Periodontal Bacterial DNA Suppresses the Immune Response to Mutans Streptococcal Glucosyltransferase

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Received 2 May 2007/Accepted 10 May 2007

Certain CpG motifs found in bacterial DNA enhance immune responses through Toll-like receptor 9 (TLR-9) and may also demonstrate adjuvant properties. Our objective was to determine if DNA from bacteria associated with periodontal disease could affect the immune response to other bacterial antigens in the oral cavity. Streptococcus sobrinus glucosyltransferase (GTF), an enzyme involved in dental caries pathogenesis, was used as a test antigen. Rowett rats were injected with aluminum hydroxide (alum) with buffer, alum-GTF, or alum-GTF together with either Escherichia coli DNA, Fusobacterium nucleatum DNA, or Porphyromonas gingivalis DNA. Contrary to expectation, animals receiving alum-GTF plus bacterial DNA (P. gingivalis in particular) demonstrated significantly reduced serum immunoglobulin G (IgG) antibody, salivary IgA antibody, and T-cell proliferation to GTF compared to animals immunized with alum-GTF alone. A diminished antibody response was also observed after administration of alum-GTF with the P. gingivalis DNA either together or separately, indicating that physical complexing of antigen and DNA was not responsible for the reduction in antibody. Since TLR triggering by DNA induces synthesis of prospective suppressive factors (e.g., suppressor of cytokine signaling [SOCS]), the effects of P. gingivalis DNA and GTF exposure on rat splenocyte production of SOCS family molecules and inflammatory cytokines were investigated in vitro. P. gingivalis DNA significantly up-regulated SOCS1 and SOCS5 expression and down-regulated interleukin-10 expression by cultured splenocytes. These results suggested that DNA from periodontal disease-associated bacteria did not enhance, but in fact suppressed, the immune response to a protein antigen from cariogenic streptococci, potentially through suppressive SOCS components triggered by innate mechanisms.

Unmethylated CpG dinucleotides are found more frequently in the genomes of bacteria and DNA viruses than in vertebrates (28, 29). These motifs have been demonstrated to enhance both systemic and mucosal antibody responses to antigen (e.g., tetanus toxoid [10]). Recognition of these motifs as short oligodeoxynucleotides (ODNs) and signaling through the Toll-like receptor 9 (TLR-9) seem to be responsible for the enhancement (10).

Host immune response can play a key role in protection against dental caries (49). Both humoral and mucosal immune pathways can be important components in response to bacterial colonization and pathogenesis (24, 36, 54). Host responses to experimental vaccines containing antigens associated with dental caries pathogenesis have been shown to block both experimental infection and disease development (49). Bacterial DNA in the vicinity of the oral antigen may modulate the response to vaccine. Although DNA as a vaccine can influence host response, genomic DNA is immunogenic (40), and epitopes encoded in such DNA can induce innate immune responses leading to enhanced or suppressed antigen-specific immunity (16, 37, 52). Bacterial DNA liberated at the site of an infection is likely to affect the local inflammatory response (17), and host responses to bacterial DNA may contribute to immunity to bacteria (11). However, the effect of bacterial DNA on a bystander antigen-elicited immune response has not been explored.

Advances have recently been made in the design of efficient mucosal adjuvants based on detoxified bacterial toxin derivatives or CpG motif-containing DNA (19, 35). DNA segment vaccines directed to a single component of a cariogenic bacterium (13, 15, 23) or a periodontal pathogen (31, 55) have been described. Since whole-genomic bacterial DNA contains many CpG motifs, we hypothesized that whole-genomic DNA from periodontal disease-associated bacteria (e.g., Porphyromonas gingivalis [39] and Fusobacterium nucleatum [12]) might enhance the immune response to a vaccine bystander antigen. In this study we used glucosyltransferase (GTF), an enzyme associated with dental caries pathogenesis (44) from dental caries-associated Streptococcus sobrinus, as antigen.

MATERIALS AND METHODS

Bacteria and DNA. All bacterial strains were obtained from the American Type Culture Collection (ATCC). P. gingivalis strain 33277 was grown in Trypticase soy broth (BD Biosciences; San Diego, CA) containing 1% yeast extract, 5 µg/ml hemin, and 2.5 µg/ml menadione (42). Escherichia coli strain DH1 33849 was grown in LB broth (Sigma, St. Louis, MO) as previously described (14). F. nucleatum strain 25586 was grown in mycoplasma broth (BD Biosciences; San Diego, CA) as previously described (30). Four liters of each bacterium was grown for 5 to 7 days at 37°C in an anaerobic chamber. Whole-genomic DNA was prepared by phenol-chloroform-isooamyl alcohol extraction (50). Protein and lipopolysaccharide (LPS) impurities were removed by anion exchange (QIAGEN, Valencia, CA). The Micro BCA protein assay (Pierce, Rockford, IL) and the Limulus amoebocyte lysate reaction (Associates of Cape Cod, East Falmouth, MA) were performed to ascertain the levels of protein and LPS components in the DNA preparations. Thus, E. coli DNA at 360 µg/ml contained <0.03 endotoxin units (EU)/µg DNA and <0.2 µg/ml protein. F. nucleatum DNA at 1.4 mg/ml contained <0.016 EU/LPS/µg DNA and <0.2 µg/ml protein.

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† Published ahead of print on 21 May 2007.
and P. gingivalis DNA at 3.4 mg/ml contained <0.01 EU LPS/µg DNA and 0.2 µg/ml protein.

Experimental protocols. Three experiments were performed with inbred female heterozygous (m/m+) Rowett rats maintained under pathogen-free conditions (experiment I, aged 1 to 2 months) in laminar flow cabinets or under conventional conditions (experiment II, aged 5 to 6 months) (10). Experiments using the animals were approved by The Forsyth Institute’s Internal Animal Care and Use Committee.

Experiment I. In order to test if bacterial genomic DNA could interfere with the host immune response to GTF, five groups (1 to 5) of rats (six to nine rats/group) were injected subcutaneously (s.c.) in the major salivary gland vicinity (sgv) with alum–phosphate–buffered saline (group I), alum-GTF (group II), alum-GTF-E. coli DNA (group III), alum-GTF-F. nucleatum DNA (group IV), and alum-GTF-p. gingivalis DNA (group V). Four milligrams of alum (which has been shown previously to increase adjuvants [10]), 15 µg of S. sobrinus strain 6715 GTF prepared as previously described (44, 48), and 100 µg of each genomic DNA were used to inject each animal according to its group assignment. We chose 100 µg of DNA for comparability to the CpG level (100 µg) which was shown to have adjuvant effects in vivo in our previous studies (10). Animals were injected on day 0 and boosted with the same ingredients on day 105 (week 15). Animals were sacrificed on day 165 [i.e., 105 days after boosting].

Determination of levels of antibody to GTF. After 165 day, rats were anesthetized with isoflurane and sacrificed. Spleen, salivary glands, and mandibular sites and alum were harvested. Blood samples were collected and the expression of suppressor of cytokine signaling (SOCS) family members (CIS and SOCS1, -2, -3, -4, and -5) and inflammatory cytokines (tumor necrosis factor alpha [TNF-α], interleukin-1β [IL-1β], IL-6, and IL-10) was determined by reverse transcription-PCR (RT-PCR) 24 h after treatments. A two-fold increase or a 50% reduction in intensity relative to control was considered the cutoff for difference. In a separate experiment, the expression of SOCS1 and SOCS5 at the RNA level (relative to the glyceraldehyde-3-phosphate dehydrogenase [GAPDH] RNA level) and protein levels were quantitated by real-time PCR and ELISA, respectively, at 24 and 48 h after treatment (n = 4).

Determination of levels of antibody to GTF. For the detection of serum IgG antibody, S. sobrinus GTF (0.15 µg/ml) was used as a coating on 96-well plates as previously described (44, 48) and rat sera (1:100 to 1:500) were applied to the GTF-coated plate (0.5 µg/ml) and mouse monoclonal anti-rat IgG antibody (1:1,000; Biosource International, Camarillo, CA) was added and incubated for 2 h. Biotin-conjugated goat anti-mouse IgG (1:1,000; Zymed Laboratories, Inc., San Francisco, CA) was then added and incubated for 2 h followed by ALCP-conjugated avidin (1:3,000; ICN Biochemicals, Aurora, OH). After incubation in p-nitrophenyl phosphate solution (1 mg/ml; Sigma, St. Louis, MO) for 30 min, the reaction was terminated by the addition of 1 N NaOH and the absorbance was determined spectrophotometrically (Biotek, Winooski, VT) at 405 nm. The antibody level was expressed as ELISA units relative to titration of a hyperimmune rat antibody-containing serum standard.

[3H]thyminide incorporation assay. At the termination of experiment I, cells from rat cervical lymph nodes were isolated and cultured in 96-well plates (1 × 10⁵ cells/well) in the presence or absence of GTF (2 µg/ml). [3H]thyminide (0.5 Ci/well) was added for the last 16 h of a total of 4 days in culture. Samples were harvested onto glass fiber filters, and radioactivity (cpm) was measured in a liquid scintillation spectrometer (TRI-CARB 2100 TR; Perkin-Elmer, Shelton, CT).

RT-PCR. Total RNA was extracted from the isolated spleen cells using an RNeasy kit (QIAGEN, Valencia, CA). Isolated RNA (0.1 µg/ml) was reverse transcribed with the SuperScript synthesis system in the presence of random primers (Invitrogen, Carlsbad, CA). The resultant cDNA was amplified by PCR using gene-specific primer pairs with Tag DNA polymerase (Invitrogen) as described by the manufacturer. The following primers were used: CIS, 5′-CCAG TTAGGCCCAGCACTA3′-C and 5′-GAGCGCAAGGTAGACGCTTCA3′-C, 486 bp; SOCS1, 5′-ACTACATCCGGCCAGCATTCC-3′ and 5′-TCCAG CAGCTGAAAGGAGCA3′-C, 300 bp; SOCS2, 5′-AGCCGCGGACACGCTTG3′ and 5′-GGGCTGTCGGTTCGCTCTT-3′, 462 bp; SOCS3, 5′-CCCGC GGGCGTTTCTTCCTT3′ and 5′-GGGGCGCCGGACCTGAC3′, 516 bp; SOCS4, 5′-CCGCTGCGGATGTTGCTTCT3′ and 5′-GCTGCGT AATCTGTTTCCACCT-3′, 471 bp; SOCS5, 5′-TCAAAGTGAATAGGGG TAAATTGG3′ and 5′-TACCTTGTGCTACTGTCCTTCTA3′-C, 1.6 kb; TNF-α, 5′-GTAGCCACGTCGATACAA3′ and 5′-CCCTTTCGCCAGTGAA3′-C, 320 bp; IL-1β, 5′-TGAATCTTACCTGAGAC3′ and 5′- GAGGGCTGATGTACGAC3′-C, 378 bp; IL-6, 5′-AGACGAGTACCTAGAGGCA3′ and 5′-TCTATGACCCATCGCTGGA3′-C, 382 bp; IL-10, 5′- CCCTGCTATGTTGCTGCT3′ and 5′-TATCTTGGCCTTCTAGGAC3′- C, 463 bp; GAPDH, 5′-TACCTGCACTCAGAAGACTGT-3′ and 5′-GCC CTCTTCCGTTCTAGA3′-C, 520 bp. PCR conditions were 94°C for 30 s, 45 s to 63°C for 30 s, and 72°C for 1 min. Amplification of the GAPDH gene was used as an internal control. For screening the initial experiment mRNA transcripts for the genes described above were evaluated semiquantitatively. The gel images were read by densitometry (AlphaImager Imaging System, Alpha Innotech), and their intensities were calculated relative to those of GAPDH controls.

Real-time PCR. PCR mixtures and cDNA were loaded into the wells of a 96-well plate with primers and probes for SOCS1, SOCS5, and IL-10 according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Each cDNA was loaded in duplicate into the plate. Primers and probes for GAPDH were used in each sample as an endogenous control and simultaneously amplified along with the target gene to normalize for the amount of RNA added to a reaction mixture. Efficiency amplification of the targets was determined previously by dilution curves and were used in the calculation of relative quantification values for each gene. PCRs for the ABI PRISM 7000 detection system were started for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 s, and 60°C for 1 min. The cycle threshold (Cₚ) was established in the linear part of the reaction curve at which a significant amount of amplified target was generated. The relative quantity of target gene normalized to the GAPDH reference is given by

\[ \frac{2^{-\Delta \Delta \text{Ct}}}}{\text{reference}} \]

and is based on the method developed previously (41).

Determination of SOCS proteins. Cellular protein fractions from cultured rat splenocytes were lysed with radioimmunooprecipitation assay buffer (Sigma, St. Louis, MO) followed by sonication and centrifugation as described previously (7). Cell lysates (50 µg/ml) were applied as a coating onto 96-well plates. The plates first were incubated with rabbit anti-rat SOCS or SOCS antibody (1: 1,000; Anaspec, San Jose, CA) for 2 h and then were incubated with ALP-conjugated goat anti-rabbit IgG antibody (1:5,000; Zymed Laboratories, Inc., San Francisco, CA) for 2 h. After the reaction mixture was incubated in p-nitrophenyl phosphate solution (1 mg/ml) for 30 min, the reaction was terminated by the addition of 1 N NaOH and the absorbance was determined spectrophotometrically at 405 nm. Serial dilutions of known amounts of SOCS1 and SOCS5 synthetic peptides (Anaspec) conjugated with bovine serum albumin were used to generate standard curves to determine the amount of SOCS1 and SOCS5 respectively (expressed as the peptide equivalent).

Statistics. Results are presented as means ± standard errors (SE). There were six to nine rats in each group for experiments I and II (n = 6 to 9). One-way analysis of variance and the Student-Newman-Keuls (SNK) multiple comparison test were used to analyze differences among groups. Statistical significance was assumed with probability values of less than 0.05.
RESULTS

Serum IgG antibody response to GTF. As expected, rats injected with alum-GTF (without DNA) showed markedly increased IgG antibody to GTF compared to (alum-buffer) controls (Fig. 1A). A second injection of alum-GTF dramatically enhanced the antibody response, with maximum mean antibody levels increasing more than 7.5-fold from 13 weeks after the first injection (Fig. 1A) to 3 weeks after boost injection at week 15 (Fig. 1B). The groups receiving alum-GTF plus F. nucleatum or P. gingivalis DNA demonstrated significantly reduced antibody levels compared to the alum-GTF-alone group at 3 and 6 weeks, and all DNA additives were associated with significantly reduced IgG antibody to GTF at 13, 18, and 21 weeks postinjection (P < 0.01, Fig. 1). In particular, rats injected with alum-GTF plus P. gingivalis DNA demonstrated the most prominently decreased IgG antibody to GTF.

Salivary IgA antibody response to GTF. Rats injected with alum-GTF also showed significantly increased salivary IgA antibody to GTF compared to controls (Fig. 2A, 6 weeks). Compared to rats receiving alum-GTF only, rats injected with alum-GTF plus E. coli, F. nucleatum, or P. gingivalis DNA demonstrated significantly decreased IgA antibody to GTF at 6 and 13 weeks after initial injection (P < 0.05, Fig. 2A). After the second injection, there was no difference in salivary IgA antibody to GTF between groups of rats receiving alum-GTF, alum-GTF-E. coli DNA, or alum-GTF-F. nucleatum DNA at 18 and 21 weeks (Fig. 2B). However, when rats were injected with alum-GTF-P. gingivalis DNA, a significant decrease in antibody to GTF was observed at 21 weeks (P < 0.01, Fig. 2B). In particular, the most prominent suppression of IgA antibody response to GTF was observed in rats injected with alum-GTF plus P. gingivalis DNA (Fig. 2A, 6 and 13 weeks; Fig. 2B, 21 weeks). These findings were consistent in general with the...
results of serum IgG antibody response to GTF observed in Fig. 1.

**T-cell response to GTF.** In order to determine if the effect of bacterial DNA on antibody could be extended to T-cell response to GTF, cells from rat submandibular/submental and cervical lymph nodes were isolated and cultured in 96-well plates in the presence or absence of GTF at the termination of experiment I. Rats injected with alum-GTF showed markedly increased T-cell proliferation when GTF was added to the cultured lymphocytes compared to controls. However, rats injected with alum-GTF along with DNA from *E. coli*, *F. nucleatum* or *P. gingivalis* demonstrated significantly decreased [3H]thymidine incorporation of cultured lymphocytes (Fig. 3). All bacterial DNA additives showed comparable levels of inhibition of T-cell response to GTF (Fig. 3), although DNA from *P. gingivalis* tended to have the strongest ability to suppress IgG and IgA antibody responses (Fig. 1A and B and Fig. 2A and B). Therefore, we used the *P. gingivalis* DNA for further in vitro studies.

**Serum IgG antibody response to GTF with different injection procedures.** It was conceivable that administration of alum-GTF with the bacterial DNA might result in formation of a complex between GTF and DNA, rendering the protein less immunogenic. Therefore, rats were injected with alum-GTF either combined with or separate from *P. gingivalis* DNA to test if the suppression of antibody response to GTF observed in the bacterial DNA-injected groups was related to DNA interference with antigen presentation by the possible formation of GTF-DNA complexes (experiment II). The results (Fig. 4) showed that at all intervals tested (3, 4.5, and 6 weeks), a significantly reduced antibody response was achieved by the administration of alum-GTF either combined with or separate from the *P. gingivalis* DNA injection (Fig. 4; $P < 0.05$ or $P < 0.01$). However, no differences in antibody response between combined and separate groups were observed. Therefore, the diminished antibody response was not attributed to formation of a complex between alum-GTF and bacterial DNA.
Screening of SOCS and inflammatory cytokine expression in rat splenocytes in vitro. Interactions between DNA and TLR-9 effectively bridge innate and acquired immunity (51). Since SOCS proteins play an important role in the TLR-induced negative immune regulatory processes (16), we determined the effect of P. gingivalis DNA on the expression of SOCS and inflammatory cytokines. The levels of mRNA transcripts from GTF-stimulated splenocytes were compared with the levels of transcripts from splenocytes treated with GTF and P. gingivalis DNA (5 μg). After 24 h, the mRNA transcript levels of six SOCS family members (CIS and SOCS1, -2, -3, -4, and -5) and four representative pro- and anti-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-10) were determined by RT-PCR (Fig. 5A). In this screening analysis, the levels of SOCS1 and SOCS5 transcripts (normalized to GAPDH) were increased by a factor of 2.4-fold (SOCS1) or 3.2-fold (SOCS5) compared to the GTF-alone group. The level of IL-10 transcripts was decreased to less than 20% compared to the GTF-alone group (Fig. 5B). The transcript levels of the other genes were not changed beyond the cutoff values.

Expression of SOCS1 and SOCS5 mRNA transcripts by rat splenocytes in vitro. SOCS1 and SOCS5, negative feedback regulators of Th1- and Th2-type cytokines, respectively (20, 43), seemed to be up-regulated after the addition of P. gingivalis DNA (Fig. 5). To investigate the effect of P. gingivalis DNA on the production of SOCS1 and SOCS5 by rat spleen cells in vitro, we further evaluated these two cytokine-inhibitory molecules by quantitative real-time PCR. SOCS1 mRNA expression in rat spleen cells was unchanged with addition of GTF alone compared to medium control at 24 h and 48 h after treatment (Fig. 6A). An up-regulation of SOCS1 mRNA (both at 24 and 48 h) was observed in cells treated with the higher dose of P. gingivalis DNA (5 μg) and GTF compared to the GTF-alone group (Fig. 6A). In addition, the higher dose of P. gingivalis DNA (5 μg) plus GTF significantly up-regulated SOCS5 mRNA expression in rat spleen cells at 24 h and expression remained up-regulated 48 h after treatment (Fig. 6B) compared to the GTF-alone group. The lower dose of P. gingivalis DNA (1 μg) plus GTF did not significantly up-regulate SOCS5 mRNA expression in rat spleen cells until 48 h after treatment (Fig. 6B).

Expression of SOCS1 and SOCS5 proteins by rat splenocytes in vitro. The protein levels of SOCS1 and SOCS5 were evaluated by ELISA. At 24 h, addition of GTF plus the lower dose of P. gingivalis DNA (1 μg) did not change the expression of SOCS1 protein in cultured rat splenocytes compared to the GTF-only group. However, SOCS1 protein was markedly increased in cultured splenocytes after the addition of the higher dose (5 μg) of P. gingivalis DNA along with GTF (Fig. 7A). At 48 h, there was no significant difference in SOCS1 expression between groups (Fig. 7A). Similarly, GTF together with the lower dose of P. gingivalis DNA (1 μg) did not change the expression of SOCS5 protein in cultured splenocytes compared to the GTF-only group at either 24 or 48 h (Fig. 7B). P. gingivalis DNA at the higher dose (5 μg) significantly increased SOCS5 protein expression in cultured splenocytes at 24 h, whereas this increase was not discernible 48 h after treatment (Fig. 7B).

Effect of P. gingivalis DNA on expression of IL-10 by rat splenocytes in vitro. SOCS1 and SOCS5 have been implicated in the inhibition of IL-10-mediated immune responses (2, 9). We investigated the effect of P. gingivalis DNA on the expression of IL-10 mRNA transcripts. The results demonstrated that there was no change in the expression of IL-10 mRNA transcripts in GTF-treated spleen cells compared to the medium control (Fig. 8). However, IL-10 transcript levels were significantly down-regulated in the splenocytes treated with GTF plus P. gingivalis DNA compared to the GTF-only group, at both 24 and 48 h (Fig. 8). Such reduction was most effectively induced with the higher dose of P. gingivalis DNA (5 μg/well) at 48 h (Fig. 8, last column).
In general, bacterial DNA is considered immunostimulatory, conferring a Th1-type immunity to infection (33). Genomic DNA released by dying cells was shown to provide a stimulus for antigen-presenting cell (APC) maturation, enhanced APC function in vitro, and improved cellular and humoral immune responses in vivo (21). Unmethylated CpG ODN motifs in these immunostimulatory DNAs can also be detected through binding to TLR-9 and activation of macrophages and dendritic cells involved in innate immunity (45, 46). However, CpG ODNs not only act as immunostimulatory agents but also can induce strong immune suppression depending on the anatomical location of the application (53). It has also been suggested that suppressive GpG ODNs effectively counteract CpG-induced, Th1-mediated autoimmune disease by skewing both the autoaggressive T-cell and B-cell responses toward a protective Th2 phenotype (18, 34). Whether such immunosuppressive ODN motifs exist in periodontal bacterial genomic DNA is not clear.

In the studies described herein, we tested the hypothesis that bacterial (*E. coli*, *F. nucleatum*, or *P. gingivalis*) whole-genomic DNA would demonstrate adjuvant properties for GTF as antigen. This hypothesis was generated to account for possible stimulatory effects of periodontal disease-associated bacteria on host immune response to dental caries-associated and other microbiota. With dental caries vaccines demonstrating considerable potential importance (49), periodontal bacterial DNAs might theoretically enhance response to caries-relevant antigens by acting as natural adjuvants (positive regulators of innate and adaptive immune response). Contrary to expectation, rats that received the DNA from any of three bacteria (*E. coli*, *F. nucleatum*, and *P. gingivalis*), tended to have the most potent suppressive effect on the immune response to GTF compared to those effects produced by DNA from *E. coli* or *F. nucleatum.*
The extent of effects of P. gingivalis DNA on response to other antigens in the oral cavity remains to be determined. However, there was no indication that the suppressive effects were only GTF specific and thus might be generalized to other antigens in the oral cavity. Furthermore, the clinical relevance of such observations remains to be determined. Both primary and secondary responses to GTF were shown to be suppressed, as was salivary IgA antibody to GTF when P. gingivalis DNA was administered, indicating that the mucosal immune response can also be modulated by bacterial DNA components (10). In separate experiments, endpoint titration of antibody also indicated that the levels of serum IgG and salivary IgA antibody were significantly reduced in animal groups receiving bacterial DNA (data not shown). Furthermore, immunization of rats with GTF and 100 μg of the CpG ODN as previously described (10) also enhanced serum IgG and salivary IgA antibody compared to GTF alone (data not shown). This finding indicated that the animals were capable of producing systemic and mucosal adjuvanted responses to GTF.

This study indicated that periodontal bacterial DNA reduced immune response to a bystander dental caries-relevant antigen (GTF). This was not attributable to formation of a complex between alum-GTF and DNA since P. gingivalis DNA administered at the same site together or at separate sites with GTF resulted in similar, significant suppression of the immune response. Taken together, this led us to the hypothesis that a mechanism of negative regulation of cytokine signaling pathways could be involved in the control, regulation, and initiation of innate immune responses with subsequent effects on adap-

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**FIG. 6.** Determination of mRNA transcript levels of SOCS1 and SOCS5 in vitro. Rat splenocytes were cultured in six-well plates with the following treatments: (i) medium only, (ii) GTF (2 μg/well), (iii) DNA1 (1 μg/well P. gingivalis DNA), (iv) DNA5 (5 μg/well P. gingivalis DNA), (v) GTF plus DNA1, and (vi) GTF plus DNA5. Twenty-four and 48 h after treatments, total cellular RNA was isolated and reverse transcribed, and the mRNA levels of SOCS1 (A) and SOCS5 (B) were determined by real-time PCR. Expression of GAPDH was used as an internal reference (n = 4; **, P < 0.01, compared to GTF-only group, SNK multiple comparison test).

**FIG. 7.** Determination of protein levels of SOCS1 and SOCS5 in vitro. Rat splenocytes were cultured in six-well plates with the following treatments: (i) medium only, (ii) GTF (2 μg/well), (iii) GTF plus DNA1 (1 μg/well P. gingivalis DNA), and (iv) GTF plus DNA5 (5 μg/well P. gingivalis DNA). Twenty-four and 48 h after treatments, cell lysates were collected to evaluate the protein level of SOCS1 (A) and SOCS5 (B) by ELISA (n = 4; **, P < 0.01, compared to GTF-only group, SNK multiple comparison test).

**FIG. 8.** Determination of mRNA transcript levels of IL-10 in vitro. Rat splenocytes were cultured in six-well plates with the following treatments: (i) medium only, (ii) GTF (2 μg/well), (iii) DNA1 (1 μg/well P. gingivalis DNA), (iv) DNA5 (5 μg/well P. gingivalis DNA), (v) GTF plus DNA1, and (vi) GTF plus DNA5. Twenty-four and 48 h after treatments, total cellular RNA was isolated and reverse transcribed, and the mRNA level of IL-10 was determined by real-time PCR. Expression of GAPDH was used as an internal reference (n = 4; *, P < 0.05, and **, P < 0.01, compared to GTF-only group, SNK multiple comparison test).
tive immunity. SOCS proteins seem to play an important role in such processes (1, 16, 32). In vitro studies indicated that P. gingivalis DNA (5 μg) inhibits IL-10-mediated immune responses (16). SOCS proteins seem to play an important role in control and regulation of initiation of innate immune response through negative regulation of cytokine signaling (1). SOCS family members have been recognized to act as negative feedback regulators that are induced by cytokine signaling itself, which then shuts down the respective signaling cascade(s) (32). For example, after binding of cytokines, the respective receptors as well as signal transducer and activator of transcription (STAT) proteins are phosphorylated by Janus kinases (JAKs). STAT proteins dimerize and translocate into the nucleus, where they initiate transcription. Among the induced proteins, SOCS proteins are also transcribed and synthesized. SOCS proteins inhibit further cytokine signaling by binding to cytokine receptors and/or by inhibiting the kinases, such as JAK (4, 38). It has been demonstrated that unmethylated CpG motifs in bacterial DNA rapidly trigger an innate immune response characterized by activation of Ig and cytokine secretion and can therefore function as potent adjuvants (5, 6). These sequences are recognized by TLR-9, which is primarily expressed by B cells and plasmacytoid dendritic cells (22, 51). TLR triggering initiates activation cascades in innate immune cells but also induces synthesis of SOCS (16). Whether SOCS proteins are capable of directly blocking TLR signaling is still under debate (16, 26).

We observed a significant down-regulation of IL-10 48 h after the addition of a high dose of P. gingivalis DNA (5 μg). IL-10 is a key cytokine in regulating inflammatory responses by controlling the production and function of various other cytokines. SOCS proteins function as negative feedback regulators of IL-10 signaling through modulation of STAT activation (3, 27). Studies have suggested that SOCS1 and SOCS5 may be important inhibitors of IL-10 signaling (2, 9, 47). Our data (Fig. 6 and 8) suggest that the reduced antibody responses observed in P. gingivalis DNA-injected animals can be associated with the inhibition of IL-10 expression by SOCS1 and/or SOCS5.

In summary, whole-genomic DNA from E. coli and from periodontal disease-implicated organisms did not enhance immune response to antigens of other bacterial species but in fact suppressed responses to the bystander antigen GTF. This diminished antibody response may be attributable to SOCS inhibition of cytokine signaling and/or possible SOCS knockdown of TLR-9 signaling (16). Thus, products of periodontal bacteria and other bacteria might serve to modulate immune responses emanating from exposure to antigen presented by a number of other natural routes including local infection. Such interference with host response to dental caries-relevant antigens has the potential to reduce the effectiveness of natural or administered dental caries vaccine.

ACKNOWLEDGMENTS

This work was supported by NIH grants DE-04733 and DE-03420 from the National Institute of Dental and Craniofacial Research (NIDCR).

REFERENCES
