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Effects of periodontal therapy on serum C-reactive protein, sE-selectin, and tumor necrosis factor-α secretion by peripheral blood-derived macrophages in diabetes. A pilot study

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Background and Objective: Diabetes is associated with an increased risk for vascular disease and periodontitis. The aim of this study was to assess the effects of periodontal treatment in diabetes with respect to alterations in the pro-inflammatory potential of peripheral blood mononuclear cells.

Material and Methods: Ten patients with diabetes and moderate to severe periodontitis received full-mouth subgingival debridement. Blood samples for serum/plasma and mononuclear cell isolation were collected prior to and 4 wk after therapy. Mononuclear cells were analyzed by flow cytometry and stimulated with lipopolysaccharide or ionomycin/phorbol ester to determine the pro-inflammatory capacity of macrophages and lymphocytes, respectively.

Results: Following periodontal treatment, all patients demonstrated a significant improvement in clinical periodontal status ($p < 0.05$), despite only modest reduction in subgingival bacterial load or homologous serum immunoglobulin G titers. $CD14^+$ blood monocytes decreased by 47% ($p < 0.05$), and the percentage of macrophages spontaneously releasing tumor necrosis factor-α decreased by 78% ($p < 0.05$). There were no significant changes in the capacity of lymphocytes to secrete interferon-γ. Among a number of serum inflammatory markers tested, high-sensitivity-C-reactive protein significantly decreased by 37% ($p < 0.01$) and soluble E-selectin decreased by 16.6% ($p < 0.05$).

Conclusion: These data suggest a reduced tendency for monocyte/macrophage-driven inflammation with periodontal therapy and a potential impact on atherosclerosis-related complications in diabetic individuals.

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Periodontal diseases are chronic infections, triggered by the bacterial biofilm of dental plaque, which result in an inflammatory loss of both soft and hard tooth-supporting structures (1). Severe forms of periodontitis can result in a state of systemic inflammation characterized by high serum levels of C-reactive protein, hyper-fibrinogenemia, moderate leukocytosis, and elevated serum interleukin-1 and interleukin-6 (2–5). Epidemiologic studies suggest an association between high levels of colonization by bacteria causative for periodontitis and subclinical atherosclerosis (6), as well as a link between periodontitis and cardiovascular disease (7–9). It has been estimated that periodontitis may increase the risk of cardiovascular disease by 20% and is associated with an increase in stroke (relative risk as high as 2.85) and peripheral vascular disease (relative risk as high as 2.27) [for review see (10)].

Although these data underscore the importance of periodontal disease as a potential contributor to a state of systemic inflammation and the development or progression of vascular disease, it is not apparent that periodontal therapy has a salutary effect on cardiovascular health. Data from recent intervention studies lend credence to this possibility, as treatment of periodontal infections was reported to result in decreased levels of C-reactive protein and interleukin-6 (11–13), as well as an improvement in endothelial dysfunction (14–16). Data regarding circulating tumor necrosis factor-α levels in periodontitis are less clear, as some studies report higher levels of tumor necrosis factor-α in the serum of chronic periodontitis patients (11,17,18), which may be reduced following therapy (11,17), while other studies have not found such an effect with therapy (19–22).

Diabetes mellitus is associated with both vascular disease and periodontitis (23,24), and patients with diabetes show higher levels of markers of systemic inflammation (24–26). Salvi and coworkers reported that monocytic secretion of tumor necrosis factor-α, prostaglandin E2 and interleukin-1β, following lipopolysaccharide challenge, was significantly elevated in type 1 diabetic individuals with severe periodontitis compared to diabetic subjects with gingivitis/mild periodontitis or systemically healthy individuals with severe periodontitis (27,28). However, studies investigating whether basic periodontal therapy in patients with diabetes results in beneficial effects on the profile of systemic inflammatory markers associated with cardiovascular risk, as well as on the pro-inflammatory potential of circulating leukocytes, are lacking. In contrast, previous studies of treatment of periodontitis in diabetes have focused on a potentially beneficial effect on the level of glycemic control of these individuals (29–31), a topic that was recently addressed by a meta-analysis of data from 10 intervention trials, including 456 patients, which revealed that the decrease in hemoglobin A1c following periodontal therapy was not statistically significant (32).

The cellular source of pro-inflammatory cytokines in the serum of individuals with periodontal disease is unclear. Circulating monocytes are probably candidate sources of cytokines such as tumor necrosis factor-α, especially in light of the disproportionately high blood flow in inflamed gingival tissue (33). Ultimately, the source of macrophages infiltrating atherosclerotic plaques is bloodborne monocytes. Activated lymphocytes are also involved in atherogenesis; by elaboration of cytokines, such as interferon-γ, they may contribute to macrophage activation (34). Therefore, the pro-inflammatory potential of these cells is likely to be a major determinant of the development and/or progression of atherosclerosis-related diseases.

The aim of the present study was to assess the effects of anti-infective periodontal treatment in patients with diabetes, and to determine whether periodontal treatment leads to alterations in the pro-inflammatory potential of peripheral blood mononuclear cells.

Material and methods

Subject sample

The study protocol was approved by the Columbia University Medical Center Institutional Review Board, and written informed consent was obtained from study participants. Patients with diabetes were recruited among those attending the Columbia University Naomi Berrie Diabetes Center, who had already agreed to participate in a case-control study examining periodontal infection profiles in type 1 diabetes (35), and among individuals attending the Columbia University College of Dental Medicine Clinic for Postdoctoral Periodontics. Sixty patients with diabetes were screened. Ten patients, seven type 1 and three type 2, participated in the study. Inclusion criteria were as follows: (i) 18–70 years of age; (ii) diagnosed with diabetes for at least 3 years; and (iii) having moderate-to-severe periodontitis, defined as at least four sites with a probing depth ≥ 6 mm and concomitant attachment loss ≥ 3 mm. Subjects were excluded if they were on regular anti-inflammatory medication, or had received periodontal therapy within 1 year or systemic antibiotics within 3 mo prior to enrollment in the study. Patients in need for antibiotic prophylaxis, according to American Heart Association recommendations, were also excluded.

Clinical periodontal examination

All subjects underwent a full-mouth clinical periodontal examination at six sites per tooth (third molars excluded) carried out by a single, calibrated periodontist (author SK), using a manual periodontal probe with Williams markings and a tip diameter of 0.45 mm. The periodontal parameters included the following assessments: (i) pocket depth: the distance of the free gingival margin to the base of the probeable pocket, recorded to the nearest mm; (ii) gingival margin: location of the free gingival margin in relation to the cemento–enamel junction (positive if located apical to the cemento–enamel junction, negative if located coronal to the cemento–enamel junction), and (iii) bleeding on probing and dental plaque, assessed dichotomously. The algebraic sum of pocket depth and gingival margin was used to compute the clinical attachment level. This examination was performed prior

to periodontal therapy (baseline) and 4 wk after completion of nonsurgical periodontal therapy (follow-up).

Diabetes related variables

The following information was collected from medical records: type of diabetes and duration (years since diagnosis); insulin regimen (multiple daily insulin injections or continuous subcutaneous insulin infusion) and/or oral hypoglycemic medications; and hemoglobin A1c results over the 2 years prior to inclusion into the study.

Periodontal treatment

All subjects received instruction on oral hygiene and full-mouth subgingival debridement, using both ultrasonic and hand instruments, by a single periodontist (author SK). Treatment was completed in two sessions within a 2-wk period, during which participants used an antiseptic mouthwash (chorhexidine gluconate 0.12%) twice daily.

Isolation of mononuclear cells and flow cytometry

Fifty-milliliter blood samples were obtained by venepuncture from each participant at approximately the same time of day, between 09:00 h and 10:00 h, at baseline and follow-up. Plain and EDTA-treated tubes were used for serum and plasma isolation, respectively. Following centrifugation at 1500 *g* for 30 min, samples were aliquoted and stored at −70°C until further analyses. To obtain mononuclear cells, blood was collected in a heparinized syringe, layered on a Histopaque-1077 gradient (Sigma, St Louis, MO, USA), and centrifuged at 400 *g* for 30 min at room temperature. Cells were washed, resuspended in phosphate-buffered saline (50-μL aliquots, final concentration 2×10^7/mL), and stained on ice using combinations of the following antibodies: CD4–fluorescein isothiocyanate, CD3–phycoerythrin, CD18–fluorescein isothiocyanate, CD49d–phycoerythrin, CD154–fluorescein isothiocyanate, CD11a–phycoerythrin, CD11b–CyChrome, CD25–phycoerythrin (all from BD Pharmingen, San Diego, CA, USA); CD8–trichrome, CD14–trichrome (Caltag, Carlsbad, CA, USA); CD36–fluorescein isothiocyanate (Fitzgerald Industries International, Concord, MA, USA); or fluorescence-conjugated isotype-matched controls. For intracytoplasmic staining, cells were seeded at 2×10^6 cells/0.5 mL in RPMI-1640 supplemented with 20% autologous serum, incubated for 16 h at 37°C in a 5% CO_2 incubator, and incubated further in the presence or absence of 100 nM 4β-phorbol 12-myristate 13-acetate + 5 μM ionomycin or 100 ng/ml of lipopolysaccharide (from *Escherichia coli* O55:B5) for 6 h at 37°C; 50 μM brefeldin A was added for the final hour to block cytokine secretion. Cells were resuspended in a final volume of 100 μl and stained with either CD4–phycoerythrin (BD Pharmingen) and CD8–CyChrome (Caltag), followed by interferon-γ–fluorescein isothiocyanate (R & D Systems, Minneapolis, MN, USA) in the presence of 0.3% saponin, or CD14–Trichrome (Caltag) followed by tumor necrosis factor-α–fluorescein isothiocyanate (R & D Systems, Minneapolis, MN, USA) in the presence of 0.3% saponin, or fluorescence-conjugated isotype-matched controls. All antibodies were used according to the manufacturers' specifications and confirmed to be at saturating concentrations. Cells were fixed in 3.7% formaldehyde and analyzed using a FACSScan and CELLQUEST software (Becton Dickinson, Rockville, MD, USA). Appropriate gates, based on forward- and side-scatter criteria, were set for monocytes and lymphocytes and confirmed by CD14 and CD3 staining. Lymphocytes appeared as a homogeneous population of cells with relatively low forward and side scatter, whereas the magnitude of forward and side scatter of monocytes was considerably greater and less uniform than that of lymphocytes.

Assessment of circulating inflammatory mediators

Levels of a number of cytokines, chemokines, and biomarkers relevant to vascular disease were assessed in baseline and follow-up samples using multiplex immunoassays for Luminex technology and commercially available kits (Linco Research, Inc., St Charles, MO, USA), according to the manufacturer's instructions. For the assessment of cytokine/chemokine levels, the human cytokine 22-plex kit was used to determine serum concentrations of interleukin 1α, interleukin 1β, interleukin-2, interleukin-1 receptor antagonist, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-10, interleukin-12, interleukin-13, interleukin-15, interleukin-17, interferon-γ, granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, tumor necrosis factor-α, eotaxin, monocyte chemotactic protein-1, macrophage inflammatory protein 1α, and the chemokine IP-10. The minimum detectable concentration in this assay was 3.2 pg/mL for each analyte. For the assessment of mediators involved in the pathogenesis of vascular disease, the CVD Human Biomarker 1 kit was used, allowing determination of soluble E-selectin, soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1, matrix metalloproteinase-9, myeloperoxidase, adiponectin, and total plasminogen activator inhibitor-1. The minimum detectable concentrations for this assay were as follows: sE-selectin, 79 pg/mL; soluble vascular cell adhesion molecule-1, 16 pg/mL; soluble intercellular adhesion molecule-1, 9 pg/mL; matrix metalloproteinase-9, 1 pg/mL; myeloperoxidase, 7 pg/mL; adiponectin, 56 pg/mL; and plasminogen activator inhibitor-1, 1 pg/mL.

C-reactive protein was measured using the Cardiophase high-sensitivity-C-reactive protein assay (Dade Behring, Deerfield, IL, USA), a particle-enhanced immunonephelometric method, on a Dade Behring BNII Nephelometer. The sensitivity of this assay is 0.175 mg/L. Fibrinogen was measured quantitatively in 3.2% citrated plasma on the STA-R analyzer (Diagnostica Stago, Parsipanny, NJ, USA) by the Clauss clotting method using electro-mechanical detection for clot detection. The sensitivity of this assay is 100 mg/dL.

Table 1. Selected subject characteristics

Age, years	
Mean (SD)	48.9 (11.0)
Range	27–62
Gender	Seven men, three women
Ethnicity	Five Hispanic, five non-Hispanic
Race	Five White, two Black, three unknown
Smoking	Seven never, one former, two current
Diabetes type	Seven type 1, three type 2
Diabetes duration, years	
Mean (SD)	17.2 (10.9)
Range	3–35
Diabetes treatment regimen	Three insulin injections, five continuous subcutaneous insulin infusion, two oral hypoglycemic agents
HbA1c, %	
Mean (SD)	7.9 (1.5)
Range	6.2–11.0

HbA1c, hemoglobin A1c.

Table 2. Selected clinical periodontal characteristics at baseline and after nonsurgical periodontal treatment

	Baseline	Post-treatment	*p*-value for difference*
Percentage of sites with plaque	34.7 (39.3)	20.0 (21.8)	NS
Percentage of sites bleeding on probing	68.3 (31.2)	33.9 (24.5)	< 0.001
Pocket depth, mm	3.5 (0.5)	3.1 (0.3)	< 0.01
No. of sites with PD ≥ 5 mm	36.2 (26.2)	18.7 (8.7)	< 0.05
Percentage of sites with PD ≥ 5 mm	24.9 (15.6)	13.3 (6.0)	< 0.01
Attachment level, mm	1.6 (0.9)	0.9 (0.5)	< 0.05
No. of sites with AL ≥ 5 mm	34.7 (26.4)	17.8 (10.1)	< 0.05
Percentage of sites with AL ≥ 5 mm	23.8 (15.6)	12.7 (6.7)	< 0.01

*Two-tailed *t*-test for paired observations, degrees of freedom = 9. Data are expressed as mean values (SD).
AL, attachment loss; PD, pocket depth.

Assessment of periodontal microbiota

Subgingival plaque samples were obtained, at baseline and follow-up, from the mesiolingual surface of each tooth at the two most posterior teeth in each quadrant (eight samples per subject) using sterile curettes. The samples were analyzed by checkerboard DNA–DNA hybridization (36) using whole genomic probes from 12 periodontal bacteria including both putative pathogens and health-related species (*Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Campylobacter rectus*, *Micromonas micros*, *Eubacterium nodatum*, *Eikenella corrodens*, *Veillonella parvula*, and *Actinomyces naeslundii*). The sampling technique and the processing of the samples have been described in detail previously (37).

Assessment of serum immunoglobulin G responses to periodontal bacteria

The presence and levels of serum immunoglobulin G against all bacteria included in the microbiological panel were assessed by checkerboard immunoblotting (38) at baseline and follow-up, as described previously (37).

Data analysis

The SAS statistical analysis package (version 9.1) was used. The analyses included descriptive statistics, and the two-tailed student's *t*-test for paired observations was used to compare baseline and post-treatment clinical periodontal parameters, levels of bacteria, serum antibodies and analytes. Flow cytometry data were analyzed by means of the Wilcoxon signed-rank test, as they were not normally distributed.

Results

Sample population

Table 1 describes selected demographic and diabetes-related characteristics of the study participants. Seven patients were male, five were Hispanic, five were White, and two were current smokers. Their age ranged between 27 and 62 years (mean ± standard deviation: 48.9 ± 11 years), and their diabetes duration between 3 and 35 years (17.2 ± 10 years). Two patients used oral hypoglycemic agents only, three used daily insulin injections, and five used continuous subcutaneous insulin infusion (insulin pump). The patients' metabolic control varied; the mean hemoglobin A1c over the 2 years preceding inclusion in the study ranged from 6.2 to 11.0% (7.9 ± 1.5%).

Effect of treatment on periodontal status

Table 2 summarizes the clinical periodontal characteristics of the patients at baseline and follow-up. Apart from the statistically insignificant reduction in dental plaque levels (from 34.7% at baseline to 20.0% after treatment), all other recorded periodontal parameters demonstrated significant improvement after nonsurgical periodontal therapy. Indeed, the percentage of sites that bled on probing, the number and percentage of deep pockets (≥ 5 mm) per subject, and the number and percentage of sites with attachment loss of ≥5 mm, were reduced to approximately 50% of the pretreatment levels after therapy.

Comparison of bacterial counts, determined by checkerboard DNA–DNA hybridization in subgingival plaque samples obtained at baseline and post-treatment (Fig. 1A), revealed a reduction for the majority of the investigated species, especially for the periodontal pathogens *P. gingivalis*, *T. forsythia*, and *T. denticola*, although none of the observed changes reached statistical significance. The majority of the serum immunoglobulin G titers remained unchanged after therapy (Fig. 1B). The mean titer to *P. gingivalis* was reduced to approximately

half of the pretreatment level, but this reduction did not reach statistical significance.

Effect of treatment on peripheral blood hematologic and inflammatory parameters

Periodontal treatment had no effect on the number of white blood cells, platelets, or the percentage of neutrophils present in the peripheral blood (data not shown). Of the serum markers examined by multiplex assays (Table 3), treatment resulted in a decrease in sE-selectin ($p < 0.05$), and there was a trend for a reduction in the levels of soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1, and plasminogen activator inhibitor-1. Serum high-sensitivity-C-reactive protein levels decreased by 37% ($p < 0.01$). There was no change in plasma fibrinogen. Thus, periodontal treatment afforded a reduction in C-reactive protein and soluble E-selectin, but not in other markers of systemic inflammation in this cohort.

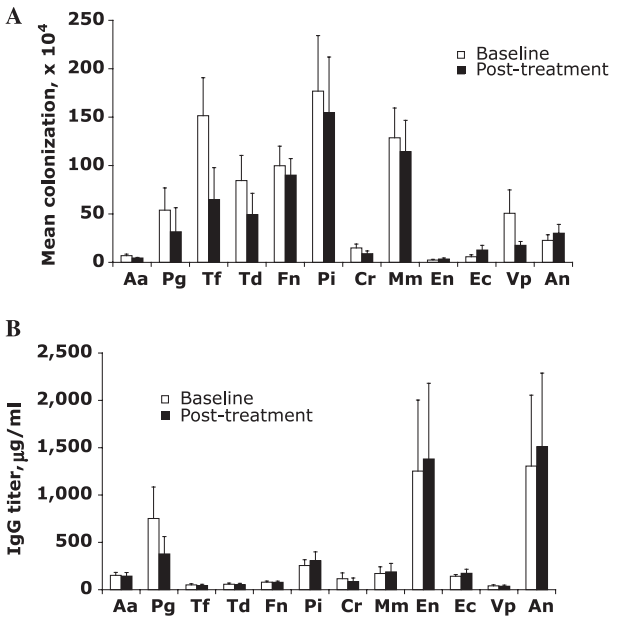

Fig. 1. (A) Mean bacterial subgingival colonization by selected bacterial species (based on eight dental plaque samples per subject) at baseline (white bars) and after nonsurgical periodontal treatment (black bars). (B) Serum immunoglobulin G responses to selected bacterial species at baseline and after periodontal treatment. Error bars represent the standard error of the mean. Aa, *Actinobacillus actinomycetemcomitans*; Pg, *Porphyromonas gingivalis*; Tf, *Tannerella forsythia*; Td, *Treponema denticola*; Fn, *Fusobacterium nucleatum*; Pi, *Prevotella intermedia*; Cr, *Campylobacter rectus*; Mm, *Micromonas micros*; En, *Eubacterium nodatum*; Ec, *Eikenella corrodens*; Vp, *Veillonella parvulla*; An, *Actinomyces naeslundii*.

Effect of treatment on the phenotype of blood mononuclear cells

The percentage of mononuclear cells that were $CD14^+$ monocytes significantly decreased following periodontal treatment (Table 4). We next examined the expression of several adhesion markers implicated in adhesion and trafficking to the endothelium underlying atherosclerotic plaques. Because the monocyte population stained uniformly positive for these antigens, and because the relative expression per cell might be expected to play a major role in influencing their adhesive behavior, we chose to express them as mean fluorescence intensities normalized to fluorescent standards. Regardless, there were no significant differences in the magnitude of mean surface expression of CD11a, CD11b, CD18, and CD49d, or for the scavenger receptor, CD36, in monocytes following treatment (Table 4). There were no changes in the percentages of lymphocytes that stained positive for CD3, CD4, and CD8, and the CD4/CD8 ratio was unchanged following therapy. CD25, a marker of lymphocyte activation, was also unaffected (Table 4).

Following an overnight culture, to allow for macrophage differentiation, we challenged the cells with lipopolysaccharide and examined the production of tumor necrosis factor-α. In unstimulated macrophages, the spontaneous production of tumor necrosis factor-α, as assessed by intracytoplasmic staining, was decreased following treatment; however, the tumor necrosis factor-α response to a maximal stimulus of lipopolysaccharide was unchanged (Fig. 2). Thus, periodontal treatment resulted in a reduction in the numbers of potentially pro-inflammatory macrophages derived from the peripheral blood. To determine the capacity of lymphocytes to synthesize interferon-γ, the principal cytokine that activates macrophages, we cultured mononuclear cells overnight (to allow for maximal capacity of interferon-γ secretion) and incubated cells in the presence or absence of 4β-phorbol 12-myristate 13-acetate/ionomycin. The production of interferon-γ was markedly increased in the presence of 4β-phorbol 12-myristate 13-acetate/ionomycin; however, there were no differences in the capacity of lymphocytes to synthesize this cytokine following periodontal treatment (Fig. 3).

Discussion

Our data demonstrated that basic nonsurgical periodontal treatment in diabetic patients with moderate to severe periodontitis results in clinically and statistically significant improvements in periodontal status, despite only a modest reduction in subgingival bacterial levels and generally marginal effects in serum immunoglobulin G titers to periodontal bacteria. Importantly, a systemic effect of periodontal therapy was evident in this setting, as a significant suppression of serum C-reactive protein and sE-selectin levels was found at follow up. Finally, the results of this study underscore the potential for peripheral blood-derived

Table 3. Effect of periodontal therapy on serum (or plasma) levels of cytokines and mediators involved in the pathogenesis of vascular disease

	Baseline	Post-treatment	*p*-value for difference*
IL-1α (ng/mL)	210.3 (286.0)	222.4 (505.5)	NS
IL-1β (ng/mL)	0.0 (0.0)	1.4 (4.6)	NS
IL-2 (ng/mL)	4.6 (8.6)	24.5 (68.6)	NS
IL-1ra (ng/mL)	207.4 (187.1)	226.7 (203.8)	NS
IL-4 (ng/mL)	212.6 (332,5)	224.0 (372.4)	NS
IL-5 (ng/mL)	0.0 (0.0)	0.0 (0.0)	NS
IL-6 (ng/mL)	79.9 (91.5)	101.9 (119.2)	NS
IL-7 (ng/mL)	11.1 (10.1)	16.8 (27.8)	NS
IL-8 (ng/mL)	39.8 (41.3)	57.8 (72.8)	NS
IL-10 (ng/mL)	14.3 (6.1)	22.0 (28.9)	NS
IL-12 p70 (ng/mL)	9.3 (17.9)	34.3 (101.0)	NS
IL-13 (ng/mL)	6.7 (10.3)	10.7 (19.1)	NS
IL-15 (ng/mL)	2.9 (5.4)	9.6 (23.1)	NS
IL-17 (ng/mL)	11.7 (18.4)	30.2 (77.1)	NS
IFN-γ (ng/mL)	1.5 (4.4)	1.2 (3.9)	NS
G-CSF (ng/mL)	89.2 (144.8)	95.1 (127.1)	NS
GM-CSF (ng/mL)	33.3 (22.2)	58.5 (98.6)	NS
TNF-α (ng/mL)	6.5 (2.6)	7.3 (3.7)	NS
Eotaxin (ng/mL)	126.7 (29.9)	138.2 (67.0)	NS
MCP-1 (ng/mL)	264.6 (96.9)	255.6 (95.7)	NS
MIP-1α (ng/mL)	32.1 (26.1)	40.7 (54.6)	NS
IP-10 (ng/mL)	132.6 (65.7)	125.5 (61.3)	NS
E-selectin (ng/mL)	27.1 (4.5)	22.6 (3.7)	< 0.05
VCAM-1 (μg/mL)	1212 (77)	1100 (65)	NS
ICAM-1 (ng/mL)	135.8 (13.8)	127.0 (12.4)	NS
MMP-9 (μg/mL)	216.9 (120.2)	260.2 (155.0)	NS
MPO (μg/mL)	125.3 (94.3)	159.6 (138.9)	NS
Adiponectin (μg/mL)	9.6 (3.8)	9.2 (3.4)	NS
PAI-1 (μg/mL)	29,061 (2214)	27,018 (2066)	NS
hs-CRP (mg/L)	2.3 (0.7)	1.5 (0.5)	< 0.01
Fibrinogen (mg/dL)	340 (21.9)	358 (35.2)	NS

*Two-tailed *t*-test for paired observations, degrees of freedom = 9. Data are expressed as mean values (SEM).
IL, interleukin; IL-1ra, interleukin 1 receptor antagonist; IFN, interferon; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1α; IP-10, interferon-inducible protein of 10 kDa; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesive molecule-1; MMP-9, matrix metalloproteinase-9; MPO, mycloperoxidase; PAI-1, plasminogen activator inhibitor-1; hs-CRP, high-sensitivity C-reactive protein.

Table 4. Effect of periodontal therapy on phenotype of peripheral blood mononuclear cells

	Baseline	Post-treatment	*p*-value for difference*
$CD14^+$ mononuclear cells, %	17.0 (6.1)	9.0 (1.2)	< 0.05
$CD11a^+$ monocytes, normalized MFI	7.6 (0.8)	8.4 (1.2)	NS
$CD11b^+$ monocytes, normalized MFI	3.6 (0.5)	2.8 (0.6)	NS
$CD18^+$ monocytes, normalized MFI	2.3 (0.3)	2.3 (0.3)	NS
$CD49d^+$ monocytes, normalized MFI	0.7 (0.1)	1.0 (0.8)	NS
$CD36^+$ monocytes, normalized MFI	5.1 (1.3)	5.3 (1.5)	NS
$CD3^+$ lymphocytes, %	60.7 (3.4)	61.8 (3.0)	NS
$CD4^+$ lymphocytes, %	40.9 (4.4)	43.7 (4.1)	NS
$CD8^+$ lymphocytes, %	25.9 (3.7)	19.9 (1.8)	NS
CD4/CD8	2.0 (0.4)	2.5 (0.4)	NS
$CD25^+$ lymphocytes, %	10.5 (3.9)	13.2 (3.3)	NS

*Wilcoxon signed-rank test. Data are expressed as mean values (SEM).
MFI, mean fluorescence intensity.

macrophages to elaborate tumor necrosis factor-α in diabetes-associated periodontitis, and also suggest that both the spontaneous production of tumor necrosis factor-α from these cells, and the number of circulating monocytes, decrease following periodontal therapy. The mechanisms underlying these changes are not apparent from the current study, and warrant further investigation. Whether the decreased number of $CD14^+$ monocytes reflects a direct systemic response to the anti-infective therapy and/or a decreased production of monocytes from bone marrow precursors, remains to be determined.

The basic, nonsurgical periodontal therapy provided within two treatment sessions had apparently only limited effects on patient behavior with respect to oral hygiene practices, and the residual levels of plaque and gingival inflammation are probable explanations for the observed nonsignificant reduction in subgingival microbial counts. The lack of any effect of therapy on serum antibodies is in accordance with our previously published observations, suggesting that immunoglobulin G titers to infecting periodontal bacteria are relatively stable in periodontitis patients, even over prolonged time periods (39). Importantly, there is no prior evidence on the time point at which systemic anti-inflammatory effects of periodontal intervention in patients with diabetes might be in effect, and the choice of a follow-up 4 wk after completion of therapy appeared a reasonable first approach. Four weeks may be somewhat early to obtain complete periodontal healing, but a longer follow-up time could lead to re-infection and/or dilute the systemic effect of the periodontal therapy we provided.

The cytokine multiplex assays revealed considerable interpatient variability in both baseline and post-treatment concentrations for the majority of analytes examined. Although the sensitivity of these assays as a limiting factor must be acknowledged, most of the inflammatory mediators assessed remained unaffected with periodontal therapy. Recent studies of periodontal therapy in sys-

temically healthy individuals have reported either no effect on circulating inflammatory mediators (19,20), or a significant reduction in a number of them (11,13,40). The discrepancy with the latter reports might be explained by the smaller number of patients involved in the present study, and methodological differences, especially the fact that we studied effects in diabetic (rather than systemically healthy) individuals, and diabetes itself causes an elevated pro-inflammatory and pro-atherogenic state. However, we did observe that nonsurgical treatment of periodontitis in diabetic individuals significantly reduced the serum levels of high-sensitivity-C-reactive protein and E-selectin. The C-reactive protein effect is important in view of prior evidence that levels of C-reactive protein are increased in diabetes (41,42) and are associated with the stage of islet cell dysfunction, insulin resistance, and elevated risk for vascular disease in diabetic individuals (43). Interestingly, even significant improvement in glycemic control appears to leave C-reactive protein levels unaffected (44,45). The same appears to be the case for E-selectin, an important marker of endothelial activation (46,47).

The effect of periodontal therapy on macrophage tumor necrosis factor-α has broad implications for inflammatory complications of diabetes, such as atherosclerosis, in which tumor necrosis factor-α may play a pathogenic role (48). The finding that serum levels of tumor necrosis factor-α were not similarly affected following therapy indicates that circulating tumor necrosis factor-α may not accurately reflect the capacity of macrophages to secrete tumor necrosis factor-α locally. As production of tumor necrosis factor-α by macrophage-derived foam cells within the atherosclerotic plaque may be more important and lead to significantly higher tissue levels compared with those found in the serum, these results are highly significant. Indeed, our findings may help to explain the apparent discordance in the literature regarding serum levels of tumor necrosis factor-α and periodontitis.

Results of the analysis of T cells in this study were less informative. Previous studies of peripheral blood T cells in periodontitis have been conflicting. One study demonstrated increased $CD3^+$ T cells, decreased $CD4^+$ T cells, and evidence of lymphocyte activation (i.e. increased CD25 expression) (49), whereas another demonstrated decreased $CD8^+$ T cells (50). Another study showed no difference in the peripheral blood T-cell phenotype of a cohort of patients with chronic periodontitis as compared with controls (51). A few studies have shown increased serum interferon-γ levels (18). However, studies have emphasized the heterogeneity in lymphocyte parameters in periodontitis patients (50), which was certainly the case in the present study. In addition, analysis of T cells derived from gingival biopsies of periodontitis patients have demonstrated a mixed pattern of T helper 1 and T helper 2 infiltration (52–54). Diabetes itself can cause alterations in lymphocyte populations derived from peripheral blood (55–57), further confounding the analysis of lymphocyte subsets in our study population.

The power of this study was limited owing to the relatively small number of patients analyzed. This consideration particularly applies to results in which trends were observed that lacked statistical significance. For example, the relative capacity of peripheral blood-derived lymphocytes to secrete inter-

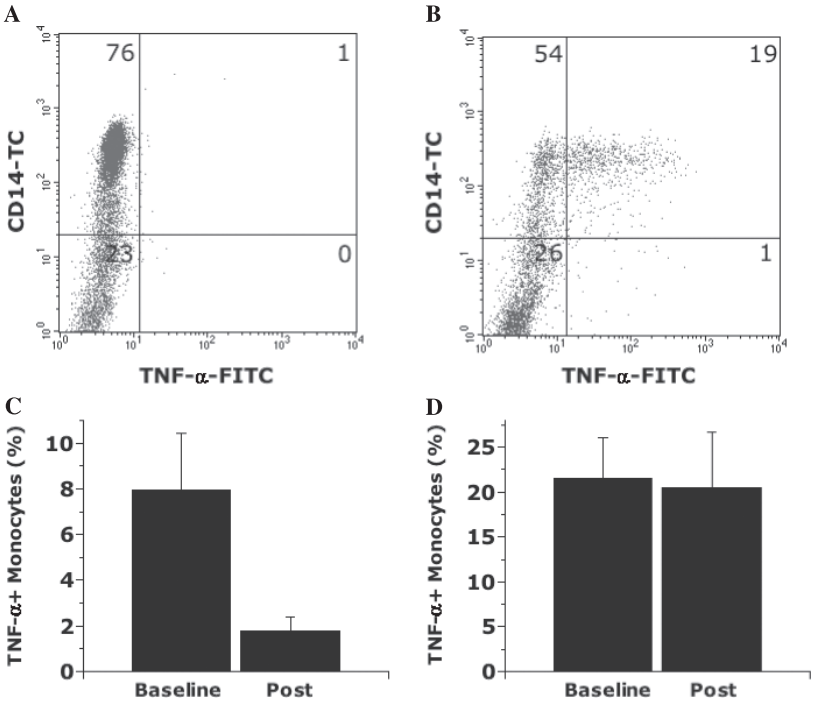

Fig. 2. Tumor necrosis factor-α production in unstimulated (A,C) and lipopolysaccharide-stimulated (B,D) macrophages. Mononuclear cells were cultured overnight and then stimulated for 6 h with 1 μg/ml of lipopolysaccharide. In the final hour, 50 μM brefeldin A was added to inhibit protein secretion, and the cells were fixed, permeabilized, and stained with tumor necrosis factor-α–fluorescein isothiocyanate and CD14–TC conjugates, and processed for flow cytometry. Typical intracytoplasmic staining profile for tumor necrosis factor-α in (A) unstimulated and (B) lipopolysaccharide-stimulated macrophages. Gating was on macrophages; numbers in the top right of each quadrant refer to the percentage of gated cells in that quadrant. In this example, mononuclear cells were derived from a patient post-treatment. In (C) note increased spontaneous tumor necrosis factor-α production in unstimulated macrophages at baseline; $p < 0.05$ (Wilcoxon signed-rank test). (D) Maximal production of tumor necrosis factor-α by lipopolysaccharide stimulation was comparable in macrophages at baseline and after periodontal treatment. Data represent the mean ± standard error of the mean, $n = 10$. FITC, fluorescein isothiocyanate; TC, trichrome; TNF-α, tumor necrosis factor-α.

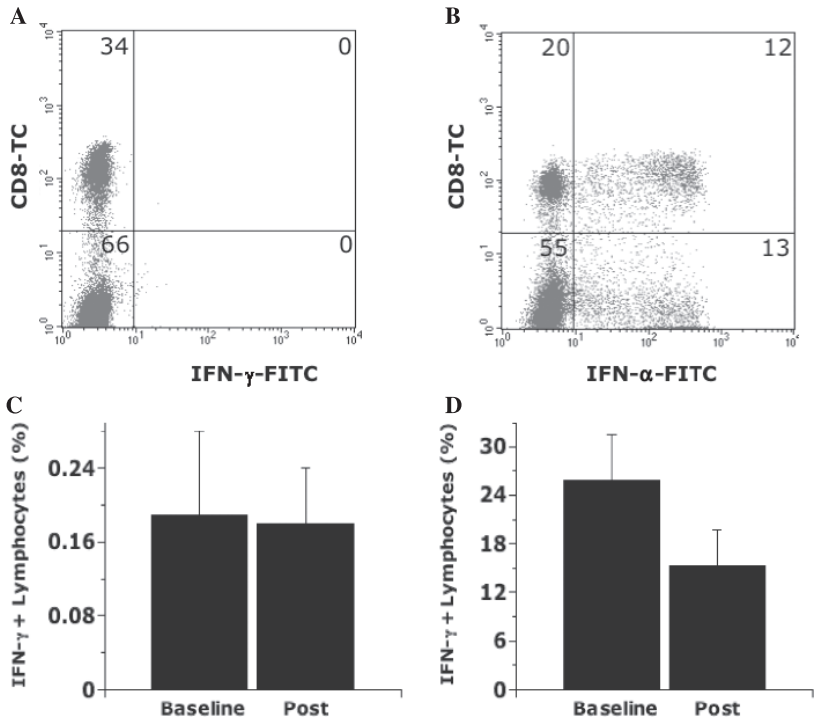

Fig. 3. Interferon-γ production in unstimulated (A,C) and 4β-phorbol 12-myristate 13-acetate/ionomycin-stimulated (B,D) lymphocytes. Mononuclear cells were cultured overnight and then stimulated for 6 h with 100 nM 4β-phorbol 12-myristate 13-acetate and 5 μM ionomycin. In the final hour, 50 μM brefeldin A was added to inhibit protein secretion, and the cells were fixed, permeabilized, and stained with interferon-γ–fluorescein isothiocyanate and either CD8–TC or CD4–phycoerythrin conjugates, and processed for flow cytometry. Typical intracytoplasmic staining profile for interferon-γ in (A) unstimulated and (B) 4β-phorbol 12-myristate 13-acetate/ionomycin-stimulated $CD8^+$ lymphocytes, respectively. Gating was on lymphocytes; numbers in the top right of each quadrant refer to the percentage of gated cells in that quadrant. In this example, mononuclear cells were derived from a patient at baseline. (C,D) No significant differences were seen in the spontaneous or 4β-phorbol 12-myristate 13-acetate/ionomycin-induced production of interferon-γ by $CD4^+$ and $CD8^+$ lymphocytes either at baseline or after periodontal therapy. Data represent the mean ± standard error of the mean, $n = 10$. FITC, fluorescein isothiocyanate; IFN-γ, interferon-γ; TC, trichrome.

feron-γ appears to have decreased following therapy. Further studies, involving a larger cohort of patients, will be required to characterize fully the pro-inflammatory potential of circulating mononuclear cells in diabetes-associated periodontitis and their response to periodontal treatment.

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