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ORIGINAL ARTICLE

TNF-α and IL-4 levels in generalized aggressive periodontitis subjects

MF Bastos, JA Lima, PM Vieira, MJ Mestnik, M Faveri, PM Duarte

Dental Research Division, Department of Periodontics, Guarulhos University, Guarulhos, São Paulo, Brazil

OBJECTIVES: The aim of this study was to evaluate tumor necrosis factor (TNF)-α and interleukin (IL)-4 levels in healthy sites and sites exhibiting signs of moderate and advanced generalized aggressive periodontitis (GAgP) in the same subject.
METHODS: The following sites were selected for crevicular fluid sampling in the same AgP subject ($n = 14$): Healthy sites (HS): no marginal bleeding or bleeding on probing (BOP) and probing depth (PD) ≤3 mm; Moderate sites (MS): BOP and PD between 4 and 6 mm; Advanced sites (AS): BOP and PD ≥7 mm. One site from periodontally healthy subjects ($n = 13$) was sampled for use as a control. TNF-α and IL-4 levels were measured using ELISA.
RESULTS: The total amount of TNF-α was lower for control sites, while there were no differences among healthy and diseased sites from GAgP subjects ($P < 0.05$). The concentration of TNF-α was higher in HS, in relation to the other sites ($P < 0.05$). There were no significant differences among the groups regarding total amounts of IL-4 ($P > 0.05$), while IL-4 concentration was significantly higher in control sites, when compared with sites from GAgP subjects ($P < 0.05$).
CONCLUSION: In conclusion, high levels of TNF-α and low levels of IL-4 were observed in both healthy and diseased sites within the same generalized AgP individuals.
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Keywords: aggressive periodontitis; cytokines; gingival crevicular fluid; IL-4; periodontal disease; tumor necrosis factor-α

Introduction

Aggressive periodontitis (AgP) is characterized by severe and rapid periodontal attachment and bone loss in young, systemically healthy individuals (Tonetti and Mombelli, 1999). Various studies have focused on the specific bacterial etiology in AgP to explain the severity of periodontal tissue destruction (Botero *et al*, 2007; Schacher *et al*, 2007). Other investigations have proposed that the association of a specific microbial environment and a modified host response could take place during the development of AgP (Garlet *et al*, 2003; Meng *et al*, 2007). As such, previous studies have investigated some factors that may increase host susceptibility to tissue destruction in AgP, including genetic factors (Meng *et al*, 2007), functional defects of polymorphonuclear leukocytes and monocytes (Liu *et al*, 2001; Gronert *et al*, 2004), and high levels of inflammatory mediators and cytokines (Liu *et al*, 2001; Garlet *et al*, 2003, 2004; Giannopoulou *et al*, 2003).

As with other inflammatory diseases, cytokines seem to play an important mediating role in AgP by controlling cellular interactions and functions (Liu *et al*, 2001; Garlet *et al*, 2003, 2004; Schenkein *et al*, 2007). The periodontal tissue destruction in response to bacterial infection is mediated by pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon-γ (IFN-γ), interleukin (IL)-1β, IL-12 and IL-6, while anti-inflammatory cytokines, including IL-10, IL-1 receptor antagonist and IL-4, are released in an attempt to control this destructive process (Seymour and Gemmell, 2001). TNF-α is a potent inflammatory cytokine that upregulates the production of collagenases, prostaglandin (PG) E_2, chemokines and cytokines, cell adhesion molecules and bone resorption-related factors (Wajant *et al*, 1998; Boyce *et al*, 2005). On the other hand, IL-4, secreted from T helper (Th)-2 subsets, has been found to suppress macrophage activation and downregulate the synthesis of pro-inflammatory mediators such as IL-1β, TNF-α, IL-6 and PGE_2 (te Velde *et al*, 1990).

The cytokine levels in the gingival crevicular fluid (GCF), observed in previous studies, suggest that an imbalance among pro- and anti-inflammatory mediators may take place in periodontal diseases. However, few studies have observed cytokine profiles with regard to the clinical state of sites within the same subject with generalized AgP (Toker *et al*, 2008). Therefore, the aim of the present study was to evaluate the levels of TNF-α

Correspondence: PM Duarte, Rua Dr. Nilo Peçanha, n. 81, Prédio U-6° Andar, Centro Guarulhos, CEP 07 023-070, São Paulo, Brazil. Tel: +55 11 64641769, Fax: +55 11 64641758, E-mail: pduarte@ung.br
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and IL-4 in healthy sites and sites exhibiting moderate and advanced AgP within the same subject.

Materials and methods

Patient selection and clinical examination

Fourteen generalized AgP and 13 periodontally healthy individuals, recruited from Guarulhos University, were enrolled in this cross-sectional study. Subjects were non-smokers, non-pregnant or lactant and systemically healthy. Exclusion criteria were use of antibiotics and anti-inflammatory drugs and local antimicrobial agents within the preceding 6 months. The selected subjects presented no history of periodontal treatment. This protocol was previously approved by the Institutional Committee of Ethics in Dental Research, Guarulhos University. Patients were informed of the characteristics of the study and gave their written consent to the described procedures.

Periodontal examinations were performed by the same examiner, who was trained and calibrated according to the procedures of Araujo *et al* (2003). Intra-examiner variability was 0.14 mm for probing depth (PD) and 0.16 mm for clinical attachment level (CAL). The following parameters were assessed at six sites in each teeth (mesio-buccal, medio-buccal, disto-buccal, mesio-lingual, medio-lingual, disto-lingual) using a manual periodontal probe (North Carolina–Hu-Friedy, Chicago, IL, USA): (1) Visible plaque accumulation (PI): presence or absence of plaque along the gingival margin (Ainamo and Bay, 1975); (2) Marginal bleeding (MB): presence or absence of bleeding recorded by running a probe along the gingival margin (Ainamo and Bay, 1975); (3) Bleeding on probing (BOP): presence or absence of bleeding of up to 15 s after gentle probing; (4) Suppuration (SUP): presence or absence of spontaneous SUP or SUP on probing; (5) PD (mm): distance between the gingival margin and the bottom of the sulcus/pocket; (6) CAL (mm): distance between cement-enamel junction and the bottom of the sulcus/pocket. Full-mouth periapical radiographs (Insight dental films; Eastman Kodak Company, SP, Brazil) were obtained for each subject using the paralleling technique. The radiographs were analyzed for periodontal bone loss by the same examiner using the cement-enamel junction as a reference point.

Generalized aggressive periodontitis was defined according to the clinical and radiographic criteria proposed by the 1999 World Workshop for Classification of Periodontal Diseases and Conditions (Armitage, 1999). In the present study, the AgP subjects included had at least 20 permanent teeth, with a minimum of six teeth including first molars and/or incisors with at least one site with PD and CAL ≥5 mm and a minimum of six teeth other than first molars and incisors with at least one site with PD and CAL ≥5 mm. In addition, at least one site exhibiting the following clinical features was required to be present in the same AgP individuals: Healthy sites (HS): No MB and BOP and, PD ≤3 mm; Moderate sites (MS): BOP and PD between 4 and 6 mm; Advanced sites (AS): BOP and PD ≥7 mm. One representative site presenting the above clinical characteristics was selected from each patient for GCF sampling.

Healthy individuals presented no PD and CAL measurements >3 mm and no radiographic evidence of alveolar bone loss. One site with no bleeding and plaque and PD <3 mm from these healthy individuals was included in the study as a control for immunological comparisons.

Collection of GCF

The above described sites were selected for GCF sampling. After removing the supragingival biofilm with sterile cotton pellets, the sites were isolated with cotton rolls and gently dried with an air syringe to eliminate the possibility of contamination with saliva. GCF was collected by inserting standard paper strips (Periopaper; Oraflow Inc., Smithtown, NY, USA) approximately 3 mm into the sulci/pocket for 30 s. Strips visually contaminated with blood were discarded. The GCF sample volume was measured by a calibrated Periotron 8000 (Proflow Inc., Amityville, NY, USA) and the readings were then converted to an actual volume (μl) by reference to the standard curve. The strips were immediately placed in a microcentrifuge tube containing 200 μl phosphate-buffered saline and protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA). The samples were stored at −20°C for subsequent assays.

ELISA

Aliquots of each sample were assayed by enzyme-linked immunosorbent assay (ELISA) for TNF-α and IL-4. The tubes were vortexed for 1 min and centrifuged for 10 min at 12 000 *g* in order to elute GCF components. The total amounts of TNF-α and IL-4 were determined by high-sensitive commercially available ELISA kits (Quantikine HS; R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturers' recommendations. The results were reported as total amount (pg) of TNF-α and IL-4 per site for 30 s. Sites with cytokine levels below the detection limit of assay were scored as 0 pg. Calculation of TNF-α and IL-4 concentration (pg/μl) in each GCF sample was performed by dividing the total amount of cytokines by the volume of the sample.

Statistical analysis

Statistical analysis was performed using statistical software (BioEstat 3.0; Sociedade Civil Mamirauá, CNPq, Brazil). Immunological and clinical data were examined for normality by the Kolmogorov–Smirnov test. As immunological data achieved normality, immunological comparisons among all sites selected for GCF sampling were performed using one-way ANOVA. When there were significant differences, according to one-way ANOVA, a pairwise comparison was performed using the Bonferroni test. PD, CAL and GCF volume comparisons were performed among all sites selected for GCF sampling (control, HS, MS and AS) using the nonparametric Kruskal–Wallis test. When there were significant differences, according to Kruskal-Wallis test,

a pairwise comparison was performed using the Dunn's method. The frequency of detection of visible plaque, MB, BOP, and SUP in the selected sites was sought using McNemar test. The mean percentage of six sites per tooth with visible plaque, MB, BOP and SUP, as well as the mean PD and CAL were computed for each tooth. Subsequently, the clinical data were averaged within subject and across subjects in AgP or periodontally healthy groups. The clinical comparisons between the full-mouth data from periodontally healthy and AgP subjects were sought using the Mann–Whitney test. Spearman's rank correlation was used to test the possible relationship between immunological and clinical findings. The significance level established for all analyses was 5% ($P < 0.05$).

Results

The GAgP subjects comprised six males and eight females (mean age, 23.4 ± 3.5 years) and the healthy subjects consisted of six males and seven females (mean age, 28.5 ± 5.0 years). Full-mouth clinical parameters of the periodontally healthy and GAgP individuals are presented in Table 1. At subject level, all full-mouth clinical parameters (PI, MB, BOP, SUP, PD and CAL) were significantly higher in GAgP when compared with the periodontally healthy group ($P < 0.05$).

Clinical parameters of the control and test sites selected for GCF sampling are presented in Table 2. PI was not detected in control sites, while it was higher in MS and AS, followed by HS ($P < 0.05$). According to the inclusion criteria, there was no MB or BOP at control sites and HS, while higher levels of MB and BOP were found in MS and AS. None of the sites selected for GCF sampling exhibited SUP. Means of PD and CAL were statistically higher in AS, followed by MS, HS and control sites, respectively ($P < 0.05$). In addition, GCF volumes were lower in control and HS groups when compared with the MS and AS groups, which did not differ from each other.

Table 1 Clinical parameters at subject level (full-mouth data) in generalized aggressive periodontitis (GAgP) and periodontally healthy individuals

	GAgP subjects (n = *14*)	*Periodontally healthy subjects* (n = *13*)
PI* (%)	45.1 ± 11.3	8.1 ± 3.3
MB* (%)	11.2 ± 10.8	3.2 ± 1.3
BOP* (%)	65.8 ± 14.8	1.8 ± 0.8
SUP* (%)	4.2 ± 2.48	0 ± 0
PD* (mm)	4.77 ± 0.9	2.3 ± 0.2
CAL* (mm)	4.59 ± 1.15	2.5 ± 0.3

PI, visible plaque accumulation; MB, marginal bleeding; BOP, bleeding on probing; SUP, suppuration; PD, probing depth; CAL, clinical attachment level. The mean percentage of six sites per tooth with visible plaque, MB, BOP and SUP, as well as the mean PD and CAL were computed for each tooth. Subsequently, the clinical data were averaged within subject and across subjects in GAgP or periodontally healthy groups.
*$P < 0.05$ – significant differences between groups (Mann–Whitney test).

Table 2 Clinical parameters in control sites from periodontally healthy individuals control and, healthy sites (HS), moderate sites (MS) and advanced sites (AS) from generalized aggressive periodontitis (GAgP) subjects

	Control (n = *13*)	*HS* (n = *14*)	*MS* (n = *14*)	*AS* (n = *14*)
PI*	0^a	7^b	11^c	11^c
MB*	0^a	0^a	7^b	8^b
BOP*	0^a	0^a	14^b	14^b
SUP	0	0	0	0
PD* (mm)	2.3 ± 0.48^a	3.0 ± 0.0^b	5.3 ± 0.5^c	7.8 ± 1.5^d
CAL* (mm)	2.4 ± 0.7^a	3.0 ± 0.0^b	5.6 ± 1.2^c	7.8 ± 1.5^d
GCF* (μl)	0.12 ± 0.15^a	0.17 ± 0.09^a	0.83 ± 0.5^b	1.08 ± 0.5^b

PI, visible plaque accumulation; MB, marginal bleeding; BOP, bleeding on probing; SUP, suppuration; PD, probing depth; CAL, clinical attachment level; GCF, gingival crevicular fluid. Control sites: No MB and/or BOP and, PD <3 mm in periodontally healthy subjects; Healthy sites (HS): No MB and/or BOP and, PD ≤3 mm in AgP subjects; Moderate sites (MS): BOP and PD between 4 and 6 mm in AgP subjects; Advanced sites (AS): BOP and PD ≥7 mm in AgP subjects.
PI, MB, BOP and SUP for control sites are presented as the number of sites out of the thirteen selected for GCF sampling. PI, MB, BOP and SUP for HS, MS and AS are presented as the number of sites out of the 14 selected for GCF sampling.
*$P < 0.05$ – significant difference among groups (McNemar test and Kruskal–Wallis). Different lower-case letters indicate difference between groups (McNemar and Dunn tests) ($P < 0.05$).

The total amounts (pg/site/30 s) and concentration (pg/μl) of TNF-α and IL-4 in the GCF of control and generalized AgP sites are presented in Figure 1. The total amount of TNF-α was lower for control sites while there were no differences among healthy and diseased sites from AgP subjects ($P < 0.05$). The concentration of TNF-α was higher in HS, in relation to the other sites ($P < 0.05$). There were no significant differences among the sites regarding total amounts of IL-4 ($P > 0.05$). In addition, the concentration of IL-4 was significantly higher in control sites when compared with the sites from GAgP individuals ($P < 0.05$), which were not different from each other.

Table 3 presents the correlation coefficients for immunological data and clinical parameters, based on the sites selected for GCF sampling. Statistically significant positive correlations were found between total amounts of TNF-α and BOP, PD and CAL. The concentration of TNF-α was positively correlated with PI. In addition, IL-4 concentration was negatively correlated with BOP, PD and CAL.

Discussion

Although various studies have demonstrated that a local imbalance of host immune response may lead to periodontal tissue destruction, few of them have investigated the role of cytokines in patients with AgP and, how this balance works in different stages of the disease (Garlet *et al*, 2003, 2004; Schenkein *et al*, 2007; Toker *et al*, 2008). It was hypothesized, in the present study, that healthy sites (shallow PD and no bleeding) and sites presenting different clinical status of GAgP within the same subject should present distinct profiles for pro- and

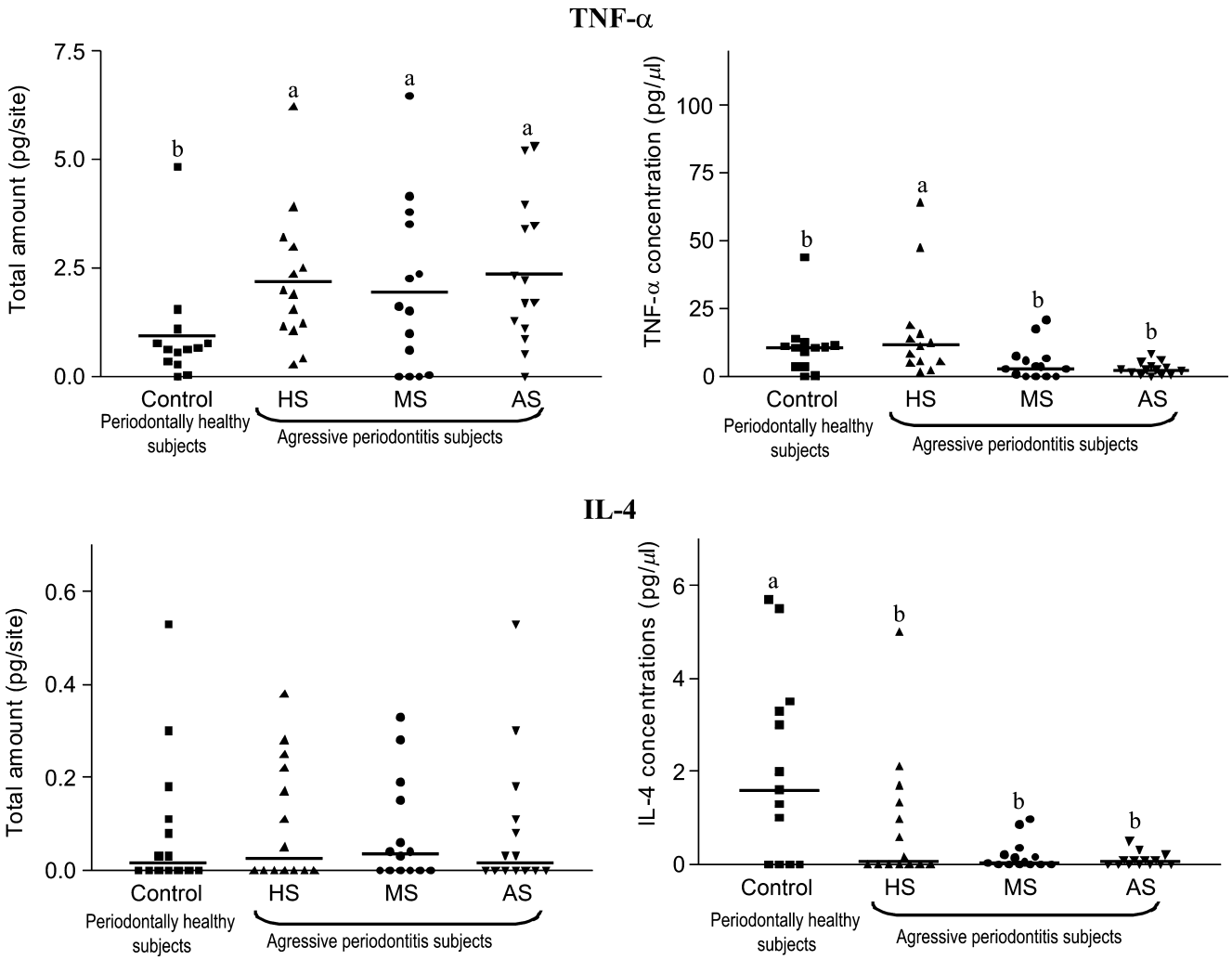

Figure 1 Distribution of the total amounts and concentrations of TNF-α and IL-4 in control sites (periodontally healthy individuals), and in healthy sites (HS), moderate sites (MS) and advanced sites (AS) from generalized aggressive periodontitis subjects. The horizontal bars show the mean value in pg/site or pg/μl. The individual dot represents the total amount or concentration of TNF-α or IL-4 at each site. Different lower-case letters indicate significant differences in the mean values of TNF-α or IL-4 between groups (ANOVA and Bonferroni test; $P < 0.05$)

anti-inflammatory cytokines. A similar study design was recently used by Toker *et al* (2008) to evaluate the levels of IL-1β (pro-inflammatory) and IL-10 (anti-inflammatory) in the GCF from GAgP subjects. Although the IL-10 levels were similar in all stages of GAgP, high levels of IL-1β were observed in moderate and deep sites compared with the shallow ones. In this study, the levels of TNF-α (pro-inflammatory) and IL-4 (anti-inflammatory) were evaluated in specific sites within the same subjects presenting clinical signs of health in some locations as well as diseased sites with moderate and advanced attachment loss.

Tumor necrosis factor-α is an important inflammatory cytokine in periodontitis, stimulating the degradation of the connective tissue matrix and bone resorption directly and indirectly (Tervahartiala *et al*, 2001; Ikezawa *et al*, 2005; Kurtiş *et al*, 2005). In this study, TNF-α total amounts were higher in sites from generalized GAgP subjects when compared with controls. In addition, TNF-α concentration was higher in healthy sites from GAgP subjects in relation to other groups (Figure 1). Furthermore, positive correlations between TNF-α and clinical parameters (i.e. BOP, PD and CAL) were more evident when total amounts were evaluated rather than concentration (Table 3). These divergences between total amount and concentration results may be attributed to the higher GCF volumes in moderate and advanced periodontitis sites when compared with healthy ones, which may result in decreased inflammatory mediator concentration in the GCF from inflamed sites. Previous studies, in which the TNF-α level in the GCF was expressed as concentration and total amount, also demonstrated a similar phenomenon (Gamonal *et al*, 2003; Ikezawa *et al*, 2005). In agreement with our results, Kurtiş *et al* (2005) demonstrated a higher total TNF-α amount in GCF from severely affected AgP sites, when compared with periodontally healthy subjects. Interestingly, both clinically healthy and diseased sites from AgP individuals presented high levels of TNF-α in our study indicating that this mediator can be

Table 3 Correlation coefficients for immunological data and clinical parameters based on the site selected for gingival crevicular fluid sampling

	TNF-α		*IL-4*	
	Total amount	*Concentration*	*Total amount*	*Concentration*
PI	0.152	0.320*	−0.014	−0.142
MB	0.092	0.251	−0.192	−0.234
BOP	0.528*	0.121	−0.154	−0.321*
PD (mm)	0.473**	0.251	−0.190	−0.378**
CAL (mm)	0.446**	0.245	−0.157	−0.345**

TNF-α, tumor necrosis factor-α; IL-4, interleukin-4; PI, visible plaque accumulation; MB, marginal bleeding; BOP, bleeding on probing; PD, probing depth; CAL, clinical attachment level.
*Correlations between TNF-α or IL-4 levels and clinical parameters at site sampled are significant at $P < 0.05$ level (Spearman rank correlation coefficients).
**Correlations between TNF-α or IL-4 levels and clinical parameters are significant at the $P < 0.01$ level (Spearman rank correlation coefficients).

expressed in sites with different clinical status. These data confirm altered inflammatory responses in diseased subjects, independently of the clinical characteristic of the site, and suggest that TNF-α may be a suitable indicator of periodontitis development. According to this hypothesis, Rossomando *et al* (1990) also demonstrated that TNF-α can be found in the GCF from periodontal sites prior to clinically observable disease. Our results from GAgP subjects are in contrast to those from a previous report using a similar experimental design for chronic periodontitis (CP), in which intra-site comparisons revealed that the total amount of TNF-α progressively rose with increasing pocket depths (Ikezawa *et al*, 2005). These divergences may be attributed to the differences between CP and AgP in relation to genetic, microbiological and immunological aspects (Garlet *et al*, 2003, 2004; Schacher *et al*, 2007; Reichert *et al*, 2008; Stein *et al*, 2008).

Although there were no statistical differences in the total amount of IL-4, the concentration of IL-4 was higher in the control sites when compared with those from GAgP individuals. In addition, an inverse correlation was noted between IL-4 concentration and BOP, PD and CAL. These data become more interesting when considering that IL-4 is a potent downregulator of macrophage function and inhibitor of the secretion of pro-inflammatory cytokines such as TNF-α (te Velde *et al*, 1990). In accordance with our results, Salvi *et al* (1998) observed that the concentration of IL-4 is very low in advanced AgP. Furthermore, Giannopoulou *et al* (2003) reported higher levels of IL-4 in GCF from healthy subjects in relation to AgP individuals, in which only >5 mm periodontal pocket was chosen for sampling. These findings confirm previous observations demonstrating that low levels of IL-4 in diseased periodontal tissues are associated with periodontal disease activity and progression (Shapira *et al*, 1992; Fujihashi *et al*, 1993; Kabashima *et al*, 1996; Giannopoulou *et al*, 2003; Tsai *et al*, 2007). It is interesting to note that there were no differences among IL-4 concentrations in healthy, moderate and advanced sites from patients with generalized AgP. Similar results, in relation to the degree of clinical or histopathological inflammation, were previously demonstrated for CP (Ukai *et al*, 2001; Tsai *et al*, 2007). Ukai *et al* (2001) demonstrated, by immunohistochemistry, that there was no significant difference in the number of IL-4-bearing cells among different histopathological stages of inflammation and PD. In addition, Tsai *et al* (2007) did not observe differences in the total amount and concentration of IL-4 between shallow and deep PD in CP.

The observed IL-4 and TNF-α profiles of healthy and diseased sites within the same AgP individuals may be related to the presence of pathogens not only in deep, but also in shallow periodontal pockets in periodontitis subjects (Socransky and Haffajee, 2005; Haffajee *et al*, 2006). The entire periodontal sites from periodontitis patients may be continually exposed to pathogenic species that could stimulate an immune response resulting in subclinical inflammation that is not detected by the available methods for clinical diagnosis. This fact reinforces the hypothesis that the commonly used clinical diagnostic procedures cannot reliably identify sites or subjects with ongoing periodontal destruction and, therefore, biochemical markers such as cytokines may be useful to assess the subclinical inflammatory process (Armitage, 1996). In addition, the similar levels of inflammatory mediators in the different sites within AgP individuals, as well as the their different amounts when compared with control sites, suggest that AgP development may be associated with host-related factors, such as genetic susceptibility, as suggested by previous investigations (for a review, see Meng *et al*, 2007).

In conclusion, high levels of TNF-α and low levels of IL-4 were observed in both healthy and diseased sites within the same generalized AgP subjects.

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Conflict of interest

There is no conflict of interest.

Author contributions

Dr. Bastos and Dr. Duarte were responsible for ELISA test, manuscript preparation and revisions. Ms. Vieira, Ms. Mestnik and Mr. Lima performed subject selection, clinical examinations and gingival crevicular fluid sampling. Dr. Faveri was responsible for the statistical analysis, tables and graphics.

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