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TLR4 and *IL-18* gene variants in aggressive periodontitis

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Abstract

Aim: We aimed to assess the association of different genotypes with increased aggressive periodontitis susceptibility by studying functional relevant variants in the pathogen-recognition receptor Toll-like receptor 4 (TLR4) and variants in the promoter region of the pro-inflammatory cytokine interleukin-18 (IL-18).

Material and Methods: One hundred and eleven patients with aggressive periodontitis and 80 periodontally healthy controls were genotyped for four functional variants in the *TLR4* gene (c.896A > G and c.1196C > T) and in the *IL-18* promoter (c. − 368G > C and c. − 838C > A). The genotype and allele frequencies, as well as the frequency of combined genotypes were compared between study groups.

Results: There were no statistical differences in genotype and allele frequencies within the four variants between the groups. All study subjects were further classified into carriers and non-carriers of at least one variant of both genes. The logistic regression analysis adjusted for gender and smoking showed no association between carrier status of at least one variant of both genes and periodontal status (OR = 1.41, 95% CI: 0.43–4.70).

Conclusions: Our results reject the hypothesis that functionally relevant *IL-18* and *TLR4* gene mutations have a major effect on aggressive periodontitis susceptibility alone or in combination.

Key words: aggressive periodontitis; genetic risk; *IL-18* gene; innate immune response; *TLR4* gene

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Aggressive periodontitis (AgP) is a rare but severe and progressive form of periodontal disease in otherwise healthy patients. It is characterized by a rapid destruction of periodontal tissue, and familial aggregation is considered (Lang et al. 1999). The pathogenic role of the subgingival microbiota in all periodontitis types is widely accepted. However, the quality and quantity of local inflammatory and immunological reactions to the bacterial challenge differ among individuals (Page et al. 1997). Several studies support the hypothesis that genetic variations in host response contribute to the multi-factorial background, particularly in AgP (Hodge & Michalowicz 2001, Kinane & Hart 2003, Loos et al. 2005, Yoshie et al. 2007). Numerous candidate genes of AgP that are involved in immunity and inflammation are under consideration. For example, the risk of having AgP has been related to the carriage of functionally relevant allelic variants of interleukins (ILs) such as IL-4 or IL-6 (Gonzales et al. 2007, Nibali et al. 2008). Recognition of invading pathogens by the innate immune system is an essential pre-requisite for triggering inflammatory response and developing acquired immunity. Among the receptors that recognize and bind pathogen-associated molecular pattern (PAMP), Toll-like receptors (TLRs) act as a key-receptor of the innate immune system. Because Toll was first identified as a critical molecule for embryonic development in the fruit fly *Drosophila* (Anderson et al. 1985a, b), a homologue family in mammals named TLRs has been discovered. TLRs are members of the IL-1 receptor family, and the first described was TLR4 (Medzhitov et al. 1997). It was identified by acting as a receptor for PAMPs such as lipopolysaccharides (LPS), derived among other pathogens from *Aggregatibacter actinomycetemcomitans* or *Porphyromonas gingivalis*, the main pathogens in AgP (Darveau et al. 2004, Kikuchi et al. 2004). Through the recognition of LPS, TLR4 activates transcription of pro-inflammatory cytokines via the nuclear transcription factor κB (NF-κB) pathway (Medzhitov et al. 1997, Poltorak et al. 1998). Accordingly, it was shown that *A. actinomycetemcomitans* stimulates

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the TLR4-dependent production of several cytokines (Kikkert et al. 2007), and the optimal interferon-γ (IFN-γ) response induced by *A. actinomycetemcomitans*-stimulated dentridic cells was also TLR4 dependent (Kikuchi et al. 2004).

Cytokines that are produced in response to LPS-induced TLR activation play an important role in the regulation of host defence mechanisms against oral pathogens, as well as in host- mediated periodontal tissue destruction if inappropriately expressed. One of the TLR4-dependent cytokines is IL-18, firstly described as IFN-γ-inducing factor (Okamura et al. 1995). It is widely accepted that the balance of T helper 1 (Th1) and T helper 2 (Th2) cells is crucial to the immunoregulation in periodontitis (Gemmell & Seymour 2004, Orozco et al. 2007), and, depending on the immunological context, that IL-18 has the capacity to induce either Th1 or Th2 cells in response to gram-negative infections. It was shown that IL-18 together with IL-12 is associated with promoting the production of IFN-γ in Th1 cells (Nakanishi et al. 2001a, b). IFN-γ was found to be over-expressed in gingival biopsies of patients presenting with AgP (Garlet et al. 2003), and it is crucial for the anti-*A. actinomycetemcomitans* immunoglobulin G (IgG) production in AgP (Schenkein et al. 2007).

IL-18 is a member of the IL-1 family, and, together with their receptors, it is also a member of the IL-1R/TLR superfamily (Dinarello et al. 1998) involved in the regulation of both the innate as well as the acquired immune response. Because Kornman et al. (1997) have firstly described a periodontitis-associated genotype in the IL-1 gene cluster, the *IL-1* genotypes appear to be the most studied genetic association with periodontal diseases. However, unlike chronic periodontitis, no association was found for any *IL-1* gene variant and the aggressive form of periodontitis as shown in a recent meta-analysis (Nikolopoulos et al. 2008). Thus, studying functionally relevant gene variants of other members of the IL-1 family in AgP was of interest. The role of IL-18 in AgP as well as the function of TLR4 in cytokine activation implies that both genes coding for these proteins could be candidate genes for an increased periodontitis risk. Recently, two missense mutations in tight linkage disequilibrium (LD) (p.Asp299Gly and p.Thr399Ile) have been identified and reported as impairing the LPS response in humans (Arbour et al. 2000), hence associated with increased susceptibility to gram-negative infections (Bochud et al. 2007). Genetic variations of the *IL-18* gene have also been shown to influence protein production (Giedraitis et al. 2001), affecting individual susceptibility to different chronic diseases such as diabetes mellitus or rheumatoid arthritis (Thompson & Humphries 2007).

There is one previous study that failed to show an association of IL-18 polymorphisms and chronic periodontitis (Folwaczny et al. 2005). Several studies on the association of single-nucleotide polymorphisms (SNPs) in the *TLR4* gene with periodontitis have been performed, with inconsistent results in different, mainly chronic periodontitis populations (D'Aiuto et al. 2004, Folwaczny et al. 2004, Brett et al. 2005, Laine et al. 2005, Schröder et al. 2005, Izakovicova Holla et al. 2007, James et al. 2007, Tervonen et al. 2007). Thus, the aim of this study was to evaluate the role of known functional variants in *TLR4* and *IL-18* in patients with generalized AgP compared with healthy controls.

Material and Methods

Study population

A total of 191 unrelated subjects volunteered to participate in this case–control association study. All of them were Germans of Caucasian origin. Each study participant received clinical and genetic counselling, an appropriate description of the study protocol, and signed a consent form, approved by the local ethics committee. A thorough history of systemic and dental diseases and smoking history was obtained by interviews. Packyears (Grossi et al. 1995) as well as the categories of smoking history were recorded as: never-smoker, smoker or former-smokers (who have quit smoking for at least 5 years). Persons suffering from severe systemic disorders (diabetes mellitus, immunological disorders, chronic inflammatory diseases, or increased risk for bacterial endocarditis), as well as pregnant or lactating women, were excluded from the study.

One hundred and eleven patients with generalized AgP met the following criteria for case-definition based on the recommendations of the 1999 international classification workshop of periodontal diseases and conditions (Lang et al. 1999):

1. Generalized interproximal attachment loss (AL) at least on three teeth other than first molars and incisors. This loss of clinical attachment had to be $\geqslant 5$ mm, and interproximal bone loss had to be $\geqslant 50\%$ of the root length as assessed by full mouth radiographs or orthopantomographs.
2. Proximal AL of $\geqslant 5$ mm in $\geqslant 30\%$ of teeth was used for threshold level in case definition to include only cases with substantial periodontitis extent and severity like proposed to identify risk factors at the 5th European Workshop on Periodontology (Tonetti & Claffey 2005).

Although the current classification system of periodontal diseases is not based on the age of disease onset, in most cases AgP is characterized by an early age of clinical manifestation. Thus, all AgP patients were younger than 40 years (mean age at the time of diagnosis 34.0 years $\pm$ 6.1).

The control group comprised a total of 80 unrelated individuals. To minimize the likelihood of a late onset of AgP, and as recommended for genetic studies regarding diseases with early onset (''Sample-Based Case–Control Design with hypernormal controls''; Morton & Collins 1998), only controls who were older than the oldest patient (mean age 52.7 years $\pm$ 10.3, age of youngest control 40.25 years, age of oldest patient 39.75 years, respectively) were enrolled. Control subjects were selectively recruited, that is, they were screened and refuted as a case. Thus, subjects of the control group were periodontally healthy or showed only minimal signs of periodontal AL or gingivitis. They did not suffer from any form of AgP or severe chronic periodontitis. As recommended, subjects with gingivitis or mild form of AL have not been excluded from the study due to the high prevalence of gingivitis and chronic periodontitis in the general population (Yoshie et al. 2007). All controls were selected based on the following criteria: at least 20 teeth in situ (no history of tooth loss because of increased tooth mobility due to periodontitis), at least 90% of measured tooth sites with probing pocket depth (PPD) <4 mm and AL $\leqslant 1$ mm, no proximal PPD $\geqslant 5$ mm and no

AL > 2 mm, as well as no vertical bone defects on the radiographs.

Blood samples and DNA isolation

Peripheral blood was obtained from all subjects and genomic DNA was purified from whole blood using the QiaAmp blood DNA purification kit (Qiagen, Hilden, Germany).

Sequence analysis

DNA samples were amplified by polymerase chain reaction (PCR) using specific primers and protocols for each examined polymorphism as described previously (Folwaczny et al. 2004, 2005). Two SNP blocks in the *IL-18* promoter region in almost complete LD have been described to date (Giedraitis et al. 2001, Tiret et al. 2005). Thus, SNPs at position c. − 838C > A and c. − 368G > C from the translation start side were selected for genotyping, representing the two main *IL-18* promoter haplotypes. The two known SNPs in the extracellular domain of the *TLR4* gene (c.896A > G, p.Asp299Gly and c.1196C > T, p.Thr399Ile) were genotyped after amplification of exon 3. Purified PCR products were directly sequenced using the ABI PRISM® BigDye® Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase v3.0 and v3.1 (Applied Biosystems, Forster City, CA, USA) and the PCR primers on capillary sequencing devices (ABI 3730 DNA Analyser, Applied Biosystems). The sequences were generated by the Sequencher 4.8 software (Gene Codes Corp., Ann Arbor, MI, USA) and aligned with the published *IL-18* gene sequence (GenBank accession no. NT_033899) and *TLR4* gene sequence (GenBank accession no. NM_138554).

Data analysis and statistical methods

Before association analysis, sample size for each group was calculated for the two-sided allelic χ^2-test on the basis of an alpha significance level of 0.05 as well as of a power level of 80%. In two previous studies, minimum carrier frequencies of variant *TLR4* alleles in control groups were 0.066 and 0.071, respectively (Folwaczny et al. 2004, Schröder et al. 2005). When assuming these frequencies of a risk allele and a relative risk of 2.5, a minimum of 110 cases and 78 controls were required if 2% of genotypes were missing non-differentially. The reported minimum *IL-18* SNP carrier frequencies have exceeded 0.06 by far (approximately 0.3) (Folwaczny et al. 2005, Gracie et al. 2005, Rueda et al. 2005). Thus, the calculated sample size also appears sufficient for analysing these SNPs.

Deviation from Hardy–Weinberg equilibrium was investigated by a χ^2 goodness-of-fit test. Fisher's exact test was used to compare allele frequencies among the groups. The two-sided exact Cochran–Armitage trend test was used to compare genotype frequencies of *IL-18* and *TLR4* variants between patients with AgP and controls. Odds ratios (OR) and 95% confidence intervals (CI) were estimated using conditional maximum likelihood. The logistic regression model was used to adjust for gender and smoking. No adjustments for age were performed because all control subjects were older than the oldest patient.

Results

Periodontal and demographic characteristics of the study population are given in Table 1. The groups differed in periodontal findings and age according to the case and control inclusion criteria. Smoking is an established risk factor for periodontitis, and the percentage of smokers was significantly higher in the case group than in the control group.

Two *TLR4* and two *IL-18* promoter variants were genotyped in all patients and controls (Table 2). The genotype frequencies in both groups did not differ from Hardy–Weinberg equilibrium.

TLR4 c.896A > G and c.1196C > T were in complete LD in our study population and all carriers were heterozygous in the AgP as well as in the control group (9.9% and 11.3%, respectively). There was no association between the *TLR4* genotype and the AgP phenotype, for example, no statistically significant differences in genotype and allele distribution were detected between AgP and control group (Table 2).

Similar results were obtained with the *IL-18* promoter genotyping analysis as summarized in Table 2. Differences between both study groups were not statistically significant.

Because activation of IL-18 depends on TLR4 function, the frequency of a ''composite genotype'' that consisted of at least one variant allele from both genes was determined. There was no significant difference among the study groups (6.3% and 7.5% in the AgP group and control group, respectively; $p = 0.777$). The logistic regression analysis adjusted for gender and smoking showed no association between the carrier status of a composite genotype of *IL-18* gene and the *TLR4* gene variants

Table 1. Characteristics of the study population

	Case group (AgP) ($n = 111$)	Control group ($n = 80$)	p-value
No. of teeth present (mean ± SD)	25.6 ± 3.1	24.4 ± 3.9	0.02*
No. (%) of teeth with AL ⩾ 5 mm (mean ± SD)	18.4 ± 5.3 (74.6 ± 19.2)	No	
No. (%) of proximal sites with bone loss ⩾ 50% (mean ± SD)	30.4 ± 7.7 (57.0 ± 17.8)	No	
Mean age (years ± SD)	34.0 ± 6.1	52.7 ± 10.3†	< 0.0001*
No. of never smokers (%)	70 (63.1)	69 (86.3)	
No. of former smokers (%)	12 (10.8)	4 (5.0)	0.002‡
No. of smokers (%)	29 (26.1)	7 (8.4)	
No. of males (%)	47 (42.3)	28 (35.0)	0.305§

AgP, generalized aggressive periodontitis.
*Unpaired *t*-test.
†Youngest control was older than oldest patient.
‡Overall *p*-value from chi-square test, post hoc testing: Fisher's exact test, comparing current smoker and never smoker between the groups, $p = 0.001$.
§Chi-square test.

Table 2. Genotype and allele frequencies of analysed variants

refSNP ID	Gene/nucleotide position and change	Amino acid change	Genotype/ allele	Controls, *n* (%)	AgP, *n* (%)	OR*	95% CI	*p*-value	OR†	95% CI	*p*-value
rs4986790				80	111						
	TLR c.896A > G	p.D299G	A/A	71 (88.8)	100 (90.1)			0.813			0.803
			A/G	9 (11.3)	11 (9.9)	0.87	0.31–2.51		0.82	0.27–2.48	
			G/G	0 (0.0)	0 (0.0)						
			Allele A	151 (94.4)	211 (95.0)						
			Allele G	9 (5.6)	11 (5.0)			0.818‡			
rs4986791	*TLR* c.1196C > T	p.T399I	C/C	71 (88.8)	100 (90.1)			0.813			0.803
			C/T	9 (11.3)	11 (9.9)	0.87	0.31–2.51		0.82	0.27–2.48	
			T/T	0 (0.0)	0 (0.0)						
			Allele C	151 (94.4)	211 (95.0)						
			Allele T	9 (5.6)	11 (5.0)			0.818‡			
rs1946518	*IL-18* promoter/ c. − 838C > A	None	C/C	26 (32.5)	42 (37.8)			0.372			0.252
			C/A	42 (52.5)	57 (51.4)	0.80	0.50–1.27				
			A/A	12 (15.0)	12 (10.8)	0.64	0.25–1.62		0.77	0.47–1.24	
			Allele C	94 (58.75)	141 (63.5)						
			Allele A	66 (41.25)	81 (36.5)			0.394‡			
rs187238	*IL-18* promoter/ c. − 368G > C	None	G/G	41 (51.3)	56 (50.5)			0.811			0.806
			G/C	35 (43.8)	47 (42.3)	1.08	0.66–1.79				
			C/C	4 (5.0)	8 (7.2)	1.17	0.43–3.22		1.07	0.64–1.80	
			Allele G	117 (73.1)	159 (71.6)						
			Allele C	43 (26.9)	63 (28.4)			0.817‡			

The genotype frequencies in patients and controls were in Hardy–Weinberg equilibrium. AgP, generalized aggressive periodontitis; OR, odds ratio; 95% CI, 95% confidence interval; refSNP ID, NCBI data base reference SNP ID

*Cochran–Armitage trend test without adjustments.

†Logistic regression with adjustments for gender and smoking habits.

‡Fisher's exact test.

and periodontal status (OR = 1.45, 95% CI: 0.44–4.81).

Discussion

TLR4 recognizes LPS of oral gram-negative pathogens such as *A. actinomycetemcomitans*, *P. gingivalis* and *Fusobacterium nucleatum* (Darveau et al. 2004, Mahanonda & Pichyangkul 2007). It is predominantly expressed not only in cells of the innate immune system in diseased periodontal tissue (Mori et al. 2003) but also in gingival and periodontal fibroblasts (Hatakeyama et al. 2003, Sun et al. 2008). Additionally, periodontal therapy can result in substantial changes in TLR4 expression in monocytes (Papapanou et al. 2007), emphasizing the potential role of TLR4 in periodontitis pathogenesis.

Upon ligand binding, TLR4-mediated signalling activates signal transduction leading to the production of pro-inflammatory cytokines involved in periodontal tissue destruction (Mahanonda & Pichyangkul 2007), of which one is IL-18. It plays an important role in the regulation of IFN-γ that was found to be over-expressed in gingival biopsies, especially from AgP patients and in active periodontal pockets (Garlet et al. 2003), emphasizing a pathogenetic role of IFN-γ in AgP. *IL-18* is expressed by macrophages as well as by non-immune cells including oral epithelial cells (Sugawara et al. 2001, Orozco et al. 2006). IL-18 concentrations were found to be increased in diseased human gingiva and were positively correlated with gingival sulcus depth (Johnson & Serio 2005). Together, the two genes investigated in this study play a role in inflammation and immunity in general as well as in periodontitis.

The analysed genetic variants are proven to be functional and have effects on either the amount of protein produced or on downstream effects of each gene (Arbour et al. 2000, Giedraitis et al. 2001, Tiret et al. 2005, Ferwerda et al. 2007, Thompson & Humphries 2007), which renders them plausible candidates for an association study of periodontitis and genetic variants besides their role in immunity and inflammation. However, the study presented here failed to show an influence of *IL-18* and *TLR4* gene variants on the susceptibility of AgP, even after adjustment for smoking and gender. Because smoking is a dose-dependent risk factor for AgP, we have used for adjustment the categorical variable (never-smoker, smoker or former-smokers) or the continuous variable packyears (data not shown), obtaining no difference in the results. Furthermore, subjects with known chronic inflammatory diseases were not enrolled in the study, to minimize other potential confounder. However, considering the age of the control group (mean age 52.7 years ± 10.3) such conditions could not be completely excluded.

Our results are in accordance with three previous studies analysing the *TLR4* gene in AgP patients (Brett et al. 2005, Schröder et al. 2005, Emingil et al. 2007). Schröder et al. (2005) have found an association between *TLR4* SNPs and chronic periodontitis in a German population. Generalized AgP patients carried the variant *TLR4* allele approximately two times more often than controls (9.9% *versus* 4.9%, $n = 81$ per group). However, this difference was not statistically significant. Similar results came from a British Caucasian population (p.Asp299Gly 9.0% *versus* 3.5% and p.Thr399Ile 3% *versus* 9%) (Brett et al. 2005), as well as from a Turkish population with lower allele frequencies (p.Asp299Gly 2.2% *versus* 2.9% and p.Thr399Ile 1.1%

versus 2.3%) (Emingil et al. 2007). In these studies, the two SNPs were not in complete LD as found in our population. The variability in the prevalence of the SNPs in different populations is known from a worldwide genotyping of 15 different ethnic groups (Ferwerda et al. 2007). Accordingly, 6–14% of the Indo-European individuals were double heterozygotes for the p.Asp299Gly and p.Thr399Ile alleles (allele frequencies 3–7%) in that study. Our data (allele frequencies) agree with these data.

In contrast, one additional association study has reported a decreased AgP susceptibility in Western European Caucasians p.Asp299Gly carriers (James et al. 2007) (2.7% *versus* 8.1% carriers in the AgP group and the control group, respectively). This result has been cautiously interpreted by the authors, considering the level of significance (genotype distribution $p = 0.026$, allele frequency $p = 0.031$), the limited size of the study (73 cases and 123 controls) and the limitations resulting from using blood donors as controls with the possibility of phenotypic misclassification. The results are supported by a further recent study showing that gingival epithelial cells from subjects with p.Asp299Gly produce lower levels of pro-inflammatory IL-6 und IL-8 after challenge with the periodontal pathogen *P. gingivalis* preventing them from periodontal destruction (Kinane et al. 2006).

Here, the two analysed *IL-18* promoter SNPs c.−838C>A and c.−368G>C are known to have influence on transcription factor binding sites resulting in an impaired *IL-18* promoter activity and an impaired IL-18 level (Giedraitis et al. 2001, Sivalingam et al. 2003). For our knowledge, the data presented here are the first study analysing *IL-18* SNPs in AgP patients. However, we could not find an association between the presence of a variant allele and AgP susceptibility. This is in agreement with Folwaczny et al. (2005), who reported no significant association between the two SNPs and chronic periodontitis. IL-18 production is regulated on different levels of transcription, translation and post-translation. Thus, a direct correlation between protein production and genotype or a simple phenotype–genotype association is difficult to verify (Sugiura et al. 2002).

Because the activation of IL-18 depends on TLR4 function (Nakanishi et al. 2001a), we have determined the prevalence of subjects carrying at least one variant allele from both genes (composite genotype). However, there was also no significant difference between the study groups. Considering the low numbers of AgP patients with the composite genotype (7 out of 111 patients), a comparison of the periodontitis severity in *IL-18/TLR4* positive and negative AgP subjects yielded no significant results. The percentage of sites with approximal bone loss $\geqslant 50\%$ was 63% and 56%, respectively. This approach should be verified in future studies with larger samples.

In conclusion, our results reject the hypothesis that functionally relevant *IL-18* and *TLR4* gene mutations have a major effect in AgP susceptibility. Assuming that periodontitis is a multifactorial disease, it would be interesting to study the impact of more complex genetic backgrounds of bacterial recognition and host inflammation in periodontitis. In the future, genome-wide association studies will be a promising approach for mapping periodontitis-susceptibility genes.

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Clinical Relevance

Scientific rationale for the study: TLRs are key-receptors of the innate immune system. IL-18 is produced in response to bacterially induced TLR activation and plays an important role in the regulation of host-mediated periodontal tissue destruction when inappropriately expressed. Sequence variants in both genes are known to increase the susceptibility to inflammatory diseases.

Principal findings: No association was found between AgP susceptibility and the gene variants.

Practical implications: Based on our data, *TLR4* and *IL-18* gene variants are no major genetic risk markers for AgP, resulting in no practical implications for disease prevention and treatment.