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An Interleukin-1β (IL-1β) Single-Nucleotide Polymorphism at Position 3954 and Red Complex Periodontopathogens Independently and Additively Modulate the Levels of IL-1β in Diseased Periodontal Tissues▽

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Inflammatory cytokines such as interleukin-1β (IL-1β) are involved in the pathogenesis of periodontal diseases. A high individual variation in the levels of IL-1β mRNA has been verified, which is possibly determined by genetic polymorphisms and/or by the presence of periodontopathogens such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans*. In this study, we investigated the role of an IL-1β promoter single-nucleotide polymorphism at position 3954 [IL-1β(3954) SNP] and the presence of the periodontopathogens in the determination of the IL-1β levels in the periodontal tissues of nonsmoking chronic periodontitis (CP) patients ($n = 117$) and control (C) subjects ($n = 175$) and the possible correlations with the clinical parameters of the disease. IL-1β(3954) SNP was investigated by restriction fragment length polymorphism, while the IL-1β levels and the presence of the periodontopathogens were determined by real-time PCR. Similar frequencies of IL-1β(3954) SNP were found in the C and CP groups, in spite of a trend toward a higher incidence of T alleles in the CP group. The IL-1β(3954) SNP CT and TT genotypes, as well as *P. gingivalis*, *T. forsythia*, and *T. denticola*, were associated with higher IL-1β levels and with higher values of the clinical parameters of disease severity. Concomitant analyses demonstrate that IL-1β(3954) and the red complex periodontopathogens were found to independently and additively modulate the levels of IL-1β in periodontal tissues. Similarly, the concurrent presence of both factors was associated with increased scores of disease severity. IL-1β(3954) genotypes and red complex periodontopathogens, individually and additively, modulate the levels of IL-1β in the diseased tissues of nonsmoking CP patients and, consequently, are potentially involved in the determination of the disease outcome.

Periodontal diseases are infectious diseases in which periodontopathogens trigger chronic inflammatory and immune responses that are thought to determine the clinical outcome of the disease (26). The presence of periodontopathogens, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* (called the red complex) and *Aggregatibacter actinomycetemcomitans*, considered the major etiologic agents in periodontitis (8), triggers the expression of proinflammatory cytokines, such as interleukin-1 (IL-1), which have been associated with the immunopathology of periodontitis (14). IL-1β has been particularly studied as a critical determinant of tissue destruction due to its proinflammatory and bone resorptive properties, and indeed, increased levels of IL-1β in gingival crevicular fluid were correlated with the severity of periodontal disease (13, 16, 54).

Interestingly, variation in cytokine levels among periodontitis patients is well documented (33), and it is supposed to account for disease susceptibility. Indeed, heritable differences are reported in IL-1β production, and single-nucleotide polymorphisms (SNPs), especially the T allele in the loci of the IL-1β gene at position 3954, have been associated with increased cytokine production (3, 6, 41). Indeed, studies have indicated a role for such polymorphisms in the risk assessment for diverse inflammatory diseases due to the increased IL-1β production (3, 7, 41, 52). Regarding periodontal disease, while some studies demonstrate that subjects with the T allele in the IL-1β gene at position 3954 have an increased risk for developing severe periodontitis, other studies were not able to demonstrate such an association (12, 17, 27, 48).

In addition to the influence of the host genetic background, specific periodontopathogens may also account for higher IL-1β levels in periodontal tissues, since the red complex periodontopathogens and *A. actinomycetemcomitans* characteristically induce IL-1β expression (2, 23). However, the putative influences of these periodontopathogens and of the IL-1β promoter SNP at position 3954

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TABLE 1. Clinical features of the C and CP individual groups

Feature	C group (n = 175)[a]	CP group (n = 117)[a]	P value
Gender distribution	84 females/ 91 males	57 females/ 60 males	
Age	41.5 ± 7.38	46.4 ± 7.46	
Ethnic group			
Caucasoid	141 (80.57)	90 (76.92)	
Afro-American/mulatto	34 (19.43)	27 (23.08)	0.8475[b]
Clinical parameters			
mPD	2.09 ± 0.40	4.29 ± 0.75	<0.0001[c]
PD (sampled site)	2.28 ± 0.64	7.04 ± 1.15	<0.0001[c]
CAL (sampled site)	0	3.96 ± 1.14	
% BOP sites (mean)	4.78 ± 1.58	62.75 ± 11.76	<0.0001[c]

[a] Values are given as means ± standard deviations or as numbers (percentages), except for the gender distribution.
[b] Determined by the Fisher exact test.
[c] Determined by the unpaired t test.

[IL-1β(3954) SNP] have been investigated separately, and consequently, their exact individual and/or combined contributions to the determination of IL-1β levels remain unknown.

Therefore, in the present study we investigated the roles of the IL-1β(3954) SNP, *P. gingivalis*, *T. forsythia*, *T. denticola*, and *A. actinomycetemcomitans* in the modulation of the IL-1β mRNA levels in the periodontal tissues of chronic periodontitis (CP) patients and their possible association with the clinical parameters of disease severity.

MATERIALS AND METHODS

Study population and clinical examination. Patients and controls, from the southeastern region of Brazil, scheduled for treatment at the Dentistry School of the University of Ribeirão Preto (UNAERP) were submitted to anamnesis, clinical examination (scored for bleeding on probing [BOP], probing depth [PD], and clinical attachment loss [CAL] at six sites per tooth by one trained examiner), and radiographic examination and then were categorized into control (C) or CP groups, as previously described (11). Before the beginning of the study, all subjects signed a consent form that was approved by an institutional review board and received supragingival prophylaxis. Exclusion criteria comprised patients who did not give informed consent, patients with a significant medical history indicating evidence of known systemic modifiers of periodontal disease, and patients that had been submitted to periodontal therapy in the previous 2 years, as previously described (11). Smokers and former smokers were specifically excluded. Smoking history was checked according to a standardized questionnaire, and patients were classified as either smokers (current regular daily and occasional smokers) or nonsmokers (patients who had never smoked tobacco).

After the diagnostic phase, the CP patients (n = 117), presenting moderate to advanced periodontal disease (at least one tooth per sextant with a PD of >6 mm and a CAL of >3 mm), received basic periodontal therapy. Biopsies of gingival tissue (one sample from each patient, comprising the junctional epithelium, gingival crevicular epithelium, and connective gingival tissue) were obtained during surgical therapy (open, flat debridement performed after an incision from the gingival margin to the bottom of the periodontal pocket, allowing the collection of a biopsy sample comprising the gingival epithelium, pocket epithelium, junctional epithelium, and connective gingival tissue) of the sites that exhibited no improvement in clinical condition (i.e., persistent BOP and higher PD) 3 to 4 weeks after the basic periodontal therapy, as previously described (11). The C group (n = 175) comprised subjects presenting clinically healthy gingival tissues (under 10% BOP; no sites with a PD of >3 mm or presenting a CAL) but scheduled for restorative dentistry procedures. A representative fraction of the C group (n = 58; 24 females and 34 males; average age, 42.8 ± 7.6; 46 [79.3%] Caucasoids and 12 [20.7%] Afro-Americans/mulattos) was also scheduled for surgical procedures due to restorative/prosthetic reasons, when biopsies of healthy gingival tissue (no BOP and PD of <3 mm) were taken. The clinical features of the groups are summarized in Table 1.

Analysis of genetic polymorphisms. DNA was extracted from epithelial buccal cells with a sequential phenol-chloroform solution and precipitated with a salt/ethanol solution (46). For genotyping IL-1β(3954) SNP, DNA fragments were amplified by the use of primer pairs 5′-CTCAGGTGTCCTCGAAGAAATCAAA-3′ and 5′-GCTTTTTTGCTGTGAGTCCCG-3′ (194 bp), as previously described (34). A 15-μl aliquot of the PCR product was subjected to restriction fragment length polymorphism (RFLP) with 3 U of TacI (Promega) at 65°C overnight, and the products were resolved and separated on a 2% agarose gel stained with SYBRsafe (Invitrogen Life Technologies, Carlsbad, CA) in order to yield C (85 + 97 + 12 bp) and T (182 + 12 bp) alleles, allowing, therefore, the determination of the CC, CT, and TT genotypes.

Real-time PCRs. The extraction of total RNA from periodontal tissue samples performed with Trizol reagent (Invitrogen) and the cDNA synthesis were accomplished as previously described (9). In order to allow the detection of *P. gingivalis*, *T. forsythia*, *T. denticola*, and *A. actinomycetemcomitans*, periodontal crevice/pocket biofilm samples were collected with sterile paper point ISO #40 from the same site biopsied before the surgical procedure, as previously described (37). Bacterial DNA was extracted from plaque samples by a DNA purification system (Promega), as previously described (9, 43). Real-time PCR mRNA or DNA analyses were performed with a MiniOpticon system (Bio-Rad, Hercules, CA), using SybrGreen MasterMix (Invitrogen), specific primers, and 2.5 ng of cDNA or 5 ng of DNA in each reaction. The primer sequences and reaction properties are depicted in Table 2. For the mRNA analysis, the relative level of gene expression was calculated in reference to β-actin using the cycle threshold method. The positivity of bacteria detection in each sample was determined based on the comparison with positive and negative controls.

Statistical analysis. The possible differences in the polymorphism frequencies were assessed by the Fisher exact test, and the risk associated with genotypes/alleles was calculated as the odds ratio (OR) with 95% confidence intervals. The possible differences between the genotype subgroups of the C and CP groups were evaluated by analysis of variance (ANOVA), followed by Tukey's honestly significant difference test. Two-way ANOVA was used to verify the possible interaction between genotype and periodontopathogen presence to determine IL-1β mRNA levels. In the analysis in which genotype subgroups were created, the carriers of the polymorphic T allele (CT and TT genotypes) were grouped in order to maintain a considerable N in each subgroup, and this group was called CT+TT. A multiple logistic and linear regression analysis was performed to evaluate possible associations between the variables. P values of <0.05 were

TABLE 2. Primer sequences and reaction properties[a]

Target	Sense/antisense sequences	T_a (°C)	T_m (°C)	Amplicon size (bp)
IL-1β(3954) SNP	CTCAGGTGTCCTCGAAGAAATCAAA/GCTTTTTTGCTGTGAGTCCCG	60		194
IL-1β	GGAAGATTCTGAAGAAGAGAC/TGAGATTTTTAGAGTAACAGG	58	79	329
β-actin	ATGTTTGAGACCTTCAACA/CACGTCAGACTTCATGATGG	56	75	195
P. gingivalis	TACCCATCGTCGCCTTGGT/CGGACTAAAACCGCATACACTTG	60	84	126
T. denticola	AGAGCAAGCTCTCCCTTACCGT/TAAGGGCGGCTTGAAATAATGA	59	80	105
T. forsythia	GGGTGAGTAACGCGTATGTAACCT/ACCCATCCGCAACCAATAAA	59	79	127
A. actinomycetemcomitans	ATGCCAACTTGACGTTAAAT/AAACCCATCTCTGAGTTCTTCTTC	60	78	557

[a] T_a, annealing temperature; T_m, melting temperature.

TABLE 3. Frequencies of IL-1β(3954) SNP in C and CP groups

Genotype or allele	No. of C subjects (%)	No. of CP patients (%)	*P* value[a]	OR	Confidence interval
IL-1β genotype					
CC	120 (68.57)	71 (60.69)			
CT	40 (22.86)	30 (25.64)	0.4734	1.268	0.7262–2.213
TT	15 (8.57)	16 (13.67)	0.1646	1.803	0.8404–3.868
CC + TT	55 (31.42)	46 (39.31)	0.1699	1.414	0.8664–2.306
Allele					
C	280 (80.00)	172 (73.50)			
T	70 (20.00)	62 (26.50)	0.0698	1.442	0.9751–2.132

[a] Determined by the Fisher exact test.

considered statistically significant. The statistical tests were performed with GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

RESULTS

IL-1β(3954C/T) SNP frequency analysis in C and CP groups. The subject sample of this study was similarly composed of male and female subjects (Table 1), and the distribution of the genotypes was found to be in Hardy-Weinberg equilibrium. Regarding ethnic status, Caucasians were prevalent compared with Afro-Americans/mulatto individuals; however, no further analysis was performed based on such classification in view of the high genetic miscegenation of the Brazilian population (40). The frequencies of IL-1β(3954) genotypes and alleles were found to be similar in the CP and C groups (Table 3) and were also found to be similar to those reported for the Brazilian population in previous studies (4, 34, 56).

IL-1β(3954) genotype versus IL-1β levels and clinical parameters. In order to evaluate the putative functionality of IL-1β(3954), we next correlated it with IL-1β mRNA expression in periodontal tissues (Fig. 1). Our data show the absence or a very weak IL-1β expression in periodontal tissues of C subjects, while a significantly stronger expression was found in the tissues harvested from CP patients. No differences were found between the IL-1β mRNA levels of the different genotypes in the C group, while in the CP group, a significant higher IL-1β mRNA expression was verified for both the CT and TT groups compared with that for the CC genotype (Fig. 1A). In addition, the TT and CT+TT groups presented increased CAL and mean PD (mPD) values compared to those of the CC carriers (Fig. 1C and D). Complementarily, the IL-1β levels were positively correlated with the levels of CAL ($r = 0.3118$; $r^2 = 0.09720$; $P = 0.0006$), PD ($r = 0.4132$; $r^2 = 0.1708$; $P < 0.0001$), and mPD ($r = 0.2144$; $r^2 = 0.04597$; $P = 0.0203$). In view of the very low clinical scores and IL-1β levels seen in the C subjects and in the absence of any association of these parameters with genotypes and/or periodontopathogens in the C group, this group was not included in the subsequent figures.

Periodontopathogens versus IL-1β levels and clinical parameters. The periodontopathogens were found to be significantly more prevalent in the CP group than in the C group (Table 4), and the *P. gingivalis* counts, evaluated by means of real-time PCR (as described by Nonnenmacher et al. [37]), were found to be notably higher in CP patients (data not shown). In the CP group, *P. gingivalis*, *T. forsythia*, and *T. denticola* detection was associated with significantly higher IL-1β mRNA expression (Fig. 2A). Regarding the possible association between the clinical parameters and the presence of the red complex periodontopathogens (Fig. 2B to E), *P. gingivalis* was associated with higher CAL, PD, and mPD, while *T. forsythia* and *T. denticola* were found to be associated with higher values of PD. Positive correlations were found between the *P. gingivalis* load and IL-1β mRNA expression ($r = 0.3063$; $r^2 = 0.09383$; $P = 0.0008$) and PD ($r = 0.3472$; $r^2 = 0.1205$; $P = 0.0001$).

Complementarily, we found that the detection of only one red complex bacterial species does not result in increased levels of IL-1β, while the presence of two or three red complex species was found to be associated with increased IL-1β mRNA levels in diseased tissues (Fig. 3A). Accordingly, the presence of two or three red complex species was associated with higher PD values (Fig. 3E), while the detection of three (and a trend of two) red complex bacteria was found to be associated with significantly increased CAL (Fig. 3C). Additionally, our data demonstrate the prevalence of the simultaneous occurrence of red complex periodontopathogen species in CP patients (40 [34.2%] were negative to the red complex, 19 [16.2%] were positive to one of the species, 29 [24.8%] presented two red complex species, and 44 [37.6%] presented all three). Since IL-1β(3954) SNP and red complex-positive patients presented both higher IL-1β levels and deeper pockets with more CAL, one may argue that the increased IL-1β levels are a consequence of the deeper pockets and not due the presence of the SNP and/or specific bacteria. Therefore, we next paired the periodontitis patients (CC versus CT+TT and red complex-positive versus -negative) into clinically homogeneous subgroups (similar BOP, CAL, and PD values) through the exclusion of the patients whose clinical parameters were set at the extremes of the lower and upper quartiles. After the sample pairing, the statistical analysis demonstrated that IL-1β levels remained significantly higher in the CT+TT carriers than in the CC group ($P < 0.05$) (data not shown). Similarly, in these clinically matched subgroups, the presence of red complex bacteria was associated with increased IL-1β levels ($P < 0.05$) (data not shown). *A. actinomycetemcomitans*, whose prevalence was found to be low in the CP group, was not associated with IL-1β levels nor with the clinical parameters evaluated.

IL-1β(3954) genotype and periodontopathogens versus IL-1β mRNA expression. In order to investigate the individual

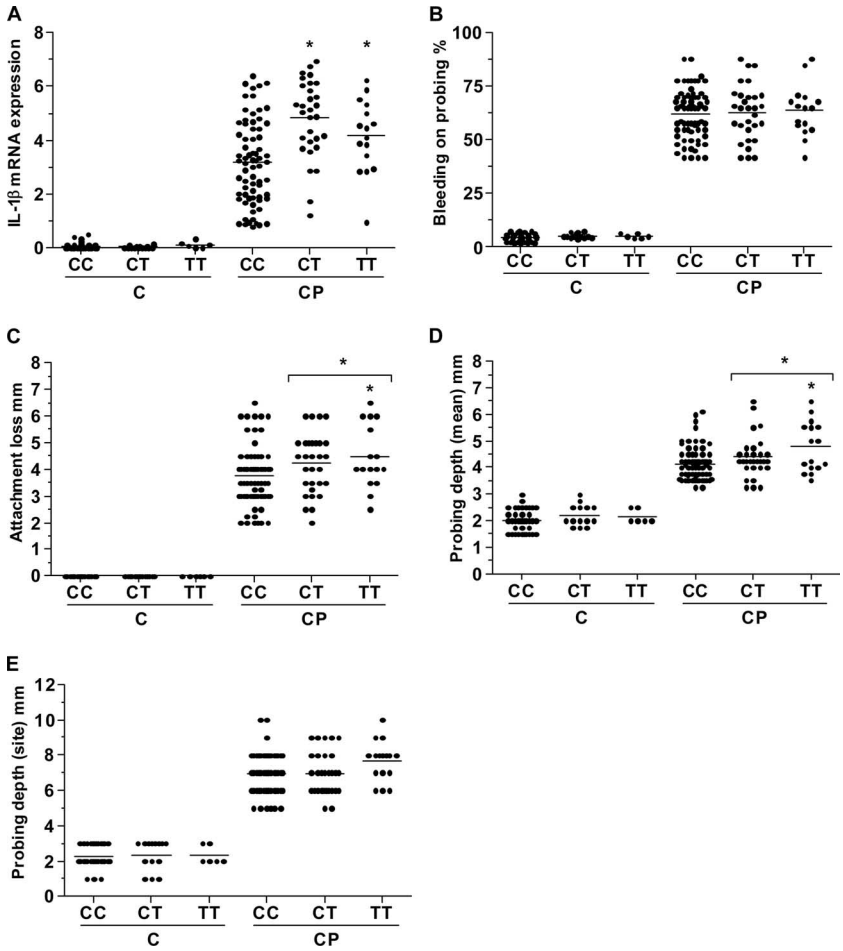

FIG. 1. IL-1β(3954) SNP genotypes: IL-1β expression and association with the clinical parameters of disease severity. C subjects and CP patients were subjected to a periodontal examination, and the genotype of IL-1β(3954) SNP was determined by RFLP. Total RNA was extracted from gingival tissues, and the levels of IL-1β mRNA were measured quantitatively by real-time PCR. The results are presented as an expression of the individual mRNAs, with normalization to β-actin. The graphs depict the expression of IL-1β and the clinical parameters of disease severity (A), BOP (B), CAL (C), mPD (D), and site PD (E) in the C and CP patients regarding their IL-1β(3954) SNP genotype. All CP patient genotype subgroups were significantly different from the C group. *, P value of <0.05 versus that of the CC genotype in the CP group.

roles of IL-1β(3954) SNP and the periodontopathogens in the determination of IL-1β levels in diseased periodontium, the CP patients were then clustered according to IL-1β(3954) genotype concurrently with the presence/absence of each target bacterium (Fig. 4). Our results demonstrate that in the absence of *P. gingivalis*, *T. forsythia*, or *T. denticola* (analyzed individually or as a complex), the presence of the T allele was associated with higher IL-1β levels. Similarly, in the presence of the red complex bacteria, the T allele was also associated with higher IL-1β levels, the simultaneous occurrence of these periodontopathogens and the polymorphic T allele being associated with the highest IL-1β levels seen in our sample. Interestingly, red complex-negative subjects bearing the T allele present similar levels of IL-1β to those of red complex-positive patients with the nonpolymorphic CC genotype. In accordance, a two-way ANOVA showed that red complex bacteria (6.12 to 10.42% of the total variation) and the IL-1β genotype (17.68 to 21.57% of the total variation) are significant sources of variation, but no interactions between these factors were verified in such an analysis, demonstrating that their coexistence results in an additive effect on IL-1β mRNA expression. The presence of *A. actinomycetemcomitans* was not found to modify the levels of IL-1β in the different genotype groups or to present associations with the clinical parameters evaluated. Complementarily, multiple regression analysis demonstrated that IL-1β expression is age ($P = 0.455$; OR = 0.85), race ($P = 0.515$; OR = 1.0), and gender ($P = 0.766$; OR = 1.2) independent, being only associated with the red complex periodontopathogens ($P = 0.008$; OR = 3.4) and with the IL-1β(3954) genotype ($P = 0.012$; OR = 3.4). Therefore, our results demonstrate that the occurrence of both the IL-1β(3954) SNP and red complex periodontopathogens can result in increased levels of IL-1β in diseased periodontal tissues and that an additive effect was verified when both factors were presented simultaneously.

Genotype versus periodontopathogen frequency and load. In order to investigate if differential responsiveness due to distinct IL-1β genotypes could interfere with the colonization of the oral cavity by periodontopathogens, we next evaluated the frequency of *P. gingivalis*, *T. forsythia*, *T. denticola*, and *A. actinomycetemcomitans* in the different IL-1β(3954) genotypes (Table 4). No

TABLE 4. Frequencies of periodontopathogen detection in C subjects and CP patients regarding their IL-1β(3954) genotype

Periodontopathogen and genotype	C subjects (n = 58)				CP patients (n = 117)				C vs CP groups[b]	
	Negative [no. (%)]	Positive [no. (%)]	P value[a]	OR[a]	Negative [no. (%)]	Positive [no. (%)]	P value[a]	OR[a]	P value	OR
P. gingivalis	50 (86.2)	8 (13.8)			43 (36.8)	74 (63.2)			<0.0001	10.756
CC	32 (84.2)	6 (15.8)	0.7015	0.5926	26 (36.6)	45 (63.4)	1.0000	0.9856		
CT+TT	18 (90.0)	2 (10.0)			17 (37.0)	29 (63.0)				
T. forsythia	53 (91.4)	5 (8.6)			49 (41.9)	68 (58.1)			<0.0001	14.71
CC	34 (89.5)	4 (10.5)	0.6502	0.4474	31 (43.7)	40 (56.3)	0.7028	1.206		
CT+TT	19 (95.0)	1 (5.0)			18 (39.1)	28 (60.9)				
T. denticola	51 (87.9)	7 (12.1)			50 (42.7)	67 (57.3)			<0.0001	9.763
CC	34 (89.5)	4 (10.5)	0.6828	1.500	28 (39.4)	43 (60.6)	0.4450	0.7104		
CT+TT	17 (85.0)	3 (15.0)			22 (47.8)	24 (52.2)				
A. actinomycetemcomitans	56 (96.6)	2 (3.4)			98 (83.8)	19 (16.2)			0.0134	5.429
CC	36 (94.7)	2 (5.3)	0.5402	0.3561	60 (83.3)	12 (16.7)	1.0000	0.9211		
CT+TT	20 (100.0)	0 (0.0)			38 (84.4)	7 (15.6)				

[a] The Fisher exact test was performed to assess the odds of periodontopathogen detection in the different genotype subgroups (CC versus CT+TT).
[b] The Fisher exact test was performed to assess the odds of periodontopathogen detection in the C and CP groups.

significant differences were found in the frequency of *P. gingivalis*, *T. forsythia*, *T. denticola*, and *A. actinomycetemcomitans* in the different genotype groups of the C subjects and CP patients. In addition, no differences were found among the *P. gingivalis* counts for the different genotype groups (data not shown). Interestingly, *A. actinomycetemcomitans* was not detected in any of the TT carriers from the CP group, but this finding did not reach statistical significance.

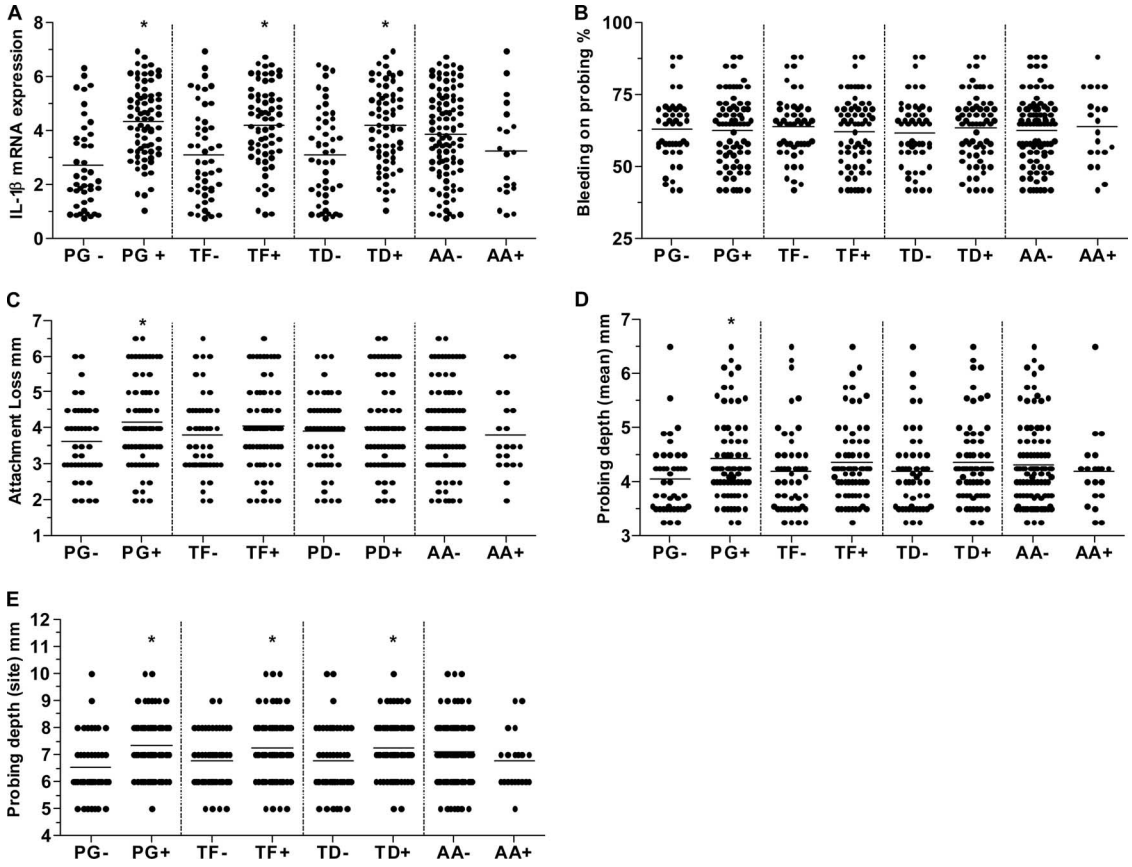

FIG. 2. Association between the presence of periodontopathogens and the expression of IL-1β mRNA and the clinical parameters of periodontal disease severity. CP patients were subjected to a periodontal examination, the total RNA was extracted from gingival tissues, and the levels of IL-1β mRNA were measured quantitatively by real-time PCR. The presence of the periodontopathogens *P. gingivalis* (PG), *T. forsythia* (TF), *T. denticola* (TD), and *A. actinomycetemcomitans* (AA) was investigated by real-time PCR. The graphs depict the expression of IL-1β and the clinical parameters of disease severity (A), BOP (B), CAL (C), mPD (D), and site PD (E) in CP patients regarding their positiveness for each bacterial detection. *, $P < 0.05$ (t test).

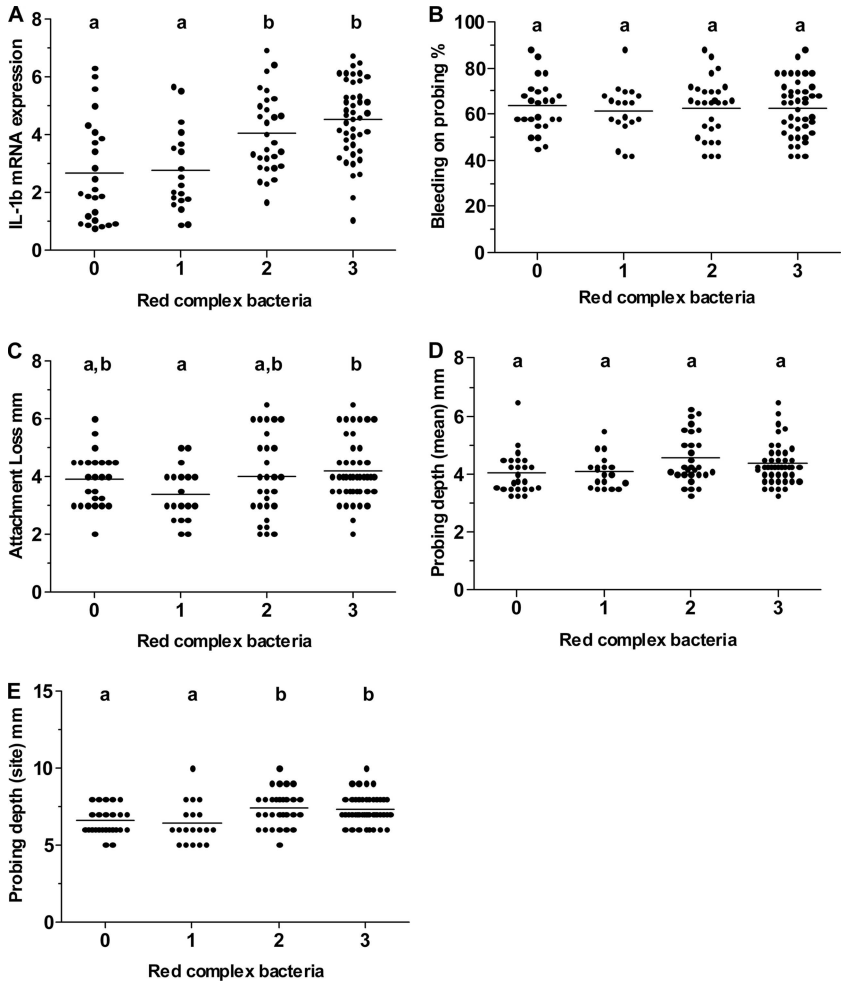

FIG. 3. Red complex periodontopathogens: association with IL-1β mRNA and with the clinical parameters of disease severity in CP patients. CP patients were subjected to a periodontal examination, and the genotype of IL-1β(3954) SNP was determined by RFLP. Total RNA was extracted from gingival tissues, and the levels of IL-1β mRNA were measured quantitatively by real-time PCR. The results are presented as the expression of the individual mRNAs, with normalization to β-actin, using the cycle threshold method. The presence of the red complex periodontopathogens *P. gingivalis*, *T. forsythia*, and *T. denticola* was investigated by real-time PCR. The graphs depict the expression of IL-1β (A) and the clinical parameters of disease severity, BOP (B), CAL (C), mPD (D), and site PD (E), in CP patients regarding the presence of zero, one, two, or three red complex periodontopathogens and also regarding their IL-1β(3954) SNP genotype concomitantly with red complex periodontopathogen detection. *, $P < 0.05$ (by ANOVA), where different letters indicate statistical significance.

DISCUSSION

Periodontal diseases are infectious diseases in which the chronic host responses raised against periodontopathogens trigger tissue destruction. The levels of the proinflammatory cytokine IL-1β are characteristically increased in diseased periodontal tissues and are thought to be a critical determinant of periodontitis outcome (13, 14, 16, 54). In accordance, our data demonstrate a higher IL-1β expression in the periodontal tissues of CP patients compared to that of the C group, but the factors involved in the determination of the IL-1β levels in diseased tissues remain unknown. The IL-1β(3954) SNP is associated with increased IL-1β synthesis in different inflammatory conditions (3, 6, 41), and specific periodontopathogens may also account for higher IL-1β levels in periodontal tissues (2, 23). However, the putative influence of periodontopathogens and genetic polymorphisms has been investigated unconnectedly, and consequently, their exact individual and/or combined contributions to the determination of IL-1β levels remain unidentified.

The results presented here demonstrate a similar frequency of the IL-1β(3954) genotypes in the C and CP groups, in spite of a trend toward a higher frequency of T allele occurrence in the diseased group. While some studies associated the IL-1β(3954) T allele with an increased risk for severe periodontitis development, others did not confirm such an association (12, 17, 27, 34, 48), and the relevance of IL-1β(3954) to the periodontitis outcome is still controversial (17, 48). Genetic studies in multifactorial complex trait diseases such as periodontitis are indeed complex, and the observance (or not) of covariates (such as smoking and specific bacteria) involved in periodontal-disease pathogenesis and the criteria to select diseased and C groups may hinder their interpretation (17, 48). Indeed, in the genetic association studies regarding periodontal disease, the C groups comprised periodontally healthy sub-

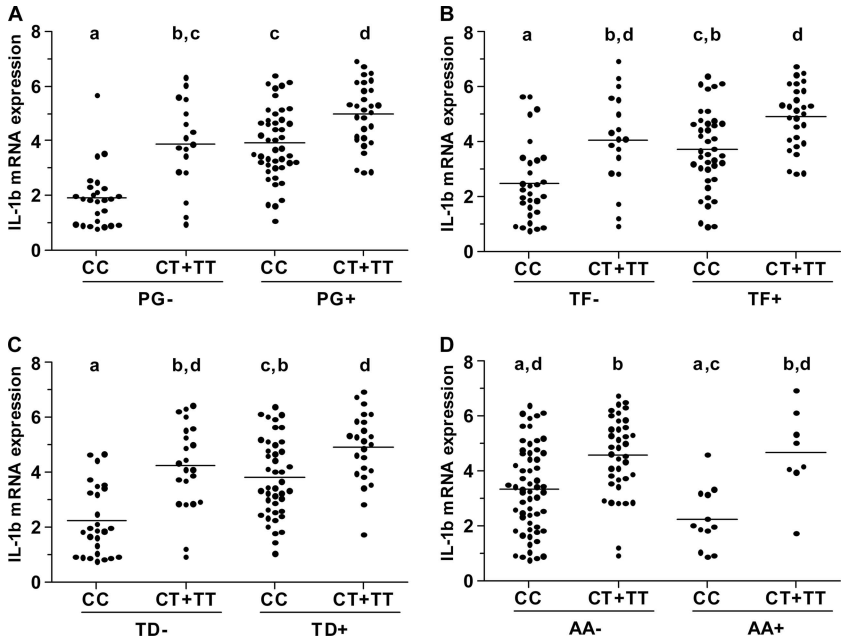

FIG. 4. IL-1β(3954) genotype and periodontopathogen contribution to IL-1β mRNA expression. CP patients were subjected to a periodontal examination, and the genotype of IL-1β(3954) SNP was determined by RFLP. Total RNA was extracted from gingival tissues, and the levels of IL-1β mRNA were measured quantitatively by real-time PCR. The results are presented as the expression of the individual mRNAs, with normalization to β-actin. The presence of the periodontopathogens was investigated by real-time PCR. The graphs depict the expression of IL-1β in the presence or absence of *P. gingivalis* (A), *T. forsythia* (B), *T. denticola* (C), and *A. actinomycetemcomitans* (D) in CP patients regarding their IL-1β(3954) SNP genotype. *, $P < 0.05$ (by ANOVA), where different letters indicate statistical significance.

jects, which does not necessarily imply a genetic resistance to development of the disease but may only reflect control of the etiologic factors of disease by proper oral hygiene. In accordance, the frequencies of periodontopathogens were strikingly lower in the C group, as previously described (8, 25).

Although there are controversies regarding the genetic association studies, the evaluation of the presumed functionality of IL-1β(3954) SNP in the periodontal environment could be helpful to unravel their putative role in this pathology. Interestingly, our results demonstrate a significantly higher IL-1β expression in the CT and TT genotypes compared to the CC subgroup. In accordance, the IL-1β(3954) T allele has been associated with increased IL-1β production in diverse pathological conditions, such as rheumatoid arthritis and osteoarthritis, and also with an increased risk for the development of such pathologies (3, 5, 15, 52). Indeed, CT and TT genotype patients also presented statistically higher values of mPD and CAL, suggesting that the genetic influence on the levels of IL-1β could be relevant to the determination of disease severity. However, no differences were found in the values of BOP and PD (of the sampled site) for the different IL-1β(3954) SNP genotype groups of CP patients, which may be due to the influence of local factors (such as restorations with subgingival margins, marginal excess, occlusal trauma, or anatomic variations) relevant to periodontitis development (38) that may mask the putative genetic influence in some specific sites.

It is also important to consider that the presence of specific periodontopathogens could also contribute to the modulation of IL-1β levels. In fact, sites positive for *P. gingivalis*, *T. forsythia*, and *T. denticola* were found to present a significantly higher expression of IL-1β compared to the level for negative sites. In accordance, *P. gingivalis* can induce IL-1β production by diverse cell types and was also associated with increased IL-1β levels in periodontal lesions (20, 28, 31, 51). Similarly, *T. forsythia* and *T. denticola* have been associated with IL-1β production in vitro and in vivo (2, 28, 51). Complementarily, *P. gingivalis*, *T. forsythia*, and *T. denticola* were associated with higher scores of disease-severity parameters, in accordance with previous studies (28, 51, 53, 57). Interestingly, the analysis of red complex periodontopathogens individually and as a group resulted in similar results, probably due to their simultaneous occurrence in most of the patients. Indeed, the red complex periodontopathogens were shown to exist as a consortium (29, 32, 49) and to exhibit synergistic virulence in a rat model of polymicrobial periodontal infection (24). In accordance, our results demonstrate an increase in IL-1β levels associated with the simultaneous occurrence of two or three red complex species, suggesting an increasing pathogenic proinflammatory role for these bacteria. Thus, the red complex periodontopathogens, similar to IL-1β(3954) SNP, may represent an important factor in the modulation of IL-1β expression. However, since IL-1β(3954) SNP and red complex-positive patients presented both higher IL-1β levels and deeper pockets with more CAL, perhaps the increased IL-1β levels are a consequence of the deeper pockets and not of the presence of the SNP and/or specific bacteria. However, when subgroups (CC versus CT+TT and red complex-positive versus -negative) of CP patients were paired into clinically homogeneous subgroups, the presence of both the T allele and red complex bacteria was still associated with increased IL-1β levels, suggesting that such variations are indeed dependent on the genetic and microbial factors investigated and independent

of variations in the disease severity between the subgroups investigated.

In view of the possible overlapping effects of IL-1β(3954) and the red complex bacteria (i.e., a red complex-positive T allele carrier), we next evaluated their putative individual roles in the modulation of IL-1β expression. Our results demonstrate that in the absence of red complex bacteria (individually and as a group), T allele carriers presented significantly higher levels of IL-1β than patients with the CC genotype. When subjects positive for red complex bacteria were analyzed, we also found that T carriers presented significantly higher levels of IL-1β than patients with the CC genotype. Interestingly, similar levels of IL-1β were found in red complex-negative subjects bearing the CT and TT genotypes and in red complex-positive patients with the nonpolymorphic CC genotype, demonstrating that both red complex bacteria and IL-1β(3954) SNP can independently increase the levels of IL-1β in diseased periodontium. Finally, T allele carriers positive for red complex bacteria presented the highest levels of IL-1β, demonstrating an additive effect of periodontopathogens and host genotype in IL-1β expression. Similar results were found when *P. gingivalis*, *T. forsythia*, and *T. denticola* were investigated individually. Taken together, our data suggest that IL-1β(3954) and the red complex bacteria, individually and combined, are associated with increased IL-1β levels in diseased periodontal tissues. Accordingly, in patients under supportive periodontal therapy, the presence of a low bacterial load in subjects positive for the IL-1β(3954) SNP results in disease severity similar to that of a high bacterial load in patients negative to the polymorphism (1). The positive association of IL-1β(3954) SNP and red complex periodontopathogens with the levels of IL-1β demonstrated herein is also reinforced by the specific exclusion of smokers from our sample. Indeed, smoke can modulate the host response, including IL-1β expression (19, 55). In addition, it is important to consider that multiple regression analysis demonstrated that IL-1β expression is age, race, and gender independent, being only associated with the red complex periodontopathogens and with IL-1β(3954), as previously discussed.

On the other hand, *A. actinomycetemcomitans* was not correlated with IL-1β expression or with the clinical parameters evaluated. While some studies describe that *A. actinomycetemcomitans* increases IL-1β production in vitro (18, 23), others describe that their virulence factors can inhibit inflammatory cytokine production (30, 39). It is important to consider that in spite of *A. actinomycetemcomitans* sometimes being detected in CP sites, it is more prevalent in aggressive periodontitis (47). However, the low *A. actinomycetemcomitans* frequency in our sample does not support strong statements, and further studies are required to evaluate its putative role in the modulation of IL-1β levels in vivo.

Interestingly, the different IL-1β(3954) genotypes were not associated with statistical differences in the frequency of red complex periodontopathogen detection or with the *P. gingivalis* load in periodontal pockets. However, none of the TT carriers were positive for *A. actinomycetemcomitans*. Recent studies have demonstrated that the IL-1β genotype was associated with higher loads of and increased odds of detecting *P. gingivalis*, *T. forsythensis*, and/or *A. actinomycetemcomitans* in periodontal pockets (28, 35, 36), an association that was not seen in our sample. While these studies suggest that a strong host inflammatory reaction could allow a wide bacterial colonization of the periodontal environment (28, 35, 36), our data could suggest that hyperinflammatory SNPs did not confer an advantage in the host response against red complex pathogens. However, a stronger host response would restrain the establishment of *A. actinomycetemcomitans* in the periodontal pocket. However, the low frequency of *A. actinomycetemcomitans* and the TT genotype in our sample does not support strong statements, and further studies are required to evaluate the possible influence of the host genetic background on the control of periodontal microbiota.

In a study designed to minimize some of the limitations of genetic association studies focused on periodontal diseases (such as the inobservance of covariates like smoking and specific bacteria) (17, 48), our data demonstrate that IL-1β(3954) genotypes and red complex periodontopathogens, individually and additively, can modulate the levels of IL-1β in the diseased tissues of nonsmoking CP patients and, consequently, are potentially involved in the determination of the disease outcome. Accordingly, meta-analysis studies demonstrate that the observance of covariates is important to unravel the possible association of IL-1β(3954) with different pathological conditions (17, 21, 22, 42, 48, 50). However, it is also important to consider that periodontitis is a complex trait disease, in which several other periodontopathogens, viruses, immunoregulatory cytokines, and systemic modifying factors not considered in this investigation may also be involved in the modulation of IL-1β levels (17, 26, 45, 48). Therefore, it is reasonable to expect that SNPs, even those proven to be functional like IL-1β(3954), potentially play a significant but not major role in the disease outcome, as described for other pathologies (21, 22, 50). Indeed, even in experimental periodontitis in knockout mice, in which a given cytokine is completely absent, the disease severity is usually found to be partially, but not completely, increased or decreased (9, 10, 44).

The first steps in solving the puzzle of the genetic and microbial contributions to the immunopathogenesis of periodontal disease have been achieved, but further studies are required to understand their exact roles in the periodontitis outcome. Such knowledge might allow us to develop diagnostic, preventive, and therapeutic strategies to improve the clinical management of periodontal disease.

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