. Significant inverse correlations were observed between SOCS1 and TNF-α, SOCS1 and RANKL, and SOCS3 and TNF-α. Increased SOCS1, -2, and -3 messenger RNA levels in periapical granulomas may be related to the downregulation of inflammatory cytokines in these lesions; therefore, SOCS molecules may play a role in the dynamics of periapical granulomas development. (J Endod 2008;34:1480–1484)

Key Words
Cytokines, inflammation, periapical granulomas, receptor activator of NF-κB ligand, suppressors of cytokine signaling, tumor necrosis factor α

Cytokines play a major role in inflammatory and immune responses within the bone microenvironment. The balance between pro- and anti-inflammatory mediators determines the outcome of resorption in bone destructive diseases, including periapical granulomas (1). The recognition of microbial structures such as lipopolysaccharides (LPS) from gram-negative bacteria through toll-like receptors (TLRs) triggers intracellular signaling pathways that culminate in an inflammatory cytokine response and bone lesions (2). Previous studies have shown that proinflammatory cytokines such as the receptor activator of NF-κB ligand (RANKL) and tumor necrosis factor α (TNF-α), members of the TNF superfamily, are established agents in the pathogenesis of chronic inflammatory diseases including pulpitis and apical periodontitis (3–5). TNF-α is a potent immunologic mediator of acute and chronic inflammatory responses (6) and has the ability to increase bone resorption (7). On the other hand, interleukin (IL)-10 is a pleiotropic cytokine with strong anti-inflammatory properties, regulating B-cell proliferation and differentiation and exhibiting immunoregulatory activities (8). IL-10 directly affects osteoclast precursors and inhibits osteoclast generation and activation (9) and has been suggested as an important suppressor factor for periodontal disease (10) and apical periodontitis (11) development in mice.

The activation of cytokine signaling via the Janus kinase (JAK) and activators of transcription (STAT) pathways may represent a key mechanism by which inflammatory cytokines contribute to osteoclast formation. Members of the suppressors of cytokine signaling (SOCS) family, which comprise eight proteins (SOCS-1 to -7 and cytokine-inducible SH2-domain-containing protein-CIS), may inhibit these pathways. The SOCS genes are activated in response to a diverse range of microbial and immunologic factors (12–14). Individual SOCS are capable of inhibiting multiple cytokines, although mechanisms of binding affinity are still unclear (13, 14). SOCS-1 can be induced by cytokines that use the JAK-STAT signaling pathway, including TNF-α, IL-6, and interferon gamma (IFN-γ), and it has been shown to inhibit the upregulation of these same inflammatory cytokines (12, 15, 16). SOCS-2 mediates the anti-inflammatory properties of aspirin-induced lipoxin in vivo (17). SOCS-3 is strongly induced by IL-1, IL-6, IL-10, and IFN-γ, and its main role appears to be the attenuation of inflammatory cytokine signaling (12, 14). Some investigators have suggested that SOCS gene expression can be induced by a range of microbial stimuli, including lipopolysaccharides and CpG oligonucleotides, and that particularly SOCS-1 is a negative regulator of the lipopolysaccharide-induced inflammatory effects via the inhibition of TLR4 signaling (13, 17–19) (Fig. 1). Although the expression of SOCS in healthy tissues is generally absent or minimal, different degrees of upregulated SOCS expression in inflamed tissues may determine the outcome of inflammatory reaction. Therefore, we hypothesized that a differential expression of SOCS in a periapical environment could interfere in the expression inflammatory cytokines such as TNF-α and RANKL, which would be relevant to the outcome of periapical granulomas.

Therefore, in this study, we investigated the expression of cytokine suppressors SOCS1, -2, and -3 in human periapical granulomas and their correlations with immunoregulatory cytokines in order to improve the understanding of some of the underlying mechanisms during periapical lesions development.
Such as cathepsin K and matrix metalloproteinases (MMPs) (5), which are involved in bone resorption. The expression of SOCS (mRNAsocs) (6), whose inducing factors in osteoclast precursors remain to be identified (7), may impair osteoclast differentiation and activation through the inhibition/degradation of TRAF6 and NF-kB (8).

Figure 1. The mechanisms of SOCS action in inflammatory cells and osteoclast precursors. A schematic representation of mechanisms of SOCS action in inflammatory cells and osteoclast precursors. (A) The inflammatory signaling triggered by lipopolysaccharides (LPS) or cytokines such as TNF-α and IL-1β (1) may involve several signaling intermediates such as JAK/STAT, TRAF6 (2), and transcription factors such as NF-kB (3), which initiate specific mRNA transcription (mRNAα, mRNAβ) at the nucleus (4). The mRNA is translated into proteins that will exert their biological roles after secretion (5). Inflammatory signaling also induces the expression of SOCS genes (mRNAsocs) (6), which is increased by IL-10 (7). The conversion of SOCS into nonsecreted proteins (8) allows their intracellular action, which consists in the inhibition of inflammatory signaling (9), therefore dampening the inflammatory effects of LPS and cytokines. (B) The interaction of RANKL with the receptor RANK (1) expressed by osteoclast precursors leads to the intracellular signaling that involves TRAF6 (2) and the transcription factor NF-kB (3), which leads to osteoclast differentiation and activation, and initiates specific mRNA transcription (mRNAα and mRNAβ) at the nucleus (4). Activated osteoclasts produce enzymes such as cathepsin K and matrix metalloproteinases (MMPs) (5), which are involved in bone resorption. The expression of SOCS (mRNAsocs) (6), whose inducing factors in osteoclast precursors remain to be identified (7), may impair osteoclast differentiation and activation through the inhibition/degradation of TRAF6 and NF-kB (8).

Material and Methods

This study was approved by the Institutional Review Board at Bauru Dental School. Fifty-seven periapical granulomas were collected from previously selected patients aged 15 to 58 years (average age, 23.15 years; 27 females and 30 males) submitted to periapical surgery and curettage of the tissues as part of their clinical treatment. The diagnosis of a periapical lesion was based on a radiographic image showing clear bone loss and disappearance of the periodontal ligament space in the periapical region. The collected lesions were divided into two parts for histopathological and molecular analyses. Routine histopathologic examination (hematoxylin-eosin staining of serially sectioned specimens) was performed, and granulomas represented a severe infiltration of inflammatory cells with no evidences of epithelial cells (ie, epithelial lining and/or epithelial infiltration within the lesion). Periapical cysts were classified as fully developed cavities lined by stratified squamous epithelium and a fibrous capsule and were excluded from the sample. Partially epithelized lesions, considered in some studies epithelized granulomas, were also excluded and will be properly analyzed comparatively with cysts and granulomas in a further study. Lesion diameter was obtained by direct measurement using an endodontic ruler onto the periapical radiograph (taken with an intraoral film positioner).

Periodontal ligaments were used as control specimens and were obtained from teeth extracted for orthodontic purposes from 38 subjects (20 males and 18 females; age, 17-23 years) presenting good oral health. Patients who used systemic modifiers of bone metabolism; patients who underwent antibiotic, anti-inflammatory, hormonal, or other assisted drug therapy within 6 months before the study; patients with primary or secondary acute periodontitis; or females who were pregnant or nursing were not included in the study.

The total RNA was extracted from samples using TRIzol reagent (Life Technologies, Grand Island, NY), and complementary DNA was synthesized by using 3 μg of RNA through a reverse transcription reaction as previously described (20). Real-time polymerase chain reaction (PCR) quantitative messenger RNA (mRNA) analyses were performed in a MiniOpticon thermocycler (BioRad, Hercules, CA) using SYBR-green chemistry (Invitrogen, Carlsbad, CA) with specific primers previously described (20). The determination of the relative levels of gene expression was performed using the cycle threshold method, in reference to β-actin, as described elsewhere (20). PCR conditions were 95°C (10 minutes), 40 cycles at 94°C (1 minute), annealing at 56°C (1 minute), and 72°C (2 minutes). Results are depicted as the mean mRNA expression from triplicate measurements normalized by internal control beta-actin. Statistical analyses included analysis of variance followed by Bonferroni correction in GraphPad Prism 4.0 (GraphPad Software Inc, San Diego, CA); p ≤ 0.05 was considered statistically significant.

Results

SOCS-1, -2, and -3; TNF-α; IL-10; and RANKL mRNA expression was significantly more frequent and intense in diseased tissues than in healthy tissues (p < 0.001) (Fig. 2). The levels of IL-10 showed a positive correlation with SOCS1 (r² = 0.1302, p = 0.0058) and SOCS3 (r² = 0.1085, p = 0.0124), whereas the levels of SOCS1 and TNF-α (r² = 0.1857, p = 0.0008), SOCS1 and RANKL (r² = 0.0923, p = 0.0116), and SOCS3 and TNF-α (r² = 0.1857, p = 0.0008) were negatively correlated (Fig. 3). A positive correlation was also observed between TNF-α and RANKL, and a trend for negative correlation was found for IL-10 and RANKL (data not shown). SOCS1 (r² = 0.0705, p = 0.0359) and SOCS2 (r² = 0.0892, p = 0.0140) mRNA levels were inversely correlated with lesion size; however, for SOCS3, a trend toward inverse correlation was verified (r² = 0.0238, p = 0.1516) (Fig. 3).

Discussion

Pronflammatory cytokines play a fundamental role in periapical bone destruction through the induction of RANKL, a gene directly involved in osteoclast activation (5, 21, 22). On the other hand, the protective mechanisms against bone resorption are not fully understood. In this study, we used samples of human periapical granulomas and investigated the expression of SOCS molecules (regarded as potentially protective) and cytokines involved in RANKL induction (TNF-α) and
anti-inflammatory effects of IL-10, which involve inhibition of inflammatory signals that lead to TNF-α production (12). Therefore, in accordance with our findings, SOCS is usually absent or minimal in healthy tissues, and their upregulated and differential expression in inflamed tissues may determine the outcome of inflammatory reaction (12). Both SOCS-1 and SOCS-3 negatively regulate the innate and adaptive immune mechanisms in inflammatory arthritis (23), and their deficiency results in increased tissue destruction (24, 25). SOCS-2 is a key intracellular mediator of the anti-inflammatory actions of lipoxins, and its induction has been suggested to represent a general anti-inflammatory pathway responsible for controlling several innate responses (26).

Furthermore, the expression of SOCS has been shown to tune proinflammatory signals (like TLRs and cytokines signaling) to prevent excessive inflammatory damage to the host tissues (14, 17, 19, 27). Both LPS (which is a TLR ligand) and several cytokines are released in root canal infection and are involved in the development of periapical lesions (28). Therefore, we speculate that SOCS may contribute to the regulation of inflammatory molecules in the periapical microenvironment. Accordingly, our results showed an inverse correlation between mRNA levels of SOCS-1 and TNF-α, SOCS-1 and RANKL, and SOCS-3 with TNF-α in periapical granulomas. TNF exacerbates bone resorption through the upregulation of RANKL expression (29). On the other hand, we found a positive correlation between SOCS1, SOCS3, and IL-10. The anti-inflammatory effects of IL-10, which involve inhibition of inflammatory mediators of mRNA transcription, may involve the participation of SOCS proteins, mainly SOCS1 and SOCS3 (30–32). Interestingly, IL-10 attenuates experimental periapical lesion progression (11), suggesting that IL-10–induced SOCS may downregulate the inflammatory signaling that leads to TNF-α mRNA transcription, which, in turn, could downregulate RANKL levels and interfere with lesion progression. Corroborating our findings, a similar pattern of coexpression of SOCS and cytokines was described in periodontal diseases in which increased levels of SOCS-1 and -3 were associated with nonprogressive gingivitis lesions (33).

SOCS-1 and -3 levels showed a trend toward negative correlation with lesion size. This could be explained in part by the association of SOCS-1 and -3 with lower RANKL expression. We have recently shown that RANKL/osteoprotegerin ratio is possibly associated with stable or progressive stages of periapical granulomas (34). Surprisingly, SOCS-2, not associated with RANKL expression, presented a significant negative correlation with lesion size. SOCS-2 was described to mediate the ubiquitinylation and proteasome-mediated degradation of TRAF6 (26), a RANKL-induced transcription factor essential to osteoclast development. Therefore, in addition to modulating inflammatory signaling, it is also possible that SOCS proteins influence signaling pathways involved in osteoclast differentiation and activation (27, 35).

To our knowledge, this is the first demonstration of a differential expression of SOCS-1, -2, and -3 mRNA expression in healthy and diseased human periapical tissues. These SOCS proteins were found to be differentially associated with molecules involved in the exacerbation or attenuation of bone loss, suggesting their possible involvement in the pathogenesis of the periapical lesions, as follows: (1) the expression of SOCS in inflammatory cells, induced as a negative feedback in response to inflammatory cytokines and microbial signaling and upregulated by IL-10, blocks further proinflammatory signaling and consequently dampens the inflammatory reaction (as exemplified in Fig. 1A); (2) the decrease in inflammatory mediators expression, such as TNF-α, resulting thereafter in a lower expression of RANKL, which, in tum, attenuates the bone resorption associated with periapical granulomas; and (3) SOCS also can directly act on osteoclasts precursors through the inhibition of the TRAF6 and NF-κB, molecules with an essential role in the differentiation and activation of osteoclasts (as exemplified in Fig. 1B). Therefore, we can consider that SOCS can attenuate periapical lesions progression at multiple steps.

Taken together, our data suggest that SOCS may potentially downregulate signaling events involved in osteoclast differentiation and activation, which consequently could interfere in lesion outcome, pointing to a promising role of SOCS as therapeutic targets to clinically interfere in the bone resorption process associated with these endodontic lesions.

Our results showed that human periapical granulomas presented significantly higher levels of SOCS-1, -2, and -3 mRNA when compared with healthy periapical tissues, indicating a possibly active role for SOCS in the pathogenesis of periapical granulomas. SOCS proteins are negative regulators of the inflammatory signaling triggered by TLRs and proinflammatory cytokines and attenuate signal transduction as part of a negative feedback loop to inhibit the response to subsequent stimuli (12). Therefore, in accordance with our findings, SOCS is usually absent or minimal in healthy tissues, and their upregulated and differential expression in inflamed tissues may determine the outcome of inflammatory reaction (12). Both SOCS-1 and SOCS-3 negatively regulate the innate and adaptive immune mechanisms in inflammatory arthritis (23), and their deficiency results in increased tissue destruction (24, 25). SOCS-2 is a key intracellular mediator of the anti-inflammatory actions of lipoxins, and its induction has been suggested to represent a general anti-inflammatory pathway responsible for controlling several innate responses (26).

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Taken together, our data suggest that SOCS may potentially downregulate signaling events involved in osteoclast differentiation and activation, which consequently could interfere in lesion outcome, pointing to a promising role of SOCS as therapeutic targets to clinically interfere in the bone resorption process associated with these endodontic lesions.
resorption process. Indeed, experiments in a mouse model of periapical lesions, in which SOCS will be both induced and inhibited, are underway to confirm such hypothesis. Furthermore, comparative analysis of SOCS expression in different types of periapical lesions as well the elucidation of its spatial expression within the lesions certainly will contribute to the understanding of periapical lesions pathogenesis. However, it is also important to consider that the pro- and anti-inflammatory networks implicated in the immunopathogenesis of apical periodontitis are very complex. In addition to proteins of the SOCS family, other regulatory molecules such as sMyD88, interleukin-1 receptor-associated kinase M, Tollip, and TNF-related apoptosis-inducing ligand receptor have been suggested as important negative regulators of inflammatory signaling and could play important roles in apical periodontitis. It is noteworthy that understanding the role of regulators of cell signaling in the development of periapical diseases may provide the basis for future therapeutic interventions.

**Conclusion**

Our study shows that SOCS-1, -2, and -3 are differentially expressed in healthy and diseased human periapical tissues and also differentially associated with molecules involved in the exacerbation or attenuation of bone loss. Based on these findings, we suggest that SOCS may potentially downregulate signaling events involved in inflammatory reaction and osteoclast differentiation and activation and may interfere directly in the development and progression of periapical lesions. Therefore, SOCS are promising therapeutic targets to clinically interfere in the periapical bone resorption process.

**References**


**Figure 3.** Correlations between the expression of SOCS and cytokines in periapical granulomas and with the radiographic diameter of the lesions. The total RNA was extracted from periapical granulomas (n = 57) and the levels of SOCS1, SOCS2, SOCS3, TNF-α, IL-10, and RANKL mRNA were measured quantitatively by using the real-time PCR SYBR-green system. Linear regression analysis was used to test the correlations between the levels of SOCS and cytokines expression and also between SOCS and the radiographic diameter of the lesions. Values of p and r² are identified in the graphs.


