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Influence of smoking on gingival crevicular fluid cytokines in severe chronic periodontitis

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Abstract

Aim: The aim of this study was to compare the expression of 22 chemokines and cytokines in gingival crevicular fluid (GCF) from smokers and non-smokers with periodontitis and periodontally healthy control subjects.

Materials and Methods: Forty subjects with generalized severe chronic periodontitis (20 smokers and 20 non-smokers) and 12 periodontally healthy control subjects participated in this study. Four diseased and two healthy sites were selected from each of the periodontitis subjects. GCF samples were collected and cytokines analysed utilizing a multiplexed immunoassay (Luminex®). Statistical analyses employed non-parametric tests including the Mann–Whitney and Wilcoxon matched-pairs signed-rank tests.

Results: Compared with healthy control subjects, GCF in subjects with chronic periodontitis contained significantly higher amounts of interleukin (IL)-1α, IL-1β, IL-6, IL-12(p40) (pro-inflammatory cytokines); IL-8, macrophage chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, regulated on activation normal T-cell expressed and secreted (RANTES) (chemokines); IL-2, IFN-γ, IL-3, IL-4 (Th1/Th2 cytokines); IL-15 [regulator of T-cells and natural killer (NK) cells]. Smokers displayed decreased amounts of pro-inflammatory cytokines [IL-1α, IL-6, IL-12(p40)], chemokines (IL-8, MCP-1, MIP-1, RANTES), and regulators of T-cells and NK cells (IL-7, IL-15).

Conclusions: Periodontitis subjects had significantly elevated cytokine and chemokine profiles. Smokers exhibited a decrease in several pro-inflammatory cytokines and chemokines and certain regulators of T-cells and NK-cells. This reflects the immunosuppressant effects of smoking which may contribute to an enhanced susceptibility to periodontitis.

Key words: chronic periodontitis; gingival crevicular fluid; immune response; periodontal disease; periodontitis/aetiology; smoking

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Gingival crevicular fluid (GCF) in periodontitis subjects contains inflammatory cells, serum proteins, bacteria, tissue breakdown products, enzymes, antibodies, complement, and numerous inflammatory mediators (Cimasoni 1983, Armitage 1996). GCF volume increases with periodontal inflammation (Loe & Holm-Pedersen 1965, Oliver et al. 1969), its flow rate increasing up to 30-fold in periodontitis sites compared with healthy sites (Goodson 2003). Collection of GCF is relatively non-invasive, making it a convenient tool to evaluate markers of periodontal inflammation. The magnitude of inflammation can be determined by measuring pro-inflammatory and immuno-regulatory cytokines and chemokines in the GCF. Sites with clinical inflammation have elevated levels of many different cytokines and chemokines including interleukin (IL)-1, IL-6, IL-8, and tumour necrosis factor (TNF)-α that play an important role in the pathogenesis of the periodontitis (Genco 1992).

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.

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Cigarette smoking is a significant risk factor for periodontitis (Bergstrom & Preber 1994). Tobacco use induces alterations in microbial populations (Zambon et al. 1996, Umeda et al. 1998, Eggert et al. 2001, Haffajee & Socransky 2001, van Winkelhoff et al. 2001). It is well accepted that smoking alters the host response, including vascular function, neutrophil/monocyte activities, adhesion molecule expression, antibody production, as well as cytokine and inflammatory mediator release (Barbour et al. 1997, Kinane & Chestnutt 2000, Palmer et al. 2005, Ryder 2007). These changes likely contribute to the negative impact of smoking on the reparative and regenerative potential of the periodontium.

In cell culture systems, nicotine treatment increases production of IL-6 by murine osteoblasts (Kamer et al. 2006) and fibroblasts (Wendell & Stein 2001) and IL-1 by keratinocytes (Johnson & Organ 1997). Although these studies are useful in understanding mechanisms of tobacco-related periodontal destruction, nicotine is only one component of tobacco. In addition, there are a variety of cell types involved in the in vivo response to smoking. GCF provides an avenue to evaluate the multiple effects of smoking on the host response in the periodontium. The existing studies have focused on smoking's impact on a limited number of pro-inflammatory cytokines and chemokines. Smokers have elevated GCF levels of TNF-α (Bostrom et al. 1998) and IL-8 (Giannopoulou et al. 2003a, b). In other studies, smoking decreases the levels of certain cytokines in GCF, such as IL-1β, IL-1 receptor antagonist (Rawlinson et al. 2003), IL-1α (Petropoulos et al. 2004), and IL-8 (Kamma et al. 2004), although others have shown no effect on IL-1β amounts (Bostrom et al. 2000, Giannopoulou et al. 2003b). Smoking's effect on a wider spectrum of biomarkers in GCF has not been well characterized. Therefore, this study evaluated the impact of smoking on a panel of biomarkers.

GCF was obtained from smokers and non-smokers with generalized severe chronic periodontitis and periodontally healthy controls. Twenty-two different biomarkers were evaluated: Th1 cytokines [IL-2, IL-12(p70), and interferon (IFN)-γ]; Th2 cytokines (IL-3, IL-4, IL-5, IL-10, and IL-13); pro-inflammatory cytokines [IL-1α, IL-1β, IL-6, granulocyte macrophage colony stimulating factor (GM-CSF), TNF-α, and IL-12(p40)]; chemokines (IL-8, interferon inducible protein-10 (IP-10), macrophage chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, regulated on activation normal T-cell expressed and secreted (RANTES) and Eotaxin; and regulators of T- and natural killer (NK) cell activation and proliferation (IL-7 and IL-15). Through the evaluation of this extensive panel of GCF biomarkers, this study aimed to characterize the impact of smoking and periodontitis on the host response.

Materials and Methods

Study population

Fifty-two subjects, including 40 periodontally diseased subjects (20 smokers and 20 non-smokers) and 12 periodontally healthy non-smokers, participated in this study (Table 1). The mean age of all 52 subjects was 55 ± 9.6 years. All subjects were Caucasian with the exception of one Asian and one Hispanic subject. Periodontally diseased subjects had a diagnosis of generalized severe chronic periodontitis [$>30\%$ of sites with a clinical attachment level (CAL) and probing depth (PD) $\geqslant 5$ mm] (Table 2).

Subjects had not received periodontal therapy for 4 months preceding their participation in the study. Smokers were enrolled if they regularly smoked $\geqslant 20$ cigarettes/day, and non-smokers were characterized as not having smoked 100 or more cigarettes in their lifetime. Periodontally healthy subjects included non-smokers with CAL and PD $\leqslant 3$ mm and bleeding on probing (BOP) at $\leqslant 10\%$ of sites. To participate in the study, all subjects were in good general health. Subjects were excluded from participating if they were pregnant or had a history of diabetes or intake of medication, such as antibiotics and anti-inflammatory agents, due to their possible effects on the microbial flora and/or the immune or inflammatory response, for 6 months before the study.

Subject selection and data collection were performed in the Department of Periodontics at the College of Dentistry, University of Iowa. Written informed consent was obtained from each subject, and the study protocol was approved by the University of Iowa Institutional Review Board.

Site selection

Two diseased (PD and CAL $\geqslant 5$ mm with BOP) and two healthy sites (PD and CAL $\leqslant 3$ mm with no BOP) were identified in each of the 40 periodontitis subjects and two healthy sites were identified in the 12 periodontally healthy control subjects. The diseased and healthy sites in the 20 smokers were identified as SD and SH, respectively. In the 20 non-smokers, the diseased and healthy sites were designated as ND and NH, respectively (Table 2). Four healthy sites were sampled from the 20 periodontally healthy, non-smoking individuals and classified as (Con).

GCF collection

GCF collection took place at a subsequent visit following the initial examination. Before GCF sampling, the individual tooth site was isolated with cotton rolls, supragingival plaque was carefully removed and the site was gently air-dried with the air syringe. A paper strip (Periopaper, Amityville, NY, USA) was inserted into the crevice 1–2 mm for 30 s. The GCF volume was determined based on measurements made using a Periotron 8000 (Oraflow Inc., Plainview, NY, USA) as recently described (Thunell et al. 2010). In cases of visible contamination with blood, the strips were discarded and new sites sampled. Strips from each subject were placed into labelled tubes containing 300 μl 0.01 M PBS, pH 7.2 and protease inhibitor (Complete Mini, protease inhibitor cocktail tablets, Roche Applied Science, Indianapolis, IN, USA). After shaking for 20 min., the strips were removed and the eluates centrifuged for 5 min. at 5800 g to remove plaque and cellular elements. The samples were frozen at -80°C until further analysis.

Table 1. Demographic characteristics of the fifty-two subjects

	Healthy controls ($N = 12$)	Smokers ($N = 20$)	Non-smokers ($N = 20$)
Age (years, mean $\pm$ SEM)	51.2 ± 5.2	51.2 ± 7.4	61.2 ± 9.7
Female	10	8	12
Male	2	12	8

Table 2. Site characteristics (mean ± SEM) of the subjects

	NH	SH	ND	SD	Con	H (pooled)	D (pooled)
Probing depth (mm)	2.6 ± 0.6	2.8 ± 0.4	5.8 ± 0.9*	5.6 ± 0.5*	2.3 ± 0.6	2.7 ± 0.5	5.7 ± 0.8*
Recession (mm)	0.3 ± 0.7	0.3 ± 0.4	0.7 ± 0.9*	1.0 ± 1.1*	0.1 ± 0.4	0.3 ± 0.6	0.8 ± 1.0*
Attachment level (mm)	2.9 ± 0.9	3.0 ± 0.3	6.5 ± 1.3*	6.6 ± 1.3*	2.5 ± 0.7	2.9 ± 0.7	6.5 ± 1.3*
Gingival crevicular fluid (μl)	1.6 ± 1.0	1.3 ± 0.9	2.3 ± 0.8	1.9 ± 0.9#	1.1 ± 0.7	1.5 ± 1.0†	2.1 ± 0.08†,‡

*Significantly different from healthy control subjects (Con).
†Significantly different from healthy controls (Con).
‡Significantly different from healthy sites in the diseased subjects (H (pooled)).
#Significantly different from diseased sites in the non-smoking periodontitis subjects (ND).
NH, healthy sites in periodontitis non-smoking subjects; SH, healthy sites in periodontitis smoking subjects; ND, diseased sites in periodontitis non-smoking subjects; SD, diseased sites in periodontitis smoking subjects; Con, healthy control; H (pooled), healthy sites in smoking and non-smoking periodontitis subjects; D (pooled), diseased sites in smoking and non-smoking periodontitis subjects.

Table 3. *p* values of intra-group, inter-group, and pooled comparisons of pro-inflammatory cytokines

	Intra-group comparisons		Inter-group comparisons						Pooled comparisons		
	NH/ND	SH/SD	Con/NH	Con/ND	Con/SH	Con/SD	NH/SH	ND/SD	H (pooled)/D (pooled)	Con/H (pooled)	Con/D (pooled)
IL-1α	<0.0001*	0.0897	0.2425	<0.0001*	0.3986	<0.005*	0.5972	<0.05*	<0.001*	0.2408	<0.001*
IL-1β	<0.005*	<0.0001*	<0.01*	<0.0001*	<0.01*	<0.0001*	0.8749	0.2862	<0.001*	<0.01*	<0.001*
IL-6	0.0728	0.2024	<0.001*	<0.0001*	0.0767	0.6141	<0.001*	<0.001*	<0.05*	0.4365	<0.05*
IL-12(p40)	<0.01*	0.1231	<0.05*	<0.0001*	0.8974	0.1365	<0.05*	<0.001*	<0.01*	0.1708	<0.001*
GM-CSF	<0.05*	0.4954	0.7371	0.2630	0.7902	0.7453	0.5183	0.3848	0.2580	0.9792	0.3813

*p values ≤ 0.05 were considered significant.
NH, healthy sites in periodontitis non-smoking subjects; SH, healthy sites in periodontitis smoking subjects; ND, diseased sites in periodontitis non-smoking subjects; SD, diseased sites in periodontitis smoking subjects; Con, healthy control; H (pooled), healthy sites in periodontitis subjects; D (pooled), diseased sites in periodontitis subjects.

Cytokine and chemokine analysis

Th1 cytokines [IL-2, IL-12(p70), and IFN-γ], Th2 cytokines (IL-3, IL-4, IL-5, IL-10, and IL-13), pro-inflammatory cytokines [IL-1α, IL-1β, IL-6, GM-CSF, TNF-α, and IL-12(p40)], chemokines (IL-8, IP-10, MCP-1, MIP-1α, RANTES, and Eotaxin), and regulators of T- and NK cell activation and proliferation (IL-7 and IL-15) in GCF (pg/30 s) were determined as described previously (Thunell et al. 2010) using a commercial multiplexed fluorescent bead-based immunoassay (Millipore, Billerica, MA, USA) in the Luminex® 100 IS Instrument (Luminex®, Austin, TX, USA). Each sample was assayed in duplicate. Briefly, 50 μl of 0.01 M PBS, pH 7.2 containing GCF samples were incubated with anti-human multi-cytokine beads at 4°C for 18 h. Unbound material was removed by filtration. Anti-human multi-cytokine biotin reporter was added, and reactions were incubated at room temperature for 1.5 h in the dark. Streptavidin–phycoerythrin then was added, and the plates were incubated at room temperature for an additional 30 min. Stop solution was added, and the plates were read in a plate reader (Model 100 IS, Luminex®). Cytokine quantities in each sample were extrapolated based on standards utilizing Beadview software (Millipore).

Statistical analysis

Analysis of normality was conducted, and non-parametric approaches were used based on the distribution of the data. Total cytokine amounts (pg/30 s) were analysed and reported for each cytokine. Cytokine and GCF volumes were analysed using the Mann–Whitney test to compare cytokine and GCF levels. This analysis was completed for inter-group and pooled comparisons. Inter-group comparisons consisted of comparing the healthy controls (Con) to both healthy and diseased sites in smoking and non-smoking diseased subjects (SH, SD, NH, ND). Additionally, smokers and non-smokers were compared in relation to healthy and diseased sites (SH *versus* NH; SD *versus* ND). Pooled comparisons consisted of comparing the healthy controls (Con) to both healthy and diseased sites in pooled smoker and non-smoker periodontitis subjects (H pooled, D pooled). Intra-group and pooled comparisons for matched-paired groups were completed using the Wilcoxon matched-pairs signed-rank test. Intra-group matched-paired comparisons consisted of comparing healthy and diseased sites in smokers and in non-smokers (SH *versus* SD; NH *versus* ND). Pooled matched-paired comparisons consisted of comparing healthy and diseased sites in diseased subjects (H pooled *versus* D pooled). In each case the level of significance was set at $p < 0.05$ (Table 3).

In some subjects, the concentrations of cytokines were beyond the detectable capacity of the assay, which was 2.3–5000 pg/ml. For those chemokines and cytokines whose concentrations were below the detectable limit of the immunoassay, a value of 1.3 pg/ml was assigned by subtracting 1.0 from 2.3 pg/ml, the lowest value of the standard curve. When the concentrations of cytokines were higher the detectable capacity of the assay, the samples were diluted and rerun.

Results

Site characteristics among subjects

Diseased sites within the smoking and non-smoking periodontitis groups [SD,

ND, D (pooled)] had significantly ($p < 0.05$) higher values for PD, REC, and CAL compared with the sites in the healthy controls (Con) (Table 2). GCF volumes were significantly higher in both healthy and diseased sites in periodontitis subjects compared with the healthy controls. [D pooled *versus* Con; H (pooled) *versus* Con, $p = 0.0366$ and < 0.001, respectively]. GCF volumes in diseased sites of the smoking and non-smoking periodontitis subjects were also significantly higher ($p = 0.0005$) than healthy sites (D pooled *versus* H pooled). Diseased sites within smokers (SD) showed significantly lower ($p = 0.0121$) GCF volumes than diseased sites in the non-smoking population (ND).

Chemokine and cytokine concentrations

Seventeen of the 22 chemokines and cytokines assayed were detected in the GCF, including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-12(p40), IL-15, IP-10, IFN-γ, GM-CSF, MCP-1, MIP-1, RANTES, and Eotaxin. The quantities of IL-5, IL-10, IL-12(p70), IL-13, and TNF-α were below the detectable limits of the multiplex assay. Therefore, they were excluded from further analysis.

Pro-inflammatory cytokines: IL-1α, IL-1β, IL-6, IL-12(p40), GM-CSF

The quantities of pro-inflammatory cytokines ranged from 0.0 to 32,580.0 pg/30 s. The ranges for IL-1α were the broadest (ranging from 41.5 to 32,580.0 pg/30 s), followed by IL-1β (0.0 to 1662.0 pg/30 s), IL-6 (10.2 to 1632.0 pg/30 s), IL-12(p40) (7.2 to 436.2 pg/30 s), and GM-CSF (0.6 to 130.8 pg/30 s) (Fig. 1a–e). The significant differences among the chemokine and cytokine values are presented in Table 3.

Intra-group comparisons

Comparison of healthy and diseased sites in non-smokers (NH *versus* ND) showed significantly higher amounts of IL-1α, IL-1β, IL-12(p40), and GM-CSF in diseased sites (Fig. 1a, b, d, and e, Table 3). Diseased sites in the smoking population (SD) also showed significantly higher quantities of IL-1β than healthy sites (SH) (Fig. 1b).

Inter-group comparisons

Healthy sites in non-smokers with periodontitis (NH) showed significantly higher amounts of IL-1β, IL-6, and IL-12(p40) than the healthy controls (Con) (Fig. 1b–d, Table 3) Diseased sites in the non-smoking population (ND) had significantly more IL-1α, IL-1β, IL-6, and IL-12(p40) than the healthy controls (Con) (Fig. 1a–d, Table 3). Healthy sites in smokers (SH) showed significantly higher quantities of IL-1β than in healthy controls (Con) (Fig. 1b, Table 3). Diseased sites in smokers (SD) showed significantly higher values for IL-1α and IL-1β than in the healthy controls (Con) (Fig. 1a and b, Table 3). Comparison of healthy sites in smokers and non-smokers (SH *versus* NH) showed significantly less IL-6 and IL-12(p40) in the smokers (Fig. 1c and d, Table 3). Additionally, diseased sites in smokers (SD) showed significantly less IL-1α, IL-6, and IL-12(p40) than non-smokers (ND) (Fig. 1a, c and d, Table 3).

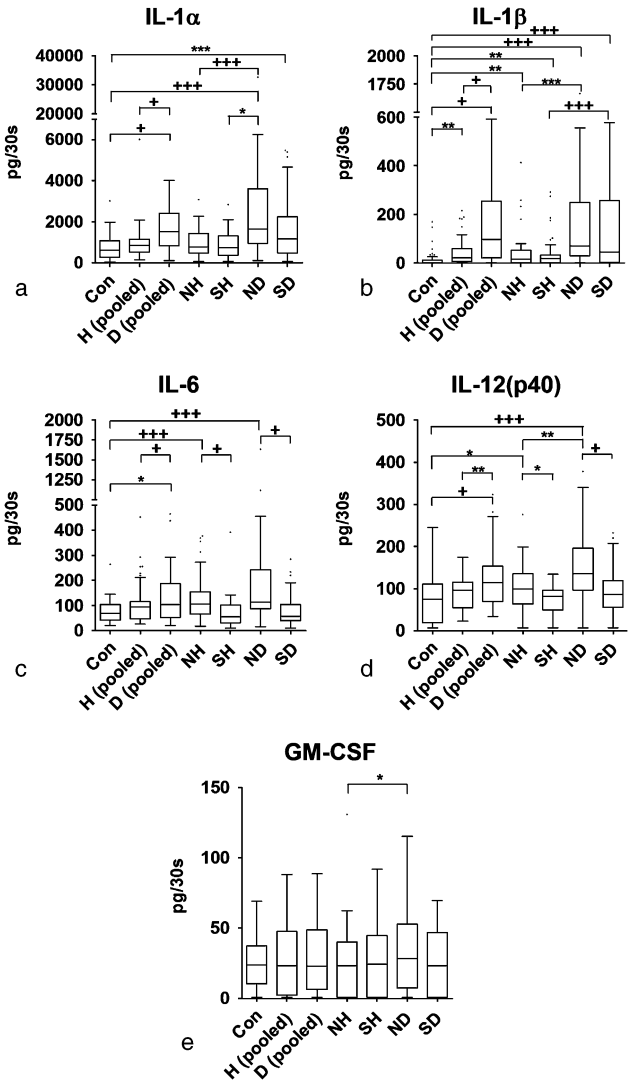

Fig. 1. Pro-inflammatory cytokines: median amounts (pg/30 s): (a) IL-1α, (b) IL-1β, (c) IL-6, (d) IL-12(p40), (e) GM-CSF. HC, healthy control; H (pooled), healthy sites in periodontitis subjects; D (pooled), diseased sites in periodontitis subjects; NH, healthy sites in periodontitis non-smoking subjects; SH, healthy sites in periodontitis smoking subjects; ND, diseased sites in periodontitis non-smoking subjects; SD, diseased sites in periodontitis smoking subjects. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.005$, $+p \leq 0.001$, $+++p \leq 0.0001$.

Pooled comparisons

Diseased sites in pooled periodontitis subjects (D pooled) demonstrated significantly higher amounts of IL-1α, IL-1β, IL-6, IL-12(p40) than healthy sites in periodontitis subjects (H pooled) (Fig. 1a–d, Table 3). When compared with healthy controls (Con), diseased sites in periodontitis subjects (D pooled) showed significantly greater quantities of IL-1α, IL-1β, IL-6, and IL-12(p40)

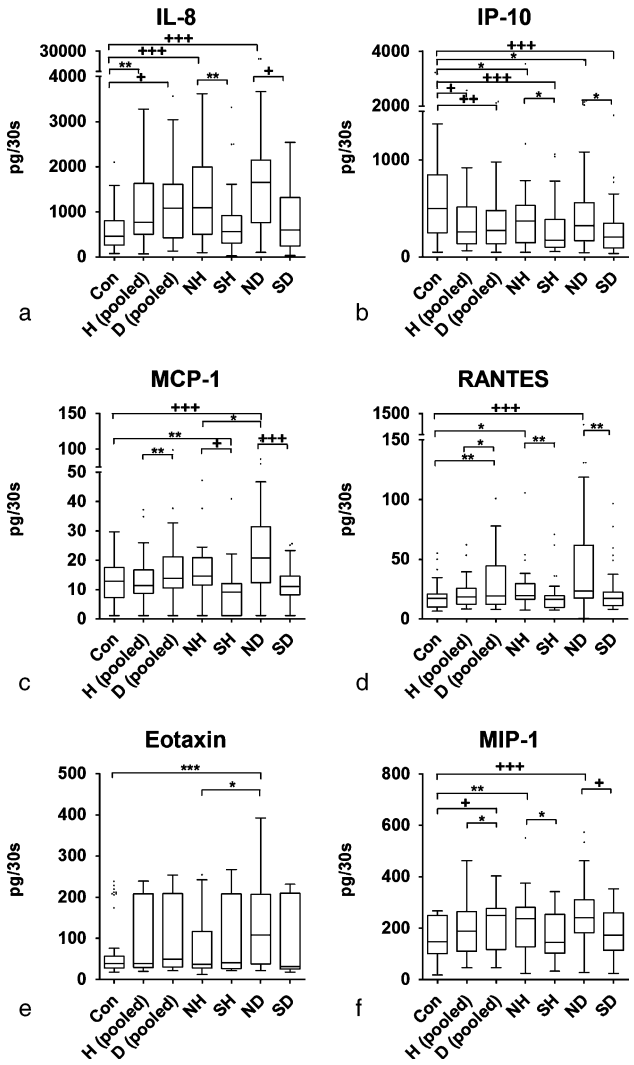

Fig. 2. Chemokines: median amounts (pg/30 s): (a) IL-8, (b) IP-10, (c) MCP-1, (d) MIP-1, (e) RANTES, (f) Eotaxin. HC, healthy control; H (pooled), healthy sites in periodontitis subjects; D (pooled), diseased sites in periodontitis subjects; NH, healthy sites in periodontitis non-smoking subjects; SH, healthy sites in periodontitis smoking subjects; ND, diseased sites in periodontitis non-smoking subjects; SD, diseased sites in periodontitis smoking subjects. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.005$, $+p \leqslant 0.001$, $++p \leqslant 0.0005$, $+++p \leqslant 0.0001$.

(Fig. 1a–d, Table 3). Healthy sites in periodontitis subjects (H pooled) also showed significantly higher IL-1β levels than that in the healthy controls (Con) (Fig. 1b, Table 3).

Chemokines: IL-8, IP-10, MCP-1, MIP-1, RANTES, and Eotaxin

Several chemokines were present in the GCF ranging in amounts from 0.6 to 24,306.0 pg/30 s. IL-8 was found in the highest amounts (ranging from 21.2 to 24,306.0 pg/30 s). The remaining chemokines had the following ranges: IP-10 (36.0–3672.0 pg/30 s); MIP-1 (18.6–573.6 pg/30 s); Eotaxin (12.0–392.4 pg/30 s); RANTES (0.6–1098.0 pg/30 s); and MCP-1 (1.2–111.6 pg/30 s). The significant differences among the chemokine and cytokine values are listed Table 4.

Intra-group comparisons

Diseased sites in non-smokers (ND) had significantly higher amounts of MCP-1 and Eotaxin than healthy sites (NH) (Fig. 2c and f, Table 4). In smokers, there were no significant differences in any of the chemokines between diseased sites (SD) when compared with healthy sites (SH) (Fig. 2a–f, Table 4).

Inter-group comparisons

Comparison of chemokine amounts in healthy sites of non-smokers (NH) compared with the healthy controls (Con) showed significantly higher values for IL-8, MIP-1, and RANTES in NH sites (Fig. 2a, d and e, Table 4). In contrast, IP-10 showed significantly lower amounts in the healthy (NH) and

Table 4. *p* values of intra-group, inter-group, and pooled comparisons of chemokines

	Intra-group Comparisons		Inter-group Comparisons						Pooled comparisons		
	NH/ND	SH/SD	Con/NH	Con/ND	Con/SH	Con/SD	NH/SH	ND/SD	H (pooled)/D (pooled)	Con/H (pooled)	Con/D (pooled)
IL-8	0.1769	0.9854	$<0.0001^{*}$	$<0.0001^{*}$	0.3364	0.3856	$<0.01^{*}$	$<0.001^{*}$	0.2734	$<0.01^{*}$	$<0.001^{*}$
IP-10	0.7562	0.8983	$<0.05^{*}$	$<0.05^{*}$	$<0.0001^{*}$	$<0.0001^{*}$	$<0.05^{*}$	$<0.05^{*}$	0.8666	$<0.001^{*}$	$<0.0005^{*}$
MCP-1	$<0.05^{*}$	0.1536	0.0703	$<0.0001^{*}$	$<0.01^{*}$	0.3054	$<0.001^{*}$	$<0.0001^{*}$	$<0.01^{*}$	0.5524	0.0649
MIP-1	0.1054	0.0826	$<0.01^{*}$	$<0.0001^{*}$	0.8359	0.2213	$<0.05^{*}$	$<0.001^{*}$	$<0.05^{*}$	0.0959	$<0.001^{*}$
RANTES	0.0583	0.3683	$<0.05^{*}$	$<0.0001^{*}$	0.4793	0.5190	$<0.01^{*}$	$<0.01^{*}$	$<0.05^{*}$	0.3726	$<0.01^{*}$
Eotaxin	$<0.05^{*}$	0.0897	0.9465	$<0.005^{*}$	0.5525	0.9719	0.5336	0.0820	0.4639	0.7495	0.0656

$^{*}p$ values $\leqslant 0.05$ were considered significant.

NH, healthy sites in periodontitis non-smoking subjects; SH, healthy sites in periodontitis smoking subjects; ND, diseased sites in periodontitis non-smoking subjects; SD, diseased sites in periodontitis smoking subjects; Con, healthy control; H (pooled), healthy sites in periodontitis subjects; D (pooled), diseased sites in periodontitis subjects.

diseased (ND) sites of non-smokers than in the healthy controls (Con) (Fig. 2b, Table 4). Comparison of chemokine amounts in diseased sites of non-smokers (ND) compared with the healthy controls (Con) showed significantly higher quantities of IL-8, MCP-1, MIP-1, RANTES, and Eotaxin in ND sites (Fig. 2a and c–f, Table 4).

Healthy (SH) and diseased (SD) sites in smokers showed significantly less IP-10 than the healthy controls (Con) (Fig. 2b, Table 4). Healthy sites in smokers also had lower amounts of MCP-1 than the healthy controls (Con) (Fig. 2c, Table 4). Healthy (SH) and diseased (SD) sites in the smoking population showed significantly less IL-8, IP-10, MCP-1, MIP-1α, and RANTES than healthy (NH) and diseased sites (ND) of non-smokers with periodontitis (Fig. 2a–e, Table 4).

Pooled comparisons

Diseased sites in pooled periodontitis subjects (D pooled) showed significantly greater amounts of MCP-1, MIP-1, and RANTES than healthy sites (H) (Fig. 2c–e, Table 4). In healthy (H) sites of periodontitis subjects, amounts of IL-8 were significantly higher than in the healthy controls (Con) (Fig. 2a, Table 4). IP-10 was significantly decreased in both H (pooled) sites and D (pooled) sites compared with healthy controls (Con) (Fig. 2b, Table 4). Diseased (D) sites in periodontitis subjects had significantly greater levels of IL-8, MIP-1, and RANTES than healthy controls (Con) (Fig. 2a, d and e, Table 4).

Th1 and Th2 cytokines: IL-2, IFN-γ, IL-3, and IL-4

Th1 and Th2 cytokines were present but generally were found in low amounts (ranging from 0.0 to 349.2 pg/30 s) for all subjects in all groups. The ranges for these cytokines were as follows: IL-2 (12.2–77.4 pg/30 s); IFN-γ (0.0–152.4 pg/30 s); IL-4 (0.6–123.0 pg/30 s), and IL-3 (22.2–349.2 pg/30 s). The GCF from the healthy control group exhibited the lowest values of each of these four cytokines (Fig. 3a–d). The significant differences among the chemokine and cytokine responses are shown in Table 5.

Intra-group comparisons

Comparison of healthy and diseased sites within smokers (SH *versus* SD) and non-smokers (NH *versus* ND) showed significantly higher amounts of IFN-γ in the diseased sites of both groups (Fig. 3b, Table 5).

Inter-group comparisons

Diseased sites in non-smokers (ND) showed significantly greater amounts of IL-2, IFN-γ IL-3, and IL-4 than in

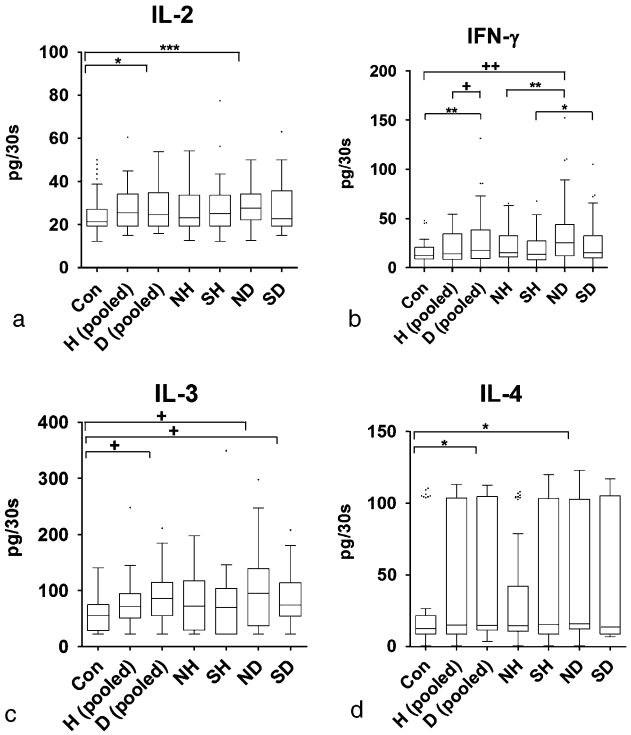

Fig. 3. Th1 and Th2 cytokines: median amounts (pg/30 s): (a) IL-2, (b) IFN-γ, (c) IL-3, (d) IL-4. HC, healthy control; H (pooled), healthy sites in periodontitis subjects; D (pooled), diseased sites in periodontitis subjects; NH, healthy sites in periodontitis non-smoking subjects; SH, healthy sites in periodontitis smoking subjects; ND, diseased sites in periodontitis non-smoking subjects; SD, diseased sites in periodontitis smoking subjects. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.005$, $+p \leqslant 0.001$, $++p \leqslant 0.0005$.

Table 5. *p* values of intra-group, inter-group, and pooled comparisons of Th1 and Th2 cytokines and regulators of T- and NK cells

	Intra-group Comparisons		Inter-group Comparisons						Pooled comparisons		
	NH/ND	SH/SD	Con/NH	Con/ND	Con/SH	Con/SD	NH/SH	ND/SD	H (pooled)/D (pooled)	Con/H (pooled)	Con/D (pooled)
IL-2	0.6226	0.4900	0.3307	$<0.005^{*}$	0.0859	0.3418	0.6380	0.1658	0.7718	0.1116	$<0.05^{*}$
IFN-γ	$<0.01^{*}$	$<0.05^{*}$	0.2133	$<0.0005^{*}$	0.7486	0.1314	0.4331	0.0802	$<0.001^{*}$	0.3687	$<0.01^{*}$
IL-3	0.4091	0.2935	0.5214	$<0.001^{*}$	0.305	$<0.05^{*}$	0.4955	0.3447	0.1650	0.1220	$<0.001^{*}$
IL-4	0.8906	0.6215	0.5214	$<0.05^{*}$	0.3645	0.2124	0.6672	0.6845	0.7377	0.3612	$<0.05^{*}$
IL-7	0.7841	0.4304	0.6034	$<0.05^{*}$	0.8451	0.6893	0.4682	$<0.01^{*}$	0.7933	0.8629	0.3039
IL-15	0.0897	0.2024	$<0.05^{*}$	$<0.0005^{*}$	0.6362	0.0877	0.0784	$<0.05^{*}$	$<0.05^{*}$	0.1157	$<0.01^{*}$

$^{*}p$ values $\leqslant 0.05$ were considered significant.

NH, healthy sites in periodontitis non-smoking subjects; SH, healthy sites in periodontitis smoking subjects; ND, diseased sites in periodontitis non-smoking subjects; SD, diseased sites in periodontitis smoking subjects; Con, healthy control; H (pooled), healthy sites in periodontitis subjects; D (pooled), diseased sites in periodontitis subjects.

the healthy controls (Con) (Fig. 3a–d, Table 5). Diseased sites in the smoking population (SD) showed significantly higher amounts of IL-3 than in the healthy controls (Con) (Fig. 3c, Table 5). Comparisons of healthy (NH *versus* SH) and diseased (ND *versus* SD) sites between smokers and non-smokers showed no significant differences in levels of any Th1 or Th2 cytokine (Fig. 3a–d, Table 5).

Pooled comparisons

Diseased sites in pooled periodontitis subjects (D pooled) which included smokers and non-smokers demonstrated significantly higher amounts of IFN-γ than in healthy sites (H pooled) (Fig. 3b, Table 3). Additionally, D (pooled) sites also showed significantly greater amounts of IL-2, IFN-γ, IL-3 and IL-4 compared with healthy controls (Con) (Fig. 3a–d, Table 3).

Regulators of T-cells and NK cells: IL-7, IL-15

The values for IL-7 in GCF ranged from 21.2 to 196.8 pg/30 s, and IL-15 ranged from 10.9 to 48.8 pg/30 s (Fig. 4a and b). The significant differences among the chemokine and cytokine responses are found in Table 3.

Intra-group comparisons

No significant differences in amounts of IL-7 or IL-15 were found when comparing healthy and diseased sites within smokers (SH *versus* SD) and non-smokers (NH *versus* ND) (Fig. 4a and b, Table 3).

Inter-group comparisons

Healthy (NH) and diseased (ND) sites in non-smokers with periodontitis showed significantly higher quantities of IL-15 than healthy controls (Con) (Fig. 4a and b, Table 3). Diseased sites in non-smokers (ND) also showed significantly higher IL-7 than healthy controls (Con) (Fig. 4a, Table 3). Comparison of cytokine amounts in diseased sites of smokers (SD) and non-smokers (ND) showed a significant decrease in IL-7 and IL-15 in smokers (Fig. 4a and b, Table 3).

Pooled comparisons

Comparison of healthy (H pooled) and diseased (D pooled) sites in pooled periodontitis subjects showed significantly higher levels of IL-15 in diseased sites (Fig. 4b, Table 3). Diseased sites in periodontitis subjects (D pooled) also showed significantly greater amounts of IL-15 when compared with healthy controls (Con) (Fig. 4b, Table 3).

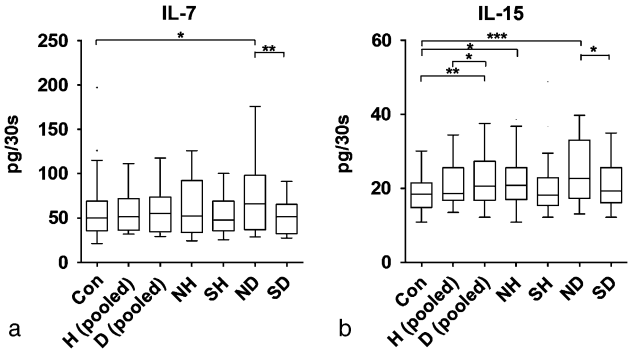

Fig. 4. Regulators of T-cells and NK cells: Median amounts (pg/30 s): (a) IL-7, (b) IL-15. HC, healthy control; H (pooled), healthy sites in periodontitis subjects; D (pooled), diseased sites in periodontitis subjects; NH, healthy sites in periodontitis non-smoking subjects; SH, healthy sites in periodontitis smoking subjects; ND, diseased sites in periodontitis non-smoking subjects; SD, diseased sites in periodontitis smoking subjects. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.005$.

Discussion

The use of the multiplex assay in this study made it possible to evaluate the levels of a comprehensive panel of cytokines and chemokines in the GCF of periodontally diseased subjects and healthy controls and the influence of smoking on these parameters. Several previous studies have evaluated GCF biomarkers in using enzyme-linked immunosorbent assays (ELISA), but a limitation is that this technology can evaluate a limited number of mediators in each sample. The most important finding of the present study was that smokers with periodontitis, as compared with non-smoking periodontitis subjects, exhibited a decrease in several pro-inflammatory cytokines, chemokines, and certain regulators of T-cells and NK cells. Consistent with this, there was a higher frequency of differences in biomarker levels between healthy and diseased sites within non-smokers than in smokers. Overall, periodontitis sites (non-smokers and smokers pooled) and diseased sites in non-smokers had significantly elevated cytokine and chemokine profiles as compared with healthy control subjects.

The impact of disease on biomarker expression

Within pooled periodontitis subjects, diseased sites as compared with healthy sites contained elevated amounts of several chemokines and pro-inflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-12(p40), MCP-1, MIP-1α, and RANTES. IFN-γ, a Th1 cytokine, and IL-15, a lymphocyte growth factor, were also elevated in these sites. All of these biomarkers as well as IL-2 IL-3, IL-4 (Th1/Th2 cytokines), and IL-8 were increased in diseased sites in periodontitis subjects relative to healthy controls.

Numerous studies have shown increased GCF amounts of pro-inflammatory cytokines in periodontitis, including IL-1 (Tsai et al. 1995, Teles et al. 2010), IL-6 (Geivelis et al. 1993), and IFN-γ (Dutzan et al. 2009). Interestingly, healthy sites in pooled periodontitis subjects exhibited increased IL-1β and IL-8 when compared with healthy control subjects. This pattern has also been reported by Teles et al. (2010) and Engebretson et al. (2002), where subjects with severe disease had higher levels of IL-1β in shallow sites compared with subjects with less severe disease. It has been suggested that this may reflect an overall patient factor such as genotype that influences host response to bacterial challenge or a pre-clinical stage of inflammation.

Although IL-12(p40) was elevated in periodontitis subjects, IL-12(p70) was below the detection level of the assay. IL-12 is a heterodimeric cytokine made up of a 40 kDa (p40) subunit and a 35 kDa (p35) subunit that comprise the bioactive form, IL-12(p70), which induces Th1 cell-mediated immunity. The IL-12(p40) subunit is shared by IL-23, another heterodimeric cytokine that has biological activities similar to,

yet distinct from, IL-12 (Belladonna et al. 2002). High amounts of the p40 component inhibit binding of the active IL-12(p70) to its receptor and therefore inhibit IL-12 bioactivity (Gately et al. 1998). Reduced levels of the bioactive form of IL-12 in GCF of periodontitis subjects have been noted by others, with a trend towards decreasing amounts from gingivitis to periodontitis (Orozco et al. 2006). A relative reduction in bioactive IL-12 could favour the predominant Th2 response observed in periodontitis. IL-15 is produced by lipopolysaccharide-stimulated fibroblasts, keratinocytes, macrophages, and endothelial cells and similar to the current study where it was increased in periodontitis sites, it has also been reported to be elevated in gingivitis sites (Buduneli et al. 2003). Other investigators, however, found that it was lower in inflamed as compared with healthy tissues (Johnson & Serio 2007).

Of the chemokines evaluated, IL-8, RANTES, MCP-1, and MIP-1α were elevated in diseased sites compared with healthy sites in pooled periodontitis subjects (smokers and non-smokers combined). This is consistent with other studies showing higher GCF IL-8 (Tsai et al. 1995, Pradeep et al. 2009, Teles et al. 2010), RANTES, and/or MCP-1 levels in patients with aggressive or chronic periodontitis as compared with healthy controls (Emingil et al. 2004, Kurtis et al. 2005, Pradeep et al. 2009). MIP-1α has not been evaluated in GCF, although it has been detected in diseased gingival tissues (Gemmell et al. 2001). In non-periodontal infections, MIP-1α and MCP-1 are consistent with monocyte infiltration that occurs during infection (Maurer & von Stebut 2004, Deshmane et al. 2009). IP-10 was the only marker that was reduced in periodontitis (in both healthy and diseased sites) when compared with healthy controls. This reduction in diseased sites consistently occurred within smokers and non-smokers, as well as between periodontitis subjects and healthy controls. This unique finding deserves further investigation. IP-10 induction is likely important in recruiting lymphocytes, cells prominent in periodontal lesions and likely participants in the adaptive immune response (Taubman & Kawai 2001). The balance among the various types of chemokines and their receptors determines the inflammatory and immune cell profile of the lesion and is proposed to play a role in the balance between disease activity and stability (Gemmell et al. 2001).

The impact of smoking on biomarker expression

In this study, smokers exhibited a decrease in several pro-inflammatory cytokines [IL-1α, IL-6, IL-12(p40)], chemokines (IL-8, IP-10, MCP-1, MIP-1α, and RANTES) and regulators of T-cells and NK cells (IL-7 and IL-15) in diseased sites as compared with diseased sites in non-smokers. To our knowledge, a significant decrease in IL-6, IL-7, IL-12(p40), IL-15, IP-10, MCP-1, MIP-1α, and RANTES within smokers has not been previously reported. In fact, the majority of these biomarkers have not been evaluated before in smokers. In contrast to the pro-inflammatory cytokines and chemokines, there were no apparent inhibitory effects of smoking on Th1 and Th2 cytokines.

Smoking's inhibition of certain pro-inflammatory cytokines in GCF is supported by other studies that have shown depressed IL-1α (Petropoulos et al. 2004) and IL-1β (Rawlinson et al. 2003) in the GCF of smokers with periodontitis compared with non-smokers with periodontitis. In contrast, other studies have shown higher GCF levels of IL-1β, IL-6, or TNF-α in smokers (Bostrom et al. 1998, 1999, Zhong et al. 2007) or no effect of smoking on these mediators (Bostrom et al. 2000, Erdemir et al. 2004, Kamma et al. 2004). Likewise, in vitro studies of the effects of nicotine or smoke on IL-1β release by various cell types have yielded conflicting outcomes (Payne et al. 1996, Johnson & Organ 1997, Bernzweig et al. 1998, Wendell & Stein 2001, Ryder et al. 2002, Kamer et al. 2006, de Heens et al. 2009). The majority of studies, however, support a depressive impact of smoking on IL-1β. These findings underscore how variables such as the model system, the stimulant (nicotine or smoke), GCF collection techniques and assays used for detection of biomarker expression contribute to the differences reported in these studies.

Regardless, we observed greater amounts of IL-1α, IL-1β, and IL-3 within diseased sites of smokers when compared with periodontally healthy controls. This relative increase in IL-1β in diseased smokers is consistent with previous research that found greater total amounts of IL-1β in smokers with ''early-onset'' periodontitis as compared with healthy, non-smoking control subjects (Kamma et al. 2004). Even though production of pro-inflammatory biomarkers is depressed in smokers, these mediators still occur at concentrations capable of pathogenesis.

IL-8 has been extensively evaluated in GCF in smokers. However, the effects of smoking vary depending on the type of periodontal disease (gingivitis, aggressive, or chronic periodontitis) (Giannopoulou et al. 2003a, b, Kamma et al. 2004). Our results demonstrate a reduction of IL-8, MIP-1α, MCP, and RANTES in smokers. The observed reduction in chemokines could contribute to the phenomena of impaired neutrophil chemotaxis and migration in the periodontium in spite of the presence of leukocytosis (Palmer et al. 2005).

Recently, others have found that exposure to nicotine suppresses the innate immune response to infection by reducing the activity of antimicrobial peptides (Radek et al. 2010). It is possible that a similar phenomenon may occur with the production of chemokines and cytokines in smokers. Such a situation would help explain the lower amount of chemokines and cytokines expressed in smokers.

Similar to other studies, smokers in this study had lower GCF volume compared with non-smokers (Apatzidou et al. 2005). One might question if this was the primary reason that smokers showed decreased levels of several of the biomarkers. The data, however, was also analysed based on concentration, which takes into consideration GCF volume, and similar findings were noted. In another study in which smokers and non-smokers had similar GCF volumes, the smokers had lower IL-1α concentrations (Petropoulos et al. 2004). Finally, Cesar-Neto and colleagues reported that both gingival tissue levels mRNA and protein expression of IL-1α, IL-10, TNF-α were depressed in smokers with periodontitis as compared with non-smokers with periodontitis (Cesar-Neto et al. 2007), further supporting a depressed host response in smokers.

There is generally good correlation between multiplex assays and ELISA's for most cytokines in biologic samples; however, the multiplex assays may either yield higher or lower quantitative values, depending on the cytokine being measured (Elshal & McCoy 2006). Therefore, trends in cytokine expression are similar when comparing the types of

assay, although actual cytokine values may not be directly comparable. Evidence suggests that these differences are minimized by the use of similar capture and reporter antibodies, diluents, and serum blockers. The inability to detect TNF-α in the present study may, in part, be related to these variables. Other investigators have also noted that TNF-α was frequently undetectable in GCF (Erdemir et al. 2004); furthermore, differences in GCF sampling techniques contribute to inter-study variation in cytokine values.

The multiplex immunoassay (Luminex®) employed in this study enabled the evaluation of a comprehensive chemokine and cytokine profile, in both smoking and non-smoking periodontitis subjects. This is the most comprehensive investigation to date of the impact of smoking and periodontitis on GCF biomarkers. A key finding was that smokers with periodontitis, as compared with non-smoking periodontitis subjects, exhibited a decrease in several pro-inflammatory cytokines, chemokines, and certain regulators of T-cells and NK cells. Compared with healthy controls, GCF in subjects with periodontitis contained significantly higher amounts of the majority of biomarkers evaluated. The non-smokers mainly accounted for these findings; compared with the smokers, they had a higher frequency of elevated biomarker quantities in diseased sites as compared with healthy controls. They also demonstrated more intra-group differences between healthy and diseased sites.

Smoking inhibited the expression of several biomarkers, including pro-inflammatory cytokines [IL-1α, IL-6, IL-12(p40)], chemokines (IL-8, MCP-1, MIP-1α, RANTES), and regulators of T-cells and NK cells (IL-7, IL-15). The Th1/Th2 cytokines were the only group of cytokines not decreased by smoking. A reduction of pro-inflammatory cytokines is consistent with lower levels of clinical inflammation in smokers. Interestingly, levels of IL-1α and IL-1β at diseased sites in smokers were still elevated relative to periodontally healthy controls, underscoring the centrality of pro-inflammatory cytokines in the pathogenesis of periodontal diseases. Chemokine levels in smokers with periodontitis were not significantly different from the healthy controls, whereas non-smokers with periodontitis had elevated chemokines relative to both smokers and the healthy controls. This suggests a major role for a diminished chemokine response in the pathogenesis of periodontitis in smokers. An inability to recruit inflammatory and immune cells could lead to an ineffective defense against periodontal pathogens and increased susceptibility to tissue destruction.

With the progression of our understanding of the pathogenesis of periodontal diseases, we are better able to identify potential diagnostic biochemical marker(s) that could be used to predict disease status and/or disease progression. Several studies have evaluated various cytokines and inflammatory mediators, intracellular and extracellular host enzymes, and byproducts of tissue breakdown as potential markers of periodontal diseases. The multi-bead array assay used in this study facilitated characterization of a broad biomarker profile in smokers and non-smokers with periodontitis. Future studies assaying these or additional cytokines using this methodology would improve our understanding of the role of the host response in periodontitis and smoking's impact in the inflammatory disease process.

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Clinical Relevance

Scientific rationale for the study: The impact of smoking on the host response in periodontitis is not fully understood.
Principal findings: Smokers with periodontitis, as compared with non-smokers with periodontitis, had decreased GCF quantities of several pro-inflammatory cytokines, chemokines and key regulators of T cell function. Within smokers and non-smokers with periodontitis, GCF from diseased sites exhibited higher levels of mediators than healthy sites.
Practical implications: Smoking suppresses the inflammatory and immune response which may contribute to increased periodontal disease susceptibility in smokers.