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Periodontal ligament and gingival fibroblasts from periodontitis patients are more active in interaction with *Porphyromonas gingivalis*

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Background and Objective: Inflammatory responses of host cells to oral pathogenic bacteria, such as *Porphyromonas gingivalis*, are crucial in the development of periodontitis. Host cells, such as periodontal ligament and gingival fibroblasts, from periodontitis patients may respond to *P. gingivalis* in a different manner compared with cells from healthy persons. The aim of this study was to investigate inflammatory responses to viable *P. gingivalis* by periodontal ligament and gingival fibroblasts from periodontitis patients and healthy control subjects.

Material and Methods: Primary periodontal ligament and gingival fibroblasts from periodontitis patients ($n = 14$) and healthy control subjects ($n = 8$) were challenged *in vitro* with viable *P. gingivalis*. Gene expression of Toll-like receptors (TLRs) 1, 2, 4, 6, 7 and 9, CD14, nuclear factor-κB1 and its putative inhibitor NF-κB inhibitor-like protein 1, and of interleukin-1β, interleukin-6, interleukin-8, tumour necrosis factor-α, monocyte chemotactic protein-1 and regulated upon activation, normal T-cel expressed, and secreted, were assessed by real-time PCR.

Results: Periodontal ligament fibroblasts from periodontitis patients had a higher mRNA expression of *TLR1*, *TLR4*, *TLR7* and *CD14*, and a lower expression of *NFKBIL1*, both before and after *P. gingivalis* challenge. In contrast, gingival fibroblasts from periodontitis patients had stronger induction of *TLR1*, *TLR2* and *TLR7* by *P. gingivalis*. Cytokine responses were not different between patients and control subjects. Interestingly, periodontal ligament, but not gingival, fibroblasts from *P. gingivalis* culture-positive persons responded more strongly to *P. gingivalis* than periodontal ligament fibroblasts from *P. gingivalis*-negative persons.

Conclusion: Periodontal ligament and gingival fibroblasts respond to *P. gingivalis* in a different manner and may play different roles in periodontitis. Both subsets of fibroblasts from patients appear more active in interaction with *P. gingivalis*. Moreover, periodontal ligament fibroblasts from *P. gingivalis*-positive donors are more responsive to an *in vitro P. gingivalis* challenge.

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Periodontitis is a chronic inflammatory disease of the tooth-supporting tissues that can lead to tissue destruction and finally tooth loss. The disease develops as a result of a continuous interaction between host cells and subgingival pathogenic bacteria, such as the gram-negative anaerobe *Porphyromonas gingivalis*, a major aetiological agent of periodontitis (1–3). The host response to oral pathogens is a crucial determinant in the development of periodontitis (4,5).

Fibroblasts play an important role in chronic infections, including periodontitis (6–8). Periodontal ligament and gingival fibroblasts are functionally distinct fibroblast types in the periodontal tissues (9–11). Periodontal ligament fibroblasts are essential in the formation and maintenance of the periodontal ligament connecting teeth to the alveolar bone; gingival fibroblasts are subepithelial and play a crucial role in the physiological turnover of the connective tissue adjacent to the epithelium attached to the tooth.

Periodontal ligament and gingival fibroblasts respond to *P. gingivalis* or its components by initiating an inflammatory response that includes the production of proinflammatory cytokines and chemokines (12,13). We previously showed that stimulation of periodontal ligament and gingival fibroblasts from healthy donors with viable *P. gingivalis* led to a strong induction of gene expression of proinflammatory cytokines interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNFα; *IL1B*, *IL6* and *TNF* genes) and chemokines IL-8, regulated upon activation, normal T-cel expressed, and secreted (RANTES) and moncyte chemotactic protein-1 (MCP-1; *IL8*, *CCL5* and *CCL2* genes). We also showed considerable heterogeneity in the scale of these responses, not only between periodontal ligament and gingival fibroblasts from different donors, but also between periodontal ligament and gingival fibroblasts obtained from a single individual (13,14).

Host cells can detect bacteria via pattern recognition receptors, such as the Toll-like receptors (TLRs) and CD14. Interaction between receptor and bacterium initiates downstream signaling, which leads to activation of the transcription factor nuclear factor-κB (NFκB), and this in turn leads to transcription and production of inflammatory factors, such as TNFα, IL-6 and IL-8 (15,16). Recognition of *P. gingivalis* or its components by host cells has been described to occur via TLR1, TLR2, TLR4, TLR6, TLR7, TLR9 and CD14 (17–28).

Although periodontitis can be initiated by bacteria, such as *P. gingivalis*, host susceptibility is crucial for the development of the disease. Properties of the cells interacting with *P. gingivalis* may partly determine susceptibility. For example, neutrophils, mononuclear cells and platelets from periodontitis patients have been shown to differ from cells from healthy donors in their interaction with periodontal pathogens (29–31).

Surprisingly little is known about whether differences between periodontitis patients and healthy control subjects also exist for periodontal ligament and gingival fibroblasts. Since the host response is an important determinant in the development of periodontitis, we hypothesized that periodontal ligament and gingival fibroblasts from periodontitis patients respond to *P. gingivalis* in a different manner compared with cells from healthy control subjects. As viable bacteria contain a complete subset of virulence factors and may interact with gingival and periodontal ligament fibroblasts in a unique way, this study aimed to investigate and compare responses of periodontal ligament and gingival fibroblasts from periodontitis patients and healthy control subjects to a challenge with viable *P. gingivalis*.

From previous research, it appeared that measuring protein expression may give biased results when working with viable *P. gingivalis*, due to its proteolytic activity (13,32–35); therefore, we measured gene expression rather than protein expression. Gene expression was assessed at receptor level (*TLR1*, *2*, *4*, *6*, *7*, *9* and *CD14*), transcription level [transcription factor *NFKB* and NF-κB inhibitor-like protein 1 (*NFkBIL1*)] and cytokine level [*IL1B, IL6, IL8, TNF, CCL5* (RANTES) and *CCL2* (MCP-1)]. Our results suggest that periodontal ligament and gingival fibroblasts from periodontitis patients may be more active in interaction with *P. gingivalis*. Moreover, the results suggest that the presence of *P. gingivalis* in subgingival plaque can render periodontal ligament fibroblasts more responsive to a new challenge with *P. gingivalis*.

Material and methods

Fibroblast donors

Gingival and periodontal ligament fibroblasts were obtained from 14 periodontitis patients (three male, 11 female; three females were current smokers) and eight healthy control subjects (two male, six female; one male was a current smoker). Donor characteristics and clinical parameters are depicted in Table 1. Periodontitis patients underwent tooth extraction as part of periodontal treatment, and displayed deepened pockets, bleeding on probing and loss of bone height visible on radiographs at the site of the tooth (Table 1). Control subjects underwent tooth extraction as part of treatment for reasons other than periodontitis and showed no signs of inflammation/periodontitis/loss of bone height (Table 1). None of the donors suffered from systemic diseases or was pregnant. Bacterial samples were taken from subgingival plaque from all donors by the paper point method, and were analysed for the presence of *P. gingivalis* by anaerobic culture. Donors were recruited at the Centre for Implantology and Periodontology Amstelveen (Amstelveen, The Netherlands). Donors had given written informed consent, and the study was approved by the VUmc Medical Ethical committee (VU University, Amsterdam).

Fibroblast isolation

Gingival and periodontal ligament fibroblasts were collected as described before (13). Briefly, free gingival remains were dissected from extracted teeth, and the periodontal ligament was scraped from exclusively the middle third of the root. Gingival and

Table 1. Fibroblast donors' characteristics and clinical parameters

Parameter	Periodontal ligament fibroblasts		Gingival fibroblasts	
	Control subjects (n = 8)	Patients (n = 12)	Control subjects (n = 7)	Patients (n = 12)
Age (years; mean ± SD)	48 ± 21	60 ± 9$^{n.s.}$	48 ± 21	58 ± 9$^{n.s.}$
Bone loss (%; mean ± SD)	0.4 ± 0.7	48 ± 30***	0.4 ± 0.8	51 ± 31***
Pocket depth (mm; mean ± SD)	2.7 ± 0.7	6.8 ± 3.1**	2.7 ± 0.7	7.0 ± 2.9**
Bleeding on probing (median; 0/1/2)	0	1*	0	1†
P. gingivalis carriers	2	5	2	4

Definitions: bone loss, percentage of loss of bone height around extracted tooth; pocket depth, probing depth of pocket around extracted tooth; *P. gingivalis* carriers, number of donors in whose subgingival plaque *P. gingivalis* was detected by anaerobic culture.
Symbols indicate significant differences between the patient group and control group, as follows: *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, †$p = 0.057$; n.s., no significant difference.

periodontal ligament fibroblasts expressed *FMOD* (fibromodulin), a marker for both gingival and periodontal ligament fibroblasts, at similar levels. Gene expression of *S100A4*, a marker for periodontal ligament fibroblasts, was expressed at very low levels in gingival fibroblasts but was highly expressed in periodontal ligament fibroblasts (36). Experiments were performed with cells at passage 5 or 6. In some cases, it was not possible to obtain both gingival and periodontal ligament fibroblasts from the same donor. Gingival fibroblasts could not be isolated from two patients and one control subject; periodontal ligament fibroblasts could not be isolated from two patients.

Bacterial strain and culture

Porphyromonas gingivalis W83 was cultured anaerobically (80% N_2, 10% H_2 and 10% CO_2) until reaching the log-growth phase in brain–heart infusion broth supplemented with haemin (5 mg/L) and menadione (1 mg/L). Purity was checked with Gram staining.

Viable *P. gingivalis* were harvested by centrifugation. Bacterial pellets were washed twice in sterile phosphate-buffered salt solution and resuspended in antibiotic-free Dulbecco's minimal essential medium (Gibco BRL, Paisley, Scotland) with 10% fetal calf serum (HyClone, Logan, UT, USA). The optical density was measured at 690 nm to establish the number of colony forming units (CFUs). A suspension of 2×10^8 CFU/mL was used to challenge gingival and periodontal ligament fibroblasts.

Bacterial challenge assays

Gingival and periodontal ligament fibroblasts were challenged with viable *P. gingivalis* as published previously (13). In short, gingival and periodontal ligament fibroblasts were grown until subconfluence in 24-well plates. Medium was removed and replaced with 0.5 mL of a *P. gingivalis* W83 suspension of 2×10^8 CFU/mL in antibiotic-free Dulbecco's minimal essential medium with 10% fetal calf serum. Only Dulbecco's minimal essential medium with 10% fetal calf serum was added to control gingival and periodontal ligament fibroblasts (nonchallenged).

Gingival and periodontal ligament fibroblasts were incubated with *P. gingivalis* for 6 h. After challenge, fibroblast morphology was checked for abnormalities or cell detachment by phase-contrast microscopy (Olympus CK2; Olympus, Tokyo, Japan). Subsequently, fibroblasts were washed with sterile phosphate-buffered salt solution (Gibco BRL) and lysed in lysis buffer as supplied with RNeasy Mini kit for RNA extraction (Buffer RLT; Qiagen, Hilden, Germany), supplemented with β-mercapto-ethanol. Experiments were performed in quadruplicate.

Messenger RNA expression

Fibroblast RNA was isolated using the Qiagen RNeasy Mini kit for RNA extraction. The RNA concentration was measured using a Nanodrop spectrophotometer (NanoDrop Technologies; Thermo-Fischer Scientific, Wilmington, DE, USA). Messenger RNA was reverse transcribed to cDNA according to the MBI Fermentas cDNA synthesis kit (Fermentas, Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers. For use of cDNA in RT^2 Profiler PCR Toll-like receptor signalling pathway (SA Biosciences, Frederick, MD, USA; see below), mRNA was reverse transcribed using the RT^2 First-Strand cDNA Synthesis kit (SA Biosciences) according to manufacturers' protocol.

Real-time PCR primers for *IL1B*, *IL6*, *IL8*, *TNF*, *CCL5*, *CCL2*, *NFKBIL1*, *CD14* and housekeeping gene porphobilinogen deaminase (*HMBS*) were designed using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA, USA; Table 2; 13,37). Primers for *TLR1*, *TLR2*, *TLR4*, *TLR6*, *TLR7* and *TLR9* were kind gifts from Dr J. Garcia-Vallejo (Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam; Table 2; 38). Primers for *NFKB* (p105) were obtained from SA Biosciences (assay ID NM_003998, SA Biosciences). The external standard curve used in the PCRs was a mixture of bone extract cDNA, peripheral blood mononuclear cell cDNA, gingival and periodontal ligament fibroblast cDNA, and the quantitative PCR human reference total RNA (Stratagene, La Jolla, CA, USA). Real-time PCR was performed as described before (13) on the ABI PRISM 7000 (Applied Biosystems).

Table 2. Real-time PCR primer sequences

Gene	Primer sequences	
	5′–3′ Forward	5′–3′ Reverse
IL1B	CTTTGAAGCTGATGGCCCTAAA	AGTGGTGGTCGGAGATTCGT
IL6	GGCACTGGCAGAAAACAACC	GGCAAGTCTCCTCATTGAATCC
IL8	GGCAGCCTTCCTGATTTCTG	CTGACATCTAAGTTCTTTAGCACTCCTT
TNF	CCCAGGGACCTCTCTCTAATCA	GCTTGAGGGTTTGCTACAACATG
CCL5	CATCTGCCTCCCCATATTCCT	TGCCACTGGTGTAGAAATACTCCTT
CCL2	CAGCCAGATGCAATCAATGC	TGCTGCTGGTGATTCTTCTATAGCT
NFKBIL1	GGACGAGTGGCAGGAAGTCA	CTGGCACTTCTGGGCATGTT
CD14	GGTCTCAACCTAGAGCCGTTTCT	AGCCTTGACCGTGTCAGCAT
HMBS	TGCAGTTTGAAATCATTGCTATGTC	AACAGGCTTTTCTCTCCAATCTTAGA
TLR1	TGCTGCCAATTGCTCATTTG*	GAAGGCCCTCAGGGTCTTCT*
TLR2	GGCTTCTCTGTCTTGTGACCG*	GAGCCCTGAGGGAATGGAG*
TLR4	CTGCAATGGATCAAGGAACCAG*	CCATTCGTTCAACTTCCACCA*
TLR6	CACAGACAGCTTTGTACACCGTG*	TGTGCTTGGTGCATGAGGA*
TLR7	GCTCCGGAAAAGGCTCTGT*	GGTGAGCTTGCGGGTTTGT*
TLR9	CCCAGCTACATCCCGATACCT*	CTGGCATTCAGCCAGGAGA*

*Primers were kind gifts from Dr. J. Garcia-Vallejo (Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam), and have been published elsewhere (38).

The PCR products were subjected to melting curve analysis to test for specificity of PCR products. The RT^2 Profiler PCR Array for TLR signalling was performed according to manufacturers' protocol on Roche LightCycler 480 (F. Hoffmann-La Roche AG, Basel, Switzerland). This array profiles the expression of 84 genes related to TLR-mediated signal transduction using real-time PCR (SA Biosciences, assay no. PAHS-018).

Relative gene expression of samples was compared with that of the housekeeping gene by calculating the ΔCt ($Ct_{\text{housekeeping gene}} - Ct_{\text{gene of interest}}$), and expression of the different genes is expressed as $2^{-(\Delta Ct)}$. The fold increase in gene expression (induction) was expressed as $2^{-(\Delta\Delta Ct)}$, wherein $\Delta\Delta Ct = \Delta Ct_{\text{challenged}} -$ average Ct value$_{\text{nonchallenged}}$.

Statistical analysis

Comparisons between total mRNA expression and induction of gene expression between groups were tested with Student's unpaired *t*-test. If variances in groups were not equal, Welch's correction for unequal variances was applied. If data in groups was not normally distributed, the Mann–Whitney *U*-test was used. Tests were performed with GraphPad Prism software (version 4, by MacKiev Software, Boston, MA, USA).

Results

The mRNA expression of *TLR1*, *TLR4*, *TLR7* and *CD14* is higher in periodontal ligament fibroblasts from patients

We measured mRNA expression levels (relative to the housekeeping gene) of *TLR1*, *TLR2*, *TLR4*, *TLR6*, *TLR7*, *TLR9* and *CD14*, by periodontal ligament and gingival fibroblasts, before and after a challenge with live *P. gingivalis*. The expression of *TLR9* was below detection limits in both periodontal ligament and gingival fibroblasts. All other receptors were expressed by the two cell types.

Periodontal ligament fibroblasts from patients consistently expressed higher mRNA levels of *TLR1*, *TLR4*, *TLR7* and *CD14* than periodontal ligament fibroblasts from control subjects, both before and after challenge with *P. gingivalis* (Fig. 1A, C–E). *TLR2* was expressed at similar levels in periodontal ligament fibroblasts from patients and control subjects (Fig. 1B), as was *TLR6* (data not shown).

Such differences did not occur in gingival fibroblasts. Before and after *P. gingivalis* challenge, all receptors analysed were expressed at similar levels in gingival fibroblasts from patients and control subjects (Fig. 2A–E).

Induction of *TLR1*, *TLR2* and *TLR7* by *P. gingivalis* is stronger in gingival fibroblasts from patients

In spite of the differences in receptor mRNA expression between periodontal ligament fibroblasts from patients and control subjects, the responses of the receptors to *P. gingivalis*, expressed as induction (the fold increase in gene expression in challenged compared with nonchallenged cells) were the same in periodontal ligament fibroblasts from patients and control subjects for *TLR1* [2.7- vs. 2.8-fold; not significant (n.s.)], *TLR2* (1.9- vs. 1.8-fold; n.s.), *TLR4* (1.9- vs. 2.1-fold; n.s.), *TLR7* (1.7- vs. 1.7-fold; n.s.) and *CD14* (1.6- vs. 2.7-fold; n.s.; Fig. 3A). This was in line with total mRNA expression; as gene expression of *TLR1*, *TLR4*, *TLR7* and *CD14* was already higher in periodontal ligament fibroblasts from patients without a *P. gingivalis* challenge, this remained true also after challenge.

In gingival fibroblasts, however, we found that the *P. gingivalis* challenge led to a stronger induction of *TLR1* (3.6- vs. 1.8-fold; $p < 0.05$), *TLR2* (2.8- vs. 1.6-fold; $p < 0.05$) and *TLR7*- (2.4 vs. 1.4-fold; $p < 0.05$) in gingival

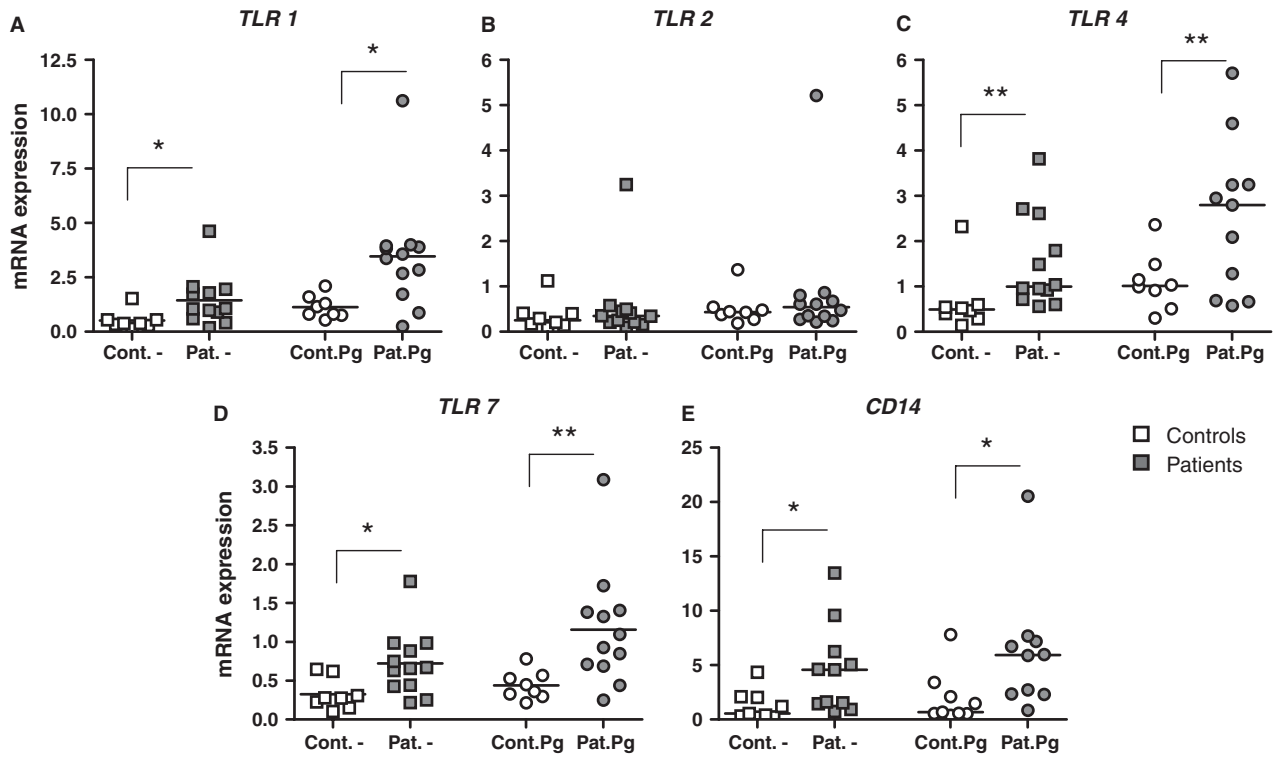

Fig. 1. Messenger RNA expression levels of *TLR1* (A), *TLR2* (B), *TLR4* (C), *TLR7* (D) and *CD14* (E) in periodontal ligament fibroblasts from control subjects ($n = 8$, open symbols) and patients ($n = 12$; shaded symbols), before (squares; –) and after (circles; Pg) a 6 h challenge with viable *P. gingivalis*. Squares/circles represent the average mRNA expression levels of each donor from bacterial challenge experiments performed in quadruplicate. Horizontal lines in groups represent means (Gaussian distribution of data; *TLR1* and *TLR7*) or medians of groups (non-Gaussian distribution; *TLR2*, *TLR4* and *CD14*). $*p < 0.05$, $**p < 0.01$, significant difference between control group and patient group.

fibroblasts from patients than in those from control subjects (Fig. 3B). Induction of *TLR4* and *CD14* was similar in gingival fibroblasts from patients and those from control subjects (2.4- vs. 1.4-fold and 1.8- vs. 1.4-fold, respectively; n.s.; Fig. 3B). The differences in responses, however, did not lead to differences in total mRNA expression of *TLR1*, *TLR2* and *TLR7* after the *P. gingivalis* challenge.

Messenger RNA expression of *NFkBIL1* is lower in periodontal ligament fibroblasts from patients

In periodontal ligament and gingival fibroblasts, *NFKB* mRNA expression was the same in fibroblasts from patients and control subjects before and after *P. gingivalis* challenge (data not shown). Also, *NFKB* induction by *P. gingivalis* was the same in patients and control subjects (data not shown).

When we analysed the induction of 84 genes involved in TLR signalling in gingival fibroblasts from one representative patient, using a PCR array (SA Biosciences), the NFκB-inhibitor-like protein, NFκBIL-1 (*NFKBIL1*), appeared to be down-regulated by *P. gingivalis* (data not shown). Analysis of expression of this gene in gingival and periodontal ligament fibroblasts from all patients using specific primers in real-time PCR showed that it was not down-regulated in cells from all donors, but it did show that before a challenge with *P. gingivalis*, the mRNA expression of *NFKBIL1* was lower in periodontal ligament fibroblasts from patients than in those from control subjects (Fig. 4A). After *P. gingivalis* challenge, mRNA expression of *NFKBIL1* still appeared lower in periodontal ligament fibroblasts from patients, but this was no longer statistically significant ($p = 0.0664$; Fig. 4A).

In gingival fibroblasts, there were no differences between patients and control subjects for *NFKBIL1* mRNA expression, or for induction (Fig. 4B and data not shown).

Induction of proinflammatory cytokines by *P. gingivalis* is not different between patients and control subjects in both periodontal ligament and gingival fibroblasts

A challenge with viable *P. gingivalis* led to induction of *IL1B*, *IL6*, *IL8*, *TNF*, *CCL5* and *CCL2* in both periodontal ligament and gingival fibroblasts from all donors (Table 3). However, these responses were not different between patients and control subjects, in either periodontal ligament or gingival fibroblasts.

Considerable donor heterogeneity existed in the scale of the responses in both periodontal ligament and gingival fibroblasts, as can be seen in the wide

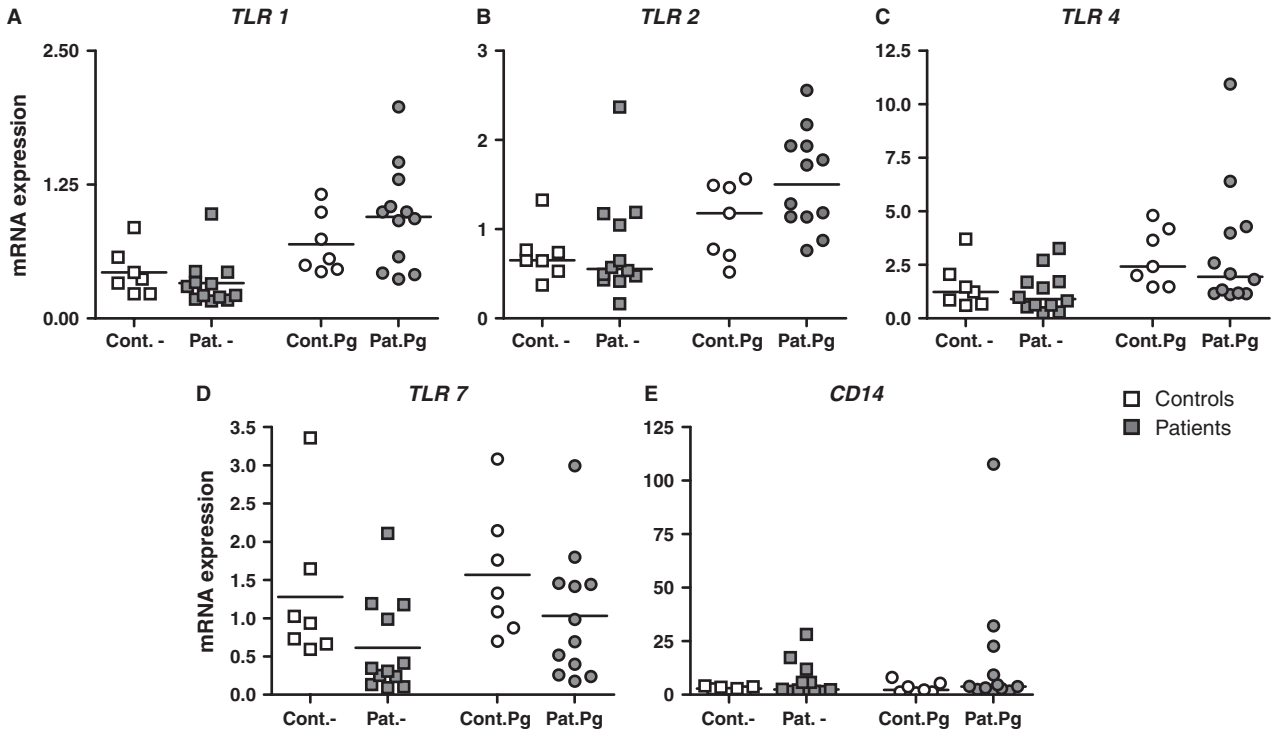

Fig. 2. Messenger RNA expression levels of *TLR1* (A), *TLR2* (B), *TLR4* (C), *TLR7* (D) and *CD14* (E) in gingival fibroblasts from control subjects ($n = 7$, open symbols) and patients ($n = 12$; shaded symbols), before (squares; –) and after (circles; Pg) a 6 h challenge with viable *P. gingivalis*. Squares/circles represent the average mRNA expression levels of each donor from bacterial challenge experiments performed in quadruplicate. Horizontal lines in groups represent means (Gaussian distribution of data; *TLR1* and *TLR7*) or medians of groups (non-Gaussian distribution of data; *TLR2*, *TLR4* and *CD14*).

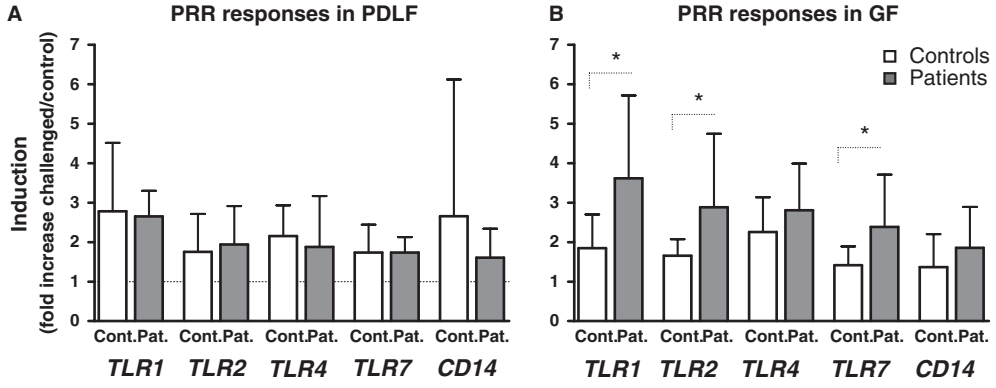

Fig. 3. Pattern recognition receptor (PRR) responses in periodontal ligament fibroblasts (PDLF; A) and gingival fibroblasts (GF; B). Induction of gene expression in response to a 6 h challenge with viable *P. gingivalis* (fold increase in gene expression in challenged compared with nonchallenged cells) of *TLR1*, *TLR2*, *TLR4*, *TLR7* and *CD14* in periodontal ligament fibroblasts (A) and gingival fibroblasts (B) from control subjects (open bars) and patients (shaded bars). Bars represent the mean induction level + SD from all donors within a group from bacterial challenge assays performed in quadruplicate; *CD14* in periodontal ligament fibroblasts (A) is represented as median + interquartile range (non-Gaussian distribution of data). *$p < 0.05$, significant difference between control group and patient group.

range of responses (Table 3). For *IL1B* and *TNF*, a fold increase in gene expression could not be calculated for all donors, because in some donors the mRNA expression was only detectable after the *P. gingivalis* challenge and not before.

Cytokine mRNA expression, before or after *P. gingivalis* challenge, was also not different between patients and control subjects in both periodontal ligament and gingival fibroblasts (data not shown).

Periodontal ligament fibroblasts from *P. gingivalis* carriers respond more strongly to a *P. gingivalis* challenge

When grouping donors not into periodontitis patients and control subjects,

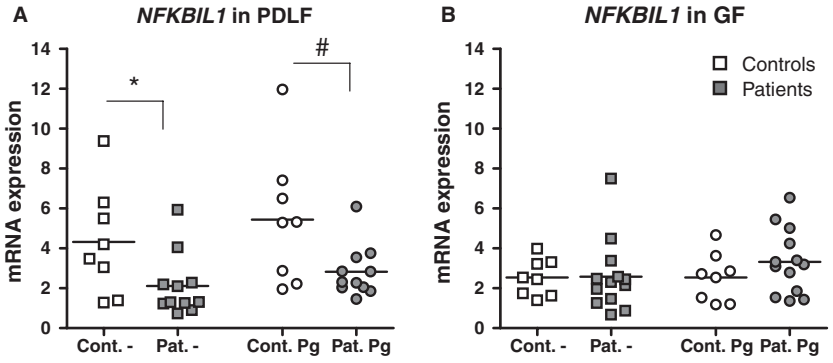

Fig. 4. Messenger RNA expression levels of *NFKBIL1* in periodontal ligament fibroblasts (PDLF; A) from control subjects ($n = 8$, open symbols) and patients ($n = 12$; shaded symbols) and gingival fibroblasts (GF; B) from control subjects ($n = 7$; open symbols) and patients ($n = 12$; shaded symbols), before (squares; –) and after (circles; Pg) a 6 h challenge with viable *P. gingivalis*. Squares/circles represent the mean mRNA expression levels of each donor from bacterial challenge experiments performed in quadruplicate. *$p < 0.05$, $p = 0.0664$, significant difference between control group and patient group.

Table 3. Induction by *P. gingivalis* of gene expression (fold increase in challenged compared with nonchallenged cells) of proinflammatory cytokines and chemokines

	Periodontal ligament fibroblasts		Gingival fibroblasts	
Gene	Control subjects ($n = 8$)	Patients ($n = 12$)	Control subjects ($n = 7$)	Patients ($n = 12$)
IL1B (mean ± SD)	61 ± 91	27 ± 44$^{n.s.}$	293 ± 433	132 ± 228$^{n.s.}$
(range)	(8–244)	(1–125)	(2–953)	(2–677)
IL6	9 ± 7	5 ± 4$^{n.s.}$	25 ± 35	21 ± 21$^{n.s.}$
	(1–21)	(1–11)	(2–93)	(2–71)
IL8	80 ± 79	49 ± 68$^{n.s.}$	59 ± 69	93 ± 80$^{n.s.}$
	(2–212)	(1–211)	(5–200)	(6–232)
TNF	23 ± 22	20 ± 22$^{n.s.}$	25 ± 28	33 ± 45$^{n.s.}$
	(0–58)	(0–58)	(1–61)	(1–143)
CCL2	4 ± 4	6 ± 7$^{n.s.}$	13 ± 19	26 ± 39$^{n.s.}$
	(1–11)	(1–27)	(2–53)	(2–140)
CCL5	12 ± 18	7 ± 16$^{n.s.}$	7 ± 8	9 ± 9$^{n.s.}$
	(1–50)	(0–57)	(1–23)	(1–31)

n.s., no significant difference between patient and control group.

but rather into *P. gingivalis* carriers (donors in whom *P. gingivalis* was detected by anaerobic culture in subgingival plaque) and noncarriers (donors who had no detectable *P. gingivalis* in subgingival plaque), interesting differences were noted between the periodontal ligament fibroblasts from these two groups.

At the receptor level, *TLR1* was induced more strongly by *P. gingivalis* in periodontal ligament fibroblasts from carriers than in those from noncarriers (3.3- vs. 2.4-fold; $p < 0.05$; Fig. 5A). *TLR7* appeared to be induced more strongly in carriers, but this was not statistically significant (2.0- vs. 1.6-fold, $p = 0.0861$; Fig. 5A).

At the transcription factor level, *NF*κB1 was induced more strongly by *P. gingivalis* in periodontal ligament fibroblasts from carriers compared with noncarriers (2.7- vs. 1.4-fold, $p < 0.05$; Fig. 5B).

The cytokine *IL1B* and chemokine *CCL5* were also induced more strongly by *P. gingivalis* in periodontal ligament fibroblasts from carriers compared with noncarriers (88.9- vs. 11.9-fold. $p < 0.05$, and 19.3- vs. 3.3-fold, $p < 0.01$, respectively; Fig. 5C). *CCL2* appeared to be induced more strongly by *P. gingivalis* in periodontal ligament fibroblasts from carriers, but this was not statistically significant (9.4- vs. 2.4-fold, $p = 0.0781$; Fig. 5C).

Interestingly, this difference between *P. gingivalis*-positive and *P. gingivalis*-negative donors was not present in gingival fibroblasts.

Discussion

In the present study we hypothesized that, as the host response is important in the development of periodontitis, periodontal fibroblasts respond in a different manner to a bacterial challenge in periodontitis patients compared with healthy control subjects. Therefore, we challenged primary periodontal ligament fibroblasts and gingival fibroblasts from patients and healthy control subjects with viable *P. gingivalis*.

Overall, the periodontal ligament fibroblasts from periodontitis patients appeared to be in a more inflammatory state compared with those from control subjects; whether challenged or nonchallenged, patients' periodontal ligament fibroblasts had higher mRNA expression of *TLR1*, *TLR4*, *TLR7* and *CD14*. This could mean that they are more prone to recognize and respond to *P. gingivalis*. In gingival fibroblasts, mRNA expression of *TLR1*, *TLR2*, *TLR4*, *TLR7* and *CD14* was similar in patients and control subjects. However, in patients' gingival fibroblasts, a *P. gingivalis* challenge led to a stronger induction of *TLR1*, *TLR2* and *TLR7*, suggesting a higher responsiveness of patients' gingival fibroblasts to *P. gingivalis* at the receptor level.

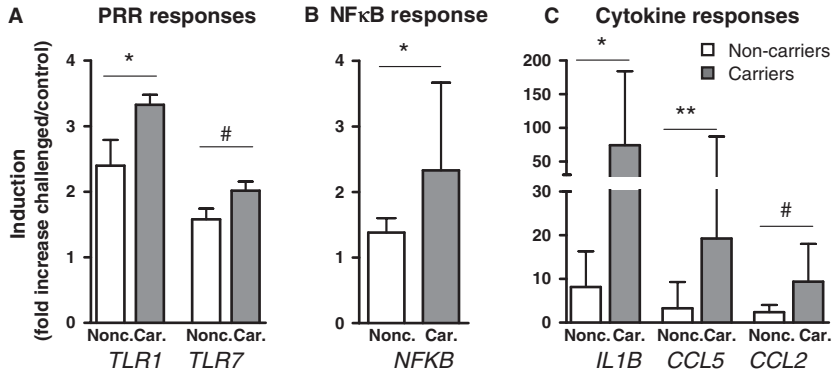

Fig. 5. Induction of gene expression in response to a 6 h challenge with viable *P. gingivalis* (fold increase in gene expression in challenged compared with nonchallenged cells) of *TLR1* and *TLR7* (A), *NFKB* (B) and *IL1B*, *CCL5* and *CCL2* (C) in periodontal ligament fibroblasts from persons who did not carry *P. gingivalis* (open bars; nonc.) and carriers of *P. gingivalis* (shaded bars; car.). Bars represent the mean induction level + SD (Gaussian distribution of data; *TLR1*, *TLR7* and *CCL2*) or median + interquartile range (non-Gaussian distribution of data; *NFKB1*, *IL-1B* and *CCL5*) from all donors within a group from bacterial challenge assays performed in quadruplicate. $*p < 0.05$, $**p < 0.01$, significant differences between noncarrier group and carrier group; $p = 0.0860$ for *TLR7* (A) and $\#p = 0.0781$ for *CCL2* (B).

In particular, TLR2, but also TLR4, are the TLRs frequently described as recognizing and interacting with *P. gingivalis* (17,18,20,22–24,39). Toll-like receptor 2 can form heterodimers with TLR1 or with TLR6 (19,21).

In our study, *TLR4* mRNA expression was higher in patients' periodontal ligament fibroblasts. In contrast, *TLR2* induction in response to *P. gingivalis* was higher in gingival fibroblasts, indicating that gingival and periodontal ligament fibroblasts interact in a different manner with *P. gingivalis* or its components at the receptor level. Furthermore, *TLR1* expression was higher in patients' periodontal ligament fibroblasts and was induced more strongly by *P. gingivalis* in patients' gingival fibroblasts. Since the TLR2–TLR1 complex is important for recognition of *P. gingivalis* (19), this suggests a stronger susceptibility to *P. gingivalis* of patients' gingival and periodontal ligament fibroblasts.

In periodontal ligament fibroblasts, but not gingival fibroblasts, from patients, mRNA expression of *NFKBIL1* before bacterial challenge was lower compared with control subjects. Also after challenge, it appeared to be lower. NFκBIL-1 (or IκBL) is a protein homologous to the IκB protein family that regulates activation of NFκB. Polymorphisms in the *NFKBIL1* gene have been linked to inflammatory diseases, such as ulcerative colitis, rheumatoid arthritis, or Chagas' cardiomyopathy (40–42). Although the exact function of NFκBIL-1 has not yet been determined, it is considered to be a putative inhibitor of NFκB (43,44). A lower expression of this inhibitor could therefore mean that NFκB is more easily activated in patients' periodontal ligament fibroblasts.

In spite of the differences between patients and control subjects at receptor and transcription factor level, cytokine responses in periodontal ligament or gingival fibroblasts from patients and control subjects were the same. This could be due to the relatively short duration of the bacterial challenge, being 6 h, as it may take more time for differences in cytokine responses to become detectable. However, as *P. gingivalis* would die during longer challenges due to the aerobic environment, we chose 6 h. The very large heterogeneity in the scale of cytokine responses between individuals within both the patient and the control group may also make it more difficult to locate differences at the cytokine level. We have previously shown that cytokine responses to viable *P. gingivalis* by periodontal ligament and gingival fibroblasts from young healthy donors (mean age 22.5 years) also vary greatly (13).

Nevertheless, it appears that fibroblasts from patients are in a more activated state than those from healthy control subjects. This might be caused by genetic, intrinsic properties of patients' periodontal ligament and gingival fibroblasts, but also by the inflammatory state of the periodontal tissue from patients. It has been previously shown that gingival and periodontal ligament fibroblasts can stably retain their phenotypes *in vitro* (45). Recently, it was described that periodontal ligament fibroblasts isolated from periodontally affected sites retained their inflammatory phenotype *in vitro*, and behaved in a different way from those isolated from healthy sites (46).

Interestingly, periodontal ligament fibroblasts from individuals who harboured *P. gingivalis* in their subgingival plaque responded more strongly to the *in vitro* challenge with *P. gingivalis* than those from individuals who did not carry *P. gingivalis*. Thus, these periodontal ligament fibroblasts might have retained a more inflammatory phenotype *in vitro*, caused by a prior (recent) *in vivo* encounter with *P. gingivalis*. This may have rendered the periodontal ligament fibroblasts more responsive to a new (*in vitro*) challenge.

In contrast to the periodontal ligament fibroblasts, we found no such increased responsiveness to *P. gingivalis* in gingival fibroblasts; all gene-expression responses were similar in gingival fibroblasts from *P. gingivalis* carriers and those from noncarriers. In line with this finding is a study by Ara *et al.* (6), who showed that pretreatment of gingival fibroblasts with

P. gingivalis lipopolysaccharide did not effect their response to a second treatment.

The mechanism by which periodontal ligament fibroblasts, but not gingival fibroblasts, seem to 'remember' *P. gingivalis* remains to be elucidated and is an interesting topic for further research. We speculate that for periodontal ligament or gingival fibroblasts to interact with *P. gingivalis*, it is possible that different TLRs and subsequent downstream signalling pathways are of importance. For instance, TLR2 and TLR4 can both induce inflammatory cytokines through the adaptor molecule myeloid differentiation primary response gene (88). In contrast, TLR4, but not TLR2, can also signal via adaptor molecule TRIF-related adaptor molecule and thereby induce different sets of genes (16). It is possible that negative regulators of TLR signalling may play a role; lower expression or activation of such regulators in periodontal ligament fibroblasts may allow for increasing responsiveness to *P. gingivalis*, whereas higher expression or activation in gingival fibroblasts might prevent inflammatory responses from becoming stronger.

Gingival and periodontal ligament fibroblasts have been shown previously to differ in various aspects, and it has been known for a long time that heterogeneous subsets of fibroblasts can play distinct roles in inflammation (45).

Owing to their location in the periodontium, gingival fibroblasts are more likely to encounter *P. gingivalis*; therefore, they can contribute to a first line of defence by providing signals to attract an infiltrate of inflammatory cells. A higher responsiveness of patients' gingival fibroblasts may then lead to a more vigorous innate immune response. Furthermore, as their responsiveness towards *P. gingivalis* is not influenced by a prior encounter, they may continuously provide inflammatory signals.

Periodontal ligament fibroblasts are located closer to the alveolar bone and appear to play a role in its remodelling (9–11). When infection spreads to the periodontal ligament, a higher responsiveness of patients' periodontal ligament fibroblasts may have more explicit consequences for osteoclast formation. Moreover, as responsiveness of periodontal ligament fibroblasts towards *P. gingivalis* seems to increase after a prior encounter, this could mean that their inflammatory responses increase in strength during the presence of bacteria. This may lead to better bacterial clearance, but also to more tissue damage.

In conclusion, we found that periodontal ligament and gingival fibroblasts, either from periodontitis patients or from healthy control subjects, respond in a different manner to a challenge with *P. gingivalis*. Moreover, the periodontal ligament fibroblasts in particular, and to a lesser extent the gingival fibroblasts, from patients seem to be in a more activated state at the receptor level than fibroblasts from healthy control subjects. In addition, periodontal ligament fibroblasts from *P. gingivalis* carriers are more responsive to an *in vitro* challenge with *P. gingivalis* than those from noncarriers. Thus, gingival and periodontal ligament fibroblasts may play different roles in periodontitis, and they may be hyper-reactive in periodontitis patients.

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