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Gingival fibroblast responsiveness is differentially affected by *Porphyromonas gingivalis*: implications for the pathogenesis of periodontitis

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SUMMARY

In periodontitis, tissue damage results mainly from aberrant host responses to oral microorganisms. Fibroblasts can play an important role in this. Gingival fibroblasts do not develop tolerance against the lipopolysaccharide of *Porphyromonas gingivalis,* a keystone pathogen in periodontitis, which may partly explain the persistence of inflammation. However, besides lipopolysaccharide, live *P. gingivalis* possess numerous virulence traits to impair host-responses. We hypothesized that fibroblast-responsiveness to a bacterial challenge could be affected by live *P. gingivalis.* We investigated if inflammatory responses of gingival fibroblasts to *P. gingivalis* were altered, when the fibroblasts had encountered *P. gingivalis* previously. On consecutive days, primary human gingival fibroblasts were challenged twice for 6 h with live *P. gingivalis*, or fibroblasts were preincubated for 24 h with a lower concentration of live *P. gingivalis* and re-challenged for 6 h with a higher concentration. As the *P. gingivalis* capsule and proteases are involved in modulating host responses, we used encapsulated *P. gingivalis* W83 and a non-encapsulated mutant, and *P. gingivalis* ATCC33277 and a lys-gingipain and arg-gingipain mutant, to challenge fibroblasts. With all *P. gingivalis*-strains, interleukin-8 and monocyte chemoattractant protein-1 responses to the second challenge were less strong in fibroblasts that had been challenged with *P. gingivalis* before. These lower responses might correspond with higher interleukin-1 receptor agonist expression. Fibroblast responses to a second challenge were not influenced by 24 h preincubation. Reduced chemokine responses after consecutive potent *P. gingivalis* challenges indicate that gingival fibroblast responsiveness is affected by a previous bacterial encounter. In periodontitis, such reduced chemokine responses may impair chemotaxis and clearance of oral microorganisms, thereby leading to prolonged inflammatory responses and tissue damage.

INTRODUCTION

Periodontitis is a complex chronic inflammatory disease of the tooth-supporting tissues that can lead to severe tissue damage, degradation of the alveolar

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bone, and tooth loss. Periodontal tissue damage results mainly from an exaggerated and prolonged host inflammatory response against pathogenic oral microflora (Feng & Weinberg, 2006; Darveau, 2009, 2010). It is now widely recognized that periodontitis is a polymicrobial and multifactorial disease, in which disease is induced by a shift in the oral microflora and ecology, rather than by a single pathogen (Wade, 2011). Nonetheless, several oral pathogens, such as *Porphyromonas gingivalis*, have long been strongly associated with periodontitis (Socransky *et al*., 1998; Darveau, 2010). As *P. gingivalis* has many advanced mechanisms to de-regulate the inflammatory responses of host cells, that may also benefit other microorganisms in the oral biofilm, it has even been indicated as a 'keystone' species in periodontitis (Darveau, 2009; Hajishengallis *et al*., 2012).

Various host cells in the periodontium are involved in the inflammatory response in periodontitis. They include gingival fibroblasts, i.e. connective-tissue cells that form a substantial part of the periodontium (Phipps *et al*., 1997). As well as playing an essential role in gingival tissue homeostasis, these fibroblasts are also equipped to recognize and respond to bacteria (Wang & Ohura, 2002; Uehara & Takada, 2007). For instance, we have previously shown that they respond to live *P. gingivalis* by upregulating the expression of a range of proinflammatory cytokines and chemokines associated with periodontitis (Scheres *et al*., 2010, 2011).

Although fibroblasts are not by nature inflammatory cells, they can be important determinants in the development of chronic inflammatory diseases (Buckley *et al*., 2001; Flavell *et al*., 2008; Buckley, 2011). They can provide both the signals to attract and regulate an infiltrate of inflammatory cells to clear an infection at the necessary site, and the signals that ensure clearance of the inflammatory cell infiltrate after the initial inflammation has been resolved. Deregulation of these processes may lead to prolonged inflammation (Buckley *et al*., 2001; Buckley, 2011).

A critical role in periodontitis may be played by gingival fibroblasts. Human gingival fibroblasts do not develop endotoxin tolerance towards the lipopolysaccharide (LPS) of *P. gingivalis* (Ara *et al*., 2009; Zaric *et al*., 2011). As a result of this lack of tolerance, gingival fibroblast may persistently provide proinflammatory signals, thereby favoring the development of a chronic inflammation (Ara *et al*., 2009). Nonetheless, gingival fibroblasts are also likely to interact with live cells and other components of *P. gingivalis* besides LPS (O'Brien-Simpson *et al*., 2004; Pathirana *et al*., 2010). As *P. gingivalis* possesses many mechanisms to subvert the host response, we hypothesized that these bacteria–host cell interactions may influence the responses of the fibroblasts towards a later encounter with *P. gingivalis*. In this study we therefore investigated whether the responsiveness of gingival fibroblasts towards a challenge with live *P. gingivalis* was affected by a previous encounter with live *P. gingivalis*. To do so, we challenged gingival fibroblasts twice with live *P. gingivalis* on two consecutive days. Fibroblasts were also preincubated with a lower concentration of live *P. gingivalis* and then directly re-challenged with a higher concentration of live *P. gingivalis*. The *P. gingivalis* capsule and its lysine-specific gingipain Kgp and arginine-specific gingipains RgpA and RgpB (Guo *et al*., 2010), can play an important role in modulating the host response. Therefore, the fibroblasts were challenged with either encapsulated strain W83 or with a non-encapsulated isogenic mutant, and with strain ATCC33277 or two isogenic gingipain mutants, lacking either the lys-gingipain, or the arg-gingipains (Shi *et al*., 1999; Brunner *et al*., 2010; Singh *et al*., 2011; Kaman *et al*., 2012). To measure fibroblast responses, we analysed the gene expression and protein expression of the chemokine interleukin-8 (IL-8), and gene expression of monocyte chemotactic protein 1 (MCP-1), the proinflammatory cytokines IL-1β and IL-6, and the anti-inflammatory mediators IL-1 receptor agonist (IL-1ra) and transforming growth factor-β (TGF-β). We found that repeated potent challenges with live *P. gingivalis* did indeed affect fibroblast responsiveness towards a new bacterial challenge: if fibroblasts had already been challenged with live *P. gingivalis* the previous day, fibroblast chemokine responses to a second challenge with *P. gingivalis* were less strong.

METHODS

Bacterial strains and culture

Porphyromonas gingivalis wild-type W83 (serotype K1) and a non-encapsulated isogenic mutant (Δ*epsC*, knockout in the epimerase-coding gene *epsC*) (Brunner

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et al., 2010), wild-type ATCC33277 and isogenic gingipain mutants KDP129 (Δ*kgp*) and KDP133 (Δ*rgpA* Δ*rgpB*) (Shi *et al.*, 1999; Kaman *et al.*, 2012) were cultured anaerobically (80% N_2, 10% H_2, 10% CO_2) until log-growth phase in brain–heart infusion (BHI) broth enriched with hemin (5 mg l^{-1}) and menadione (1 mg l^{-1}). Mutant strains KDP129 and KDP133 were kind gifts from Dr F. Bikker (Dept of Biochemistry, Academic Centre for Dentistry Amsterdam, the Netherlands).

For challenge assays, bacteria were harvested by centrifugation, washed in sterile phosphate-buffered saline and in antibiotic-free Dulbecco's minimal essential medium (DMEM; Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT); and suspended in antibiotic-free DMEM with 10% FCS at the appropriate concentrations.

Gingival fibroblasts

Primary human gingival fibroblasts from 13 donors (two male, 11 female, mean age 48 ± 16 years, 10 periodontitis patients, three non-periodontitis donors) were obtained during a previous study (Scheres *et al.*, 2011). Briefly, gingival fibroblasts were obtained by dissecting free gingival remains from extracted teeth. Gingival remains were minced, washed and cultured in DMEM with 2% antibiotics (Antibiotic antimycotic solution: 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 250 ng ml^{-1} amphotericin B; Sigma-Aldrich, St Louis, MO) and 10% FCS in a humidified atmosphere with 5% CO_2 at 37°C. Fibroblasts were expanded through passages 1–4 before storage in liquid nitrogen. Fibroblasts from passages 5–7 were used in experiments. The fibroblastic nature of the cells had been confirmed previously by examining the expression of the fibroblast marker gene *FMOD* (Scheres *et al.*, 2011).

Fibroblast donors had given written informed consent. The collection and use of the fibroblasts were approved by the VUmc Medical Ethical Committee (VU University Medical Centre, Amsterdam, the Netherlands).

Bacterial challenge assays

For bacterial challenge assays, gingival fibroblasts were seeded into 24-well plates at a concentration of 10^4 cells $well^{-1}$ in 0.5 ml antibiotic-free DMEM with 10% FCS.

Two P. gingivalis *challenges (pre-challenge and re-challenge) on consecutive days*

On day one, gingival fibroblasts were incubated in 24-well plates for 6 h with 0.5 ml of a suspension of 2×10^8 colony-forming units (CFU) ml^{-1} live *P. gingivalis* (strains as indicated in Results section) in antibiotic-free DMEM with 10% FCS (pre-challenge). Control fibroblasts were challenged with only DMEM + 10% FCS. After 6 h, they were washed with sterile phosphate-buffered saline to remove unattached bacteria, and either lysed for gene-expression analysis, or incubated in DMEM + 10% FCS and 1% antibiotics for 30 min to kill attached bacteria; according to viability counts, this concentration of antibiotics completely abolished *P. gingivalis* growth after only 5 min. The fibroblasts were then washed three times to remove killed bacteria, and incubated overnight in antibiotic-free medium. The following day, 18 h after the pre-challenge, fibroblasts were either lysed, or were re-challenged with a 2×10^8 CFU ml^{-1} suspension of the same *P. gingivalis* strain for 6 h (re-challenge). Control fibroblasts were challenged with only DMEM + 10% FCS. After 6 h, fibroblasts were washed and lysed for gene-expression analysis.

Fibroblasts were lysed in the lysis buffer supplied with the RNeasy Mini Kit for RNA extraction (Qiagen, Hilden, Germany) with β-mercaptoethanol.

Preincubation and re-challenge with P. gingivalis

On day one, gingival fibroblasts were incubated for 24 h with 0.5 ml of a suspension of 2×10^6 CFU ml^{-1} *P. gingivalis* W83 or *epsC* in antibiotic-free medium with 10% FCS (preincubation). Control fibroblasts were incubated with medium only. After 24 h, on day two, fibroblasts were washed and then directly re-challenged for 6 h with a 2×10^8 CFU ml^{-1} *P. gingivalis* suspension. Only DMEM + 10% FCS was added to control fibroblasts. After 6 h, fibroblasts were lysed for gene-expression analysis.

After all *P. gingivalis* challenges, fibroblast morphology was checked for abnormalities or cell detachment by phase-contrast microscopy (Olympus CK2, Olympus, Japan). Visual examination and Trypan Blue exclusion indicated no significant decrease in fibroblast viability after a 6-h challenge with 2×10^8 CFU ml^{-1} *P. gingivalis* (pre-/re-challenge) or

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a 24-h challenge with 2×10^6 CFU ml^{-1} *P. gingivalis* (preincubation).

mRNA expression

Fibroblast RNA was isolated using the Qiagen RNeasy Mini Kit. RNA concentrations were measured on a Nanodrop spectrophotometer (NanoDrop Technologies; Thermo-Fischer Scientific, Wilmington, DE). The mRNA was reverse-transcribed to cDNA with the MBI Fermentas RevertAid cDNA synthesis kit (Fermentas, Vilnius, Lithuania), using Oligo(dT)18 and D (N)6 primers.

Real-time polymerase chain reaction (PCR) primers for IL-1β (gene *IL1B*), IL-6 (*IL6*), IL-8 (IL8), MCP-1 (*CCL2*), TGF-β (*TGFB1*), IL-1ra (*IL1RN*), and housekeeping genes YWHAZ and β_2-microglobulin (*B2M*) were purchased from Sigma-Aldrich Co. The primer sequences are shown in Table 1.

Real-time PCR was performed on Roche LightCycler 480 (F. Hoffmann-La Roche AG, Basel, Switzerland). Reactions were performed with 2 ng of cDNA in a total volume of 11 μl containing Roche LightCycler® 480 SYBR Green I Master Mix, and 0.91 pmol $^{-1}$ of each primer. The quantitative PCR human reference total RNA (Stratagene, La Jolla, CA) was used as an external standard curve.

After an activation step of 5 min at 95°C, 40 cycles were run of 10 s at 95°C, 5 s at 58°C, 10 s at 72°C and 5 s at 78°C; in the case of IL-1β, 50 cycles were run. The PCR products were subjected to melting curve analysis for specificity. Gene expression of samples relative to that of the housekeeping genes *YWHAZ* and *B2M* was calculated by the formula $2^{-(\Delta C_t)}$, in which ΔC_t is the $C_{t_{\text{gene of interest}}} - \text{average}C_{t_{\text{housekeeping genes}}}$. Fold increase in gene expression (induction) was expressed as $2^{-(\Delta\Delta C_t)}$, wherein $\Delta\Delta C_t = \Delta C_{t_{challenged}} - \text{average}C_t - \text{value}_{\text{non-challenged}}$.

IL-8 protein expression

Protein expression of IL-8 was determined in undiluted culture supernatants of fibroblasts that were either pre-challenged and re-challenged with *P. gingivalis* W83, pre-challenged with medium and re-challenged with *P. gingivalis* W83, or never challenged, as described above. Pelikine IL-8 enzyme-linked immunosorbent assay (Sanquin Blood Supply, Division Reagents, Amsterdam, the Netherlands) were used to determine protein levels, all samples were tested in duplicate and according to manufacturer's protocol.

Statistical analysis

If data were normally distributed, comparisons between the experimental conditions and groups depicted in the figures were tested with Student's *t*-test, or, in the case of pooled groups, with paired *t*-test. In the event of unequal variances, Welch's correction was applied. For data that were not normally distributed, the Mann–Whitney test (unpaired), or Wilcoxon matched pairs test (paired), and Wilcoxon signed rank test were used. Statistical analyses were performed with GraphPad Prism 4 (version 4, by Mackiev Software). Differences were considered statistically significant at $P < 0.05$. If they provided a useful overall image, trends ($P < 0.1$) were also indicated.

RESULTS

Fibroblast IL-8 and MCP-1 responses to a re-challenge with *P. gingivalis* were reduced when fibroblasts were also pre-challenged with *P. gingivalis*

To investigate whether the sensitivity of gingival fibroblasts towards *P. gingivalis* was influenced by a previous encounter with this bacterium, gingival

Table 1 Real-time polymerase chain reaction primer sequences

Primer set	5′–3′ Forward sequence	5′–3′ Reverse sequence
IL-1β	CTTTGAAGCTGATGGCCCTAAA	AGTGGTGGTCGGAGATTCGT
IL-6	GGCACTGGCAGAAAACAACC	GGCAAGTCTCCTCATTGAATCC
IL-8	GGCAGCCTTCCTGATTTCTG	CTGACATCTAAGTTCTTTAGCACTCCTT
MCP-1	CAGCCAGATGCAATCAATGC	TGCTGCTGGTGATTCTTCTATAGCT
IL-1ra	AATCCAGCAAGATGCAAGCC	ACGCCTTCGTCAGGCATATT
TGF-β	CACCCGCGTGCTAATGGT	CTCGGAGCTCTGATGTGTTGAA
β_2-microglobulin	TCTGGCCTGGAGGCTATCCAG	AGAAAGACCAGTCCTTGCTGAA
YWHAZ	GATGAAGCCATTGCTGAACTTG	CTATTTGTGGGACAGCATGGA

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fibroblasts from eight donors were pre-challenged and re-challenged for 6 h on two consecutive days with 2×10^8 CFU ml^{-1} live *P. gingivalis* W83, or with medium alone (controls). We have previously shown that this challenge induces a strong inflammatory response in gingival fibroblasts (Scheres *et al*., 2010). The fibroblasts were chosen randomly from an available fibroblast collection, without selection for certain donor characteristics.

Fibroblasts from all donors responded to the pre-challenge with *P. gingivalis* on day one by increasing the gene expression of IL-8, MCP-1 and IL-6. However, by the time re-challenge started on the second day, gene expression had returned to basal levels (data not shown).

Re-challenge with *P. gingivalis* on day two also increased the gene expression of IL-8, MCP-1 and IL-6. Interestingly, however, when the fibroblasts had been pre-challenged with *P. gingivalis*, IL-8 gene expression in fibroblasts from six donors was significantly lower after the re-challenge on day two, than when these fibroblasts had been pre-challenged with medium (Fig. 1A). In fibroblasts from seven donors, the gene expression of MCP-1 was lower after the re-challenge when the fibroblasts had also been pre-challenged with *P. gingivalis* (Fig. 1B).

Although we also analysed the gene expression of IL-1β, these expression levels were generally very low compared with those of IL-8, MCP-1 and IL-6, and could not always be detected. But while these IL-1β results were less decisive, we still observed a trend similar to that in IL-8 and MCP-1: after re-challenge with *P. gingivalis*, the gene expression of IL-1β appeared to be not as high when fibroblasts had already been pre-challenged with *P. gingivalis* (data not shown). In contrast, except for the fibroblasts from one donor, the gene expression of IL-6 after re-challenge did not differ between fibroblasts that had been pre-challenged with *P. gingivalis* or with medium (Fig. 1C).

To determine the overall effect of a *P. gingivalis* pre-challenge on fibroblast responses to a re-challenge, and to correct for the substantial differences in cytokine expression levels between donors, the fibroblast responses, relative to control fibroblasts, of all donors together were expressed as the actual induction (fold-increase in gene expression) of IL-8, MCP-1 and IL-6 gene expression by the re-challenge (Fig. 1D,E). Although there was also heterogeneity in the induction levels between the different donors, this showed that the gene expression of IL-8 and MCP-1 was indeed induced less strongly by a re-challenge with *P. gingivalis* when fibroblasts had already been pre-challenged with *P. gingivalis* the previous day.

In Fig. 1A–C, donors E and F were non-periodontitis donors; the other donors were patients with periodontitis. Because of the relatively small number of healthy donors, a division into groups would not allow for proper comparison or statistics. All the same, responses in fibroblasts from donors E and F appear not to have differed clearly from responses in fibroblasts from the other donors.

Reduced IL-8 protein expression after pre-challenge and re-challenge with *P. gingivalis*

To confirm whether the reduced chemokine gene-expression responses also resulted in reduced chemokine protein levels, fibroblasts from five of the eight donors used in Fig. 1 were pre-challenged with 2×10^8 CFU ml^{-1} live *P. gingivalis* W83 or medium alone, and re-challenged with 2×10^8 CFU ml^{-1} live *P. gingivalis* W83. The concentration of IL-8 protein in their culture supernatant was assessed. Although IL-8 can be efficiently degraded by *P. gingivalis* proteases (Stathopoulou *et al*., 2009), we have previously found that in spite of this degradation, elevated IL-8 levels could still be detected in culture supernatant of fibroblasts that had been challenged with 2×10^8 CFU ml^{-1} live *P. gingivalis* (Scheres *et al*., 2010).

The IL-8 protein levels that were detected in the culture supernatants of pre-challenged and re-challenged fibroblasts might be an underestimation of the actual levels produced. Nonetheless, after a re-challenge with *P. gingivalis* there was significantly less IL-8 present in the supernatant of fibroblasts that were pre-challenged with *P. gingivalis*, than in the supernatants of fibroblasts that were pre-challenged with medium (Fig. 2). This indicates that the reduced IL-8 gene-expression responses after a *P. gingivalis* pre-challenge correspond with lower IL-8 protein levels.

A pre-challenge with a non-encapsulated *P. gingivalis* mutant also caused reduced responses to a re-challenge

The polysaccharide capsule of *P. gingivalis* is a virulence factor that is known to reduce the host

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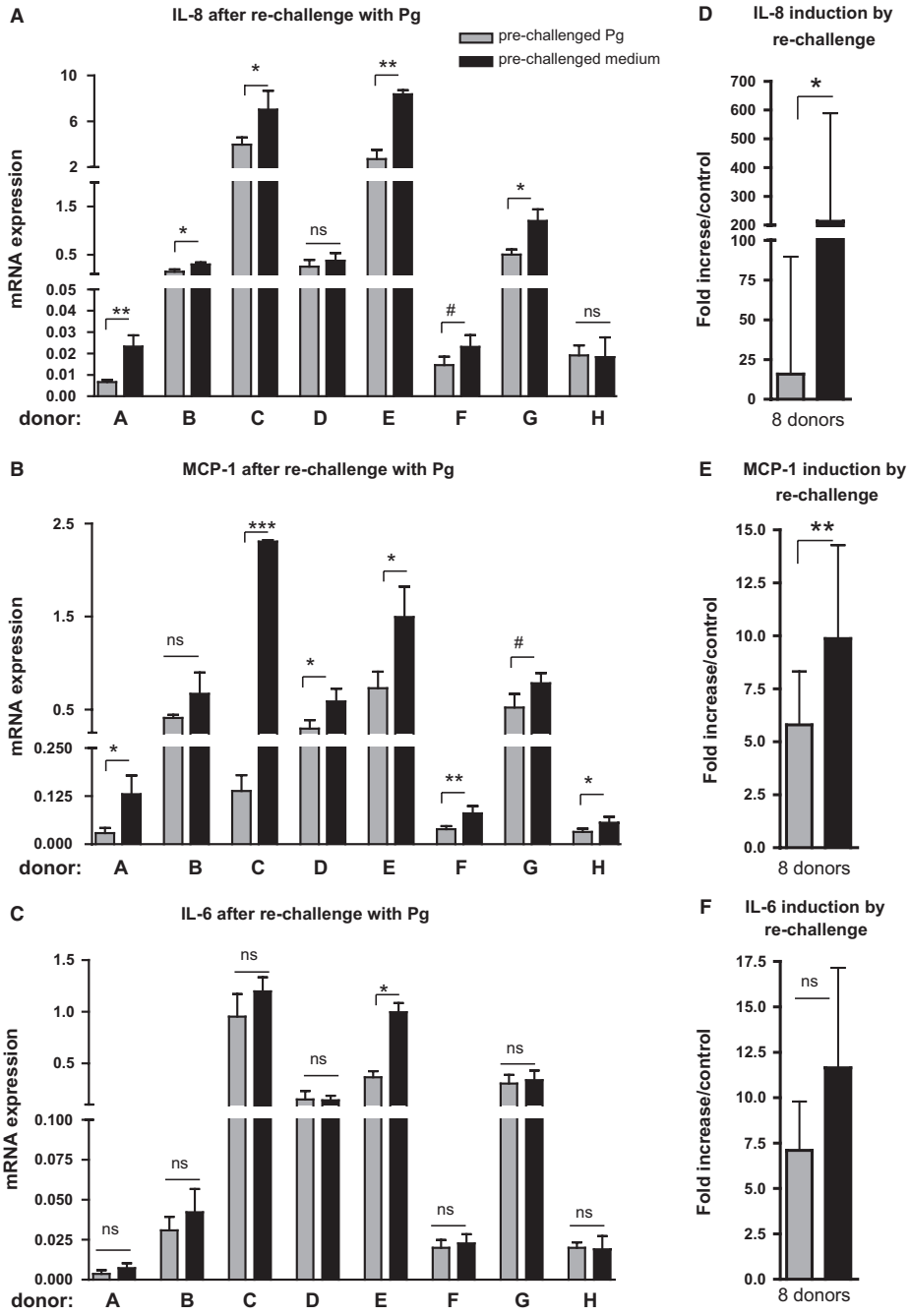

Figure 1 Interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1) and IL-6 gene expression in gingival fibroblasts after re-challenge with *Porphyromonas gingivalis* W83. (A–C) mRNA expression of IL-8 (A), MCP-1 (B) and IL-6 (C) per donor in gingival fibroblasts that were pre-challenged for 6 h with live *P. gingivalis* W83 (grey bars) or medium alone (black bars); and re-challenged the next day for 6 h with live *P. gingivalis* W83. mRNA expression was normalized for housekeeping genes β_2-microglobulin and YWHAZ; bars represent the mean gene-expression level $\pm$ SD per donor in quadruplicate measurements. Two experiments were performed using gingival fibroblasts from eight donors in total; the results were tested with Student's *t*-test. In the event of unequal variances, Welch's correction was applied. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, # $P < 0.1$ (trend; A, $P = 0.059$; B, $P = 0.0504$), ns: not significant. Pg: *P. gingivalis*. (D,E) Overall induction relative to non-challenged controls of gene expression of IL-8 (D), MCP-1 (E) and IL-6 (F) by a re-challenge with *P. gingivalis* W83 in gingival fibroblasts that were either pre-challenged with *P. gingivalis* W83 (grey bars) or with medium alone (black bars). Bars represent the average fold increase $\pm$ SEM in gene expression in fibroblasts from all donors together ($n = 8$). Results were tested with Wilcoxon matched pairs test (IL-8, MCP-1) or Student's paired *t*-test (IL-6). **$P < 0.01$, *$P < 0.05$.

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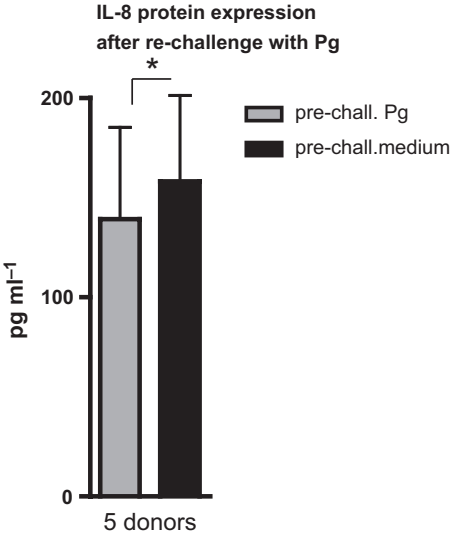

Figure 2 Interleukin-8 (IL-8) in supernatants of gingival fibroblasts after re-challenge with *Porphyromonas gingivalis* W83. IL-8 protein concentration, determined by enzyme-linked immunosorbent assay, in culture supernatants of gingival fibroblasts that were pre-challenged for 6 h with live *P. gingivalis* W83 (grey bar) or medium alone (black bar), and re-challenged the next day for 6 h with live *P. gingivalis* W83. Fibroblasts from five donors (donors A,C,D,E,F) were assayed in quadruplicate. Bars represent the average protein concentration (pg/ml) ± SEM of the five donors pooled together. Results were tested with Student's paired *t*-test. $*P < 0.05$, Pg: *P. gingivalis*.

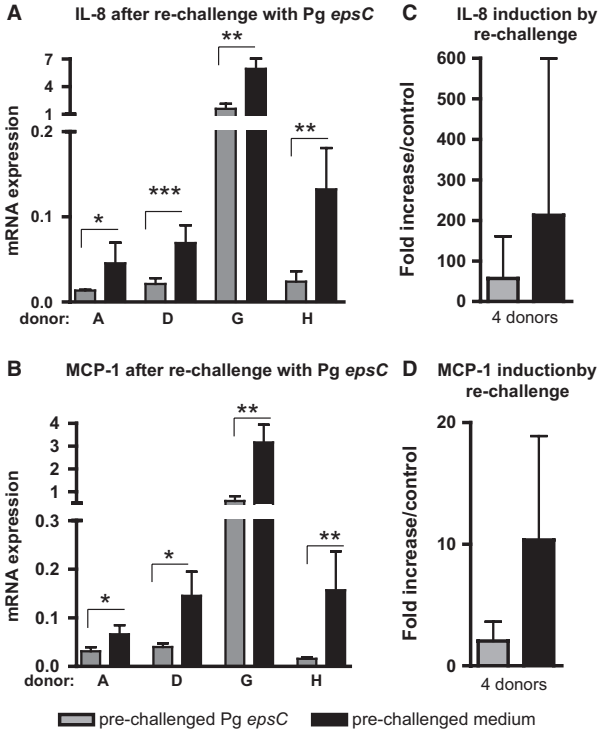

Figure 3 Interleukin-8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) gene expression in gingival fibroblasts after re-challenge with capsule mutant *epsC*. (A, B) mRNA expression of IL-8 (A) and MCP-1 (B) in gingival fibroblasts that were pre-challenged for 6 h with live *Porphyromonas gingivalis* mutant *epsC* (grey bars) or medium alone (black bars); and re-challenged the next day for 6 h with live *P. gingivalis* mutant *epsC*. Gingival fibroblasts from four donors (donors A, D, G, H) were assayed in quadruple; bars represent the mean gene-expression level ± SD per donor. Results were tested with Student's *t*-test. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. Pg: *P. gingivalis*. (C,D) Overall induction (all donors together) relative to non-challenged controls of gene-expression of IL-8 (C) and MCP-1 (D), by a re-challenge with *P. gingivalis* mutant *epsC* in gingival fibroblasts that were either pre-challenged with mutant *epsC* (grey bars) or with medium alone (black bars). Bars represent the average fold increase ± SEM in gene expression in fibroblasts from all donors together. Results were tested with Wilcoxon matched pairs test. Pg: *P. gingivalis*.

response (Singh *et al*., 2011). To investigate whether it was involved in the lower fibroblast responses to a re-challenge with *P. gingivalis*, we used non-encapsulated isogenic W83 mutant *epsC* to pre-challenge and re-challenge gingival fibroblasts from four donors that were randomly selected from the fibroblasts used in Fig. 1.

As we had observed with the wild-type, the gene expression of IL-8 and MCP-1 in fibroblasts from all donors was significantly lower after a re-challenge with mutant *epsC* if the fibroblasts had also been pre-challenged with mutant *epsC* than when they had been pre-challenged with medium (Fig. 3A,B). The expression of IL-6 after the re-challenge on day two did not differ between fibroblasts that had been pre-challenged with *P. gingivalis* and those that had been pre-challenged with medium, as was previously observed with the wild-type (data not shown).

When the overall response of the fibroblasts to a *P. gingivalis* re-challenge was expressed as the actual induction of gene expression of IL-8 and MCP-1 in the four donors together, it still appeared that the responses of the fibroblasts to a re-challenge were reduced, when they had been pre-challenged with *P. gingivalis*, compared with a pre-challenge with medium alone (Fig. 3C,D). However, statistical significance was not reached, possibly because of the lower number of donors and the inter-individual variation in the strength of fibroblast responses. Hence, it appears that pre-challenge with non-encapsulated mutant *epsC* also reduced fibroblast responses to a re-challenge.

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A pre-challenge with *P. gingivalis* gingipain mutants also caused reduced chemokine responses to a re-challenge

The *P. gingivalis* gingipains can importantly contribute to modulation of the host response, by efficiently cleaving host proteins. To specifically investigate the role of the lysine-specific Kgp, and the arginine-specific RgpA and RgpB in reducing fibroblast responses, fibroblasts were pre-challenged and re-challenged with wild-type *P. gingivalis* ATCC33277, isogenic Δ*kgp*-mutant KDP129 and isogenic Δ*rgpA* and Δ*rgpB* mutant KDP133. Here, fibroblasts from three donors were selected that had specifically expressed reduced responses with *P. gingivalis* W83.

With wild-type strain ATCC33277, the IL-8 and MCP-1 gene expression of the fibroblasts was lower after a *P. gingivalis* re-challenge when the fibroblasts had been pre-challenged with *P. gingivalis*, than when they had been pre-challenged with medium in all three donors (Fig. 4A,E). Like strain W83, pre-challenge with strain ATCC33277 also led to reduced IL-8 and MCP-1 responses.

When fibroblasts were pre-challenged and re-challenged with mutant KDP129, the gene expression of IL-8 was lower than when they had been pre-challenged with medium in one donor, and the expression of MCP-1 was lower in all three donors (Fig. 4B,F).

When fibroblasts were pre-challenged and re-challenged with mutant KDP133, the expression of both IL-8 and MCP-1 was lower than when the fibroblasts had been pre-challenged with medium in two out of three donors. (Fig. 4C,G).

When analysing the overall response of the fibroblasts to a *P. gingivalis* re-challenge in the three donors together, statistical significance was not reached, but IL-8 and MCP-1 responses of the fibroblasts to a re-challenge still seemed somewhat reduced, when they had been pre-challenged with *P. gingivalis*, compared with a pre-challenge with medium alone (Fig. 4D,H).

Interestingly, fibroblasts responded significantly less to a 6-h (pre-)challenge with either gingipain mutant than to wild-type ATCC33277 (data not shown), indicating a clear difference between the wild-type and the mutant strains. However, these differences were not retrieved as such after pre-challenge and re-challenge. Hence, also in case of the lack of Kgp, or both

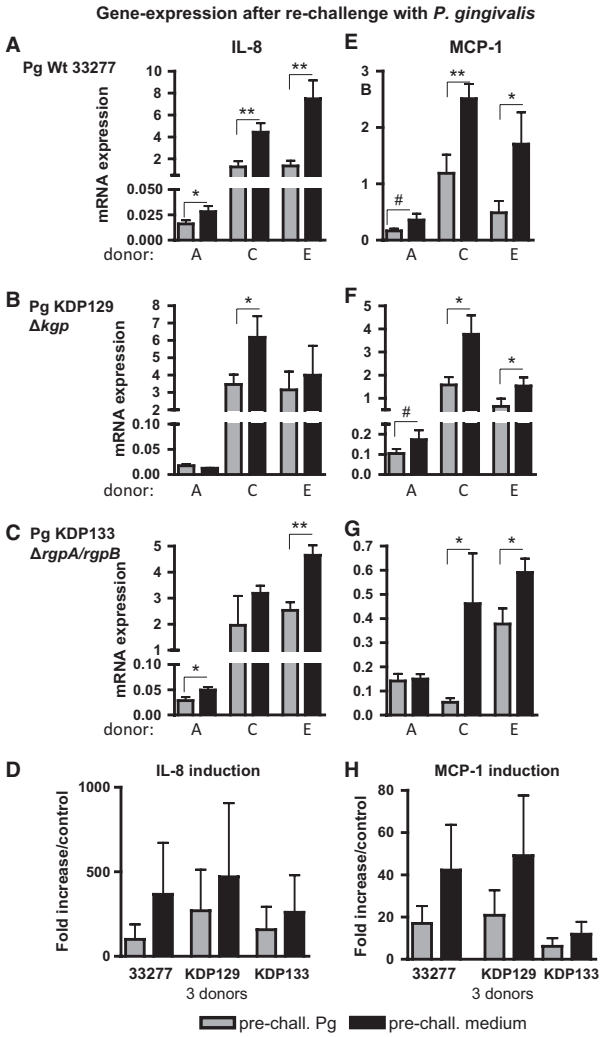

Figure 4 Interleukin-8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) gene expression in gingival fibroblasts after re-challenge with *Porphyromonas gingivalis* ATCC33277, Kgp-mutant KDP129, and RgpA/rgpB-mutant KDP133. The mRNA expression of IL-8 (A–C) and MCP-1 (E–G) in gingival fibroblasts that were pre-challenged for 6 h with live *P. gingivalis* (grey bars) or medium alone (black bars). (A,E) *P. gingivalis* ATCC33277, (B,F) mutant KDP129, (C,G) mutant KDP133. Gingival fibroblasts from three donors (donors A,C,E) were assayed in triplicate; bars represent the mean gene-expression level ± SD per donor. Results were tested with Student's *t*-test. $^{**}P < 0.01$, $^{*}P < 0.05$. $\#P < 0.1$ (trend, E $P = 0.0507$, F $P = 0.0814$) Pg: *P. gingivalis*. (D,H) Overall induction (all donors together), relative to non-challenged controls, of gene-expression of IL-8 (D) and MCP-1 (H), by a re-challenge with *P. gingivalis* ATCC33277, KDP129 and KDP133 in gingival fibroblasts that were pre-challenged with mutant Pg (grey bars) or with medium alone (black bars). Bars represent the average fold increase ± SEM in gene expression in fibroblasts from all donors together. Results were tested with Wilcoxon matched pairs test. Pg: *P. gingivalis*.

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RgpA and RgpB, a pre-challenge with *P. gingivalis* can lead to reduced IL-8 and MCP-1 responses.

Increased gene expression of IL-1ra after a pre-challenge and re-challenge with *P. gingivalis*

We hypothesized that the lower fibroblast responses observed after a pre-challenge with *P. gingivalis* might be related to a higher expression of anti-inflammatory mediators. We therefore analysed the gene expression of IL-1ra and TGF-β in fibroblasts after a pre-challenge with either medium or each *P. gingivalis* strain, and a re-challenge with each *P. gingivalis* strain.

Pre-challenge and re-challenge with *P. gingivalis* W83 did not affect TGF-β gene expression of fibroblasts from the eight donors as used in Fig. 1, in any experimental condition (data not shown).

In contrast, pre-challenge with *P. gingivalis* W83 increased the IL-1ra gene expression in fibroblasts from all eight donors; however, by the time of the re-challenge on day two, the expression of IL-1ra had returned to basal levels (data not shown).

After the re-challenge with *P. gingivalis*, the gene expression of IL-1ra appeared higher in fibroblasts from five out of eight donors when these had also been pre-challenged with *P. gingivalis* than when they had been pre-challenged with medium (see Supplementary material, Fig. S1A). Curiously, however, it was the other way round in fibroblasts from three donors: in these fibroblasts, the gene expression of IL-1ra after the re-challenge was lower when fibroblast had been pre-challenged with *P. gingivalis*, and higher when they had been pre-challenged with medium (see Supplementary material, Fig. S1B). As a result of these contrasting findings, there was no difference between a pre-challenge with *P. gingivalis* or with medium alone when analysing the induction of IL-1ra in the eight donors together (Fig. 5A). However, when fibroblasts that showed contrasting responses were separated, the overall IL-1ra response of five donors (donors B,C,D,E,G) was significantly increased after a pre-challenge with *P. gingivalis* W83 (Fig. 5B). The overall IL-1ra responses of three donors (donors A,F, H) were lower after a *P. gingivalis* pre-challenge, although statistical significance was not reached, probably because of the lower number of donors (Fig. 5C).

For mutant *epsC*, we observed that in fibroblasts from three out of four donors the gene expression of IL-1ra was higher after re-challenge when these fibroblasts had been pre-challenged with mutant *epsC* than when they had been pre-challenged with medium (see Supplementary material, Fig. S1B). This overall response to mutant *epsC* also appeared higher after a pre-challenge with mutant *epsC*, although this was no longer statistically significant (Fig. 5D).

In the fibroblasts that were pre-challenged and re-challenged with *P. gingivalis* ATCC33277 and

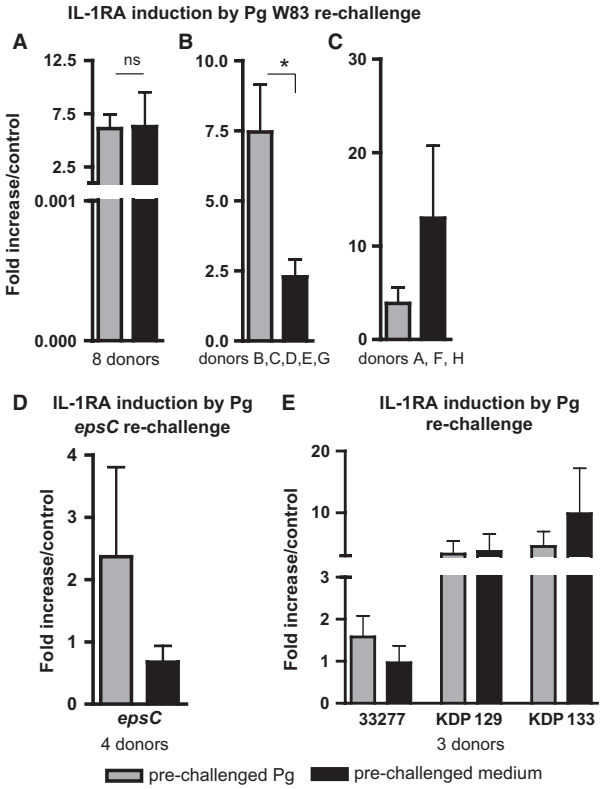

Figure 5 Induction of interleukin 1 receptor agonist (IL-1ra) gene expression in gingival fibroblasts by re-challenge with *Porphyromonas gingivalis.* (A–C) Induction of IL-1ra in gingival fibroblasts that were pre-challenged for 6 h with live *P. gingivalis* W83 (grey bars) or medium alone (black bars), and re-challenged the next day for 6 h with live *P. gingivalis* W83. (A) All donors together, (B, C) donors separated into donors with different responses. (D) Induction of IL-1ra in gingival fibroblasts that were pre-challenged with mutant *epsC* (grey bars) or medium (black bars), and re-challenged with mutant *epsC.* (E) Induction of IL-1ra in gingival fibroblasts that were pre-challenged with *P. gingivalis* wild-type ATCC33277, Kgp mutant KDP190, or RgpA/RgpB mutant KDP133 (grey bars) or medium (black bars), and re-challenged with wild-type ATCC33277, Kgp mutant KDP190, or RgpA/RgpB mutant KDP133, as indicated. Bars represent the mean gene-expression level ± SD per donor in quadruplicate measurements. Results were tested with Student's *t*-test. $*P < 0.05$, Pg: *P. gingivalis.*

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mutants KDP129 and KDP133, there were no different IL-1ra responses between fibroblasts that were pre-challenged with *P. gingivalis*, or fibroblasts that were pre-challenged with medium (Fig. 5E).

Twenty-four hours of preincubation with live *P. gingivalis* did not affect fibroblast responses to a re-challenge

The reduced responses of gingival fibroblasts as described above resulted from two relatively short and potent *P. gingivalis*-challenges that are known to induce a strong inflammatory response (Scheres *et al.*, 2010). To investigate whether fibroblast responses would also be affected by longer preincubation at a lower bacterial concentration, we preincubated gingival fibroblasts for 24 h with 2×10^6 CFU ml^{-1} *P. gingivalis* W83 (five donors), with 2×10^6 CFU ml^{-1} mutant *epsC* (five donors), or with medium alone. It has been demonstrated at similar ratios of bacteria to cells that *P. gingivalis* can adhere to and invade host cells (Pathirana *et al.*, 2007; Belanger *et al.*, 2011). A challenge with 2×10^6 CFU ml^{-1} *P. gingivalis* did not reduce fibroblast viability in previous research (Scheres *et al.*, 2010).

In the present experiments, 24 h of preincubation with 2×10^6 CFU ml^{-1} of either wild-type *P. gingivalis* or mutant *epsC* induced a slight increase in the gene expression of IL-8, MCP-1 or IL-6, relative to the expression in control fibroblasts (Fig. 6A). A following re-challenge with 2×10^8 CFU ml^{-1} *P. gingivalis* W83 or mutant *epsC*, however, induced a much stronger increase in the gene expression of IL-8, MCP-1 and IL-6 in fibroblasts from all donors, irrespective of whether they had been pre-challenged with *P. gingivalis* or with medium, although this was not always statistically significant for mutant *epsC*.

With both *P. gingivalis* W83 and mutant *epsC*, we found that the gene expression of IL-8, MCP-1 and IL-6 after the re-challenge with 2×10^8 CFU ml^{-1} *P. gingivalis* was similar in fibroblasts that had been preincubated with *P. gingivalis* to the gene expression in fibroblasts that had been preincubated only with medium (Fig. 6B–D). Hence, fibroblast responses to a re-challenge with *P. gingivalis* were not influenced by a preincubation with a lower concentration of *P. gingivalis*. Neither did we observe any clear differences between the wild-type and mutant *epsC*.

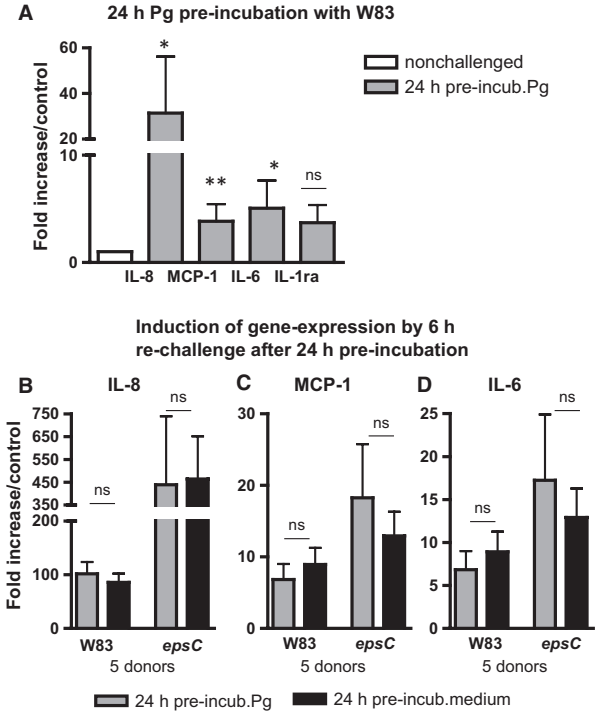

Figure 6 Interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1) and IL-6 gene expression in gingival fibroblasts after preincubation and re-challenge with *Porphyromonas gingivalis*. (A) Overall induction relative to non-challenged controls of gene-expression of IL-8, MCP-1, IL-6 and IL-1 receptor agonist (IL-1ra), by a 24-h incubation with 2×10^6 colony-forming units (CFU) ml^{-1} live *P. gingivalis* W83. Bars represent the mean ± SEM in gene expression in fibroblasts from all donors together. Results were tested with Wilcoxon signed rank test. $^{**}P < 0.01$, $^{*}P < 0.05$, ns: not significant. Pg: *P. gingivalis.* (B–D) mRNA expression of IL-8 (A), MCP-1(B) and IL-6 (C) in gingival fibroblasts that were preincubated with and re-challenged with live *P. gingivalis* (grey bars), or preincubated in medium alone and re-challenged with live *P. gingivalis* (black bars). Two experiments were performed in quadruplicate, using fibroblasts from five donors in total. Bars represent the average gene-expression levels ± SEM of all donors per group. Results were tested with Student's paired *t*-test ns: not significant. Pg: *P. gingivalis.*

DISCUSSION

In the present study, we investigated whether the responsiveness of gingival fibroblasts to live *P. gingivalis* could be affected by a previous encounter with live *P. gingivalis*. As resident tissue cells, gingival fibroblasts can play an important regulatory role in the inflammatory process in periodontitis. Their reaction to an encounter with oral microorganisms such as the periodontal pathogen *P. gingivalis* may have direct consequences for the formation of an adequate host response. Our results demonstrate that the IL-8

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and MCP-1 responses of gingival fibroblasts to a challenge with live *P. gingivalis* were less strong, when the fibroblasts had been pre-challenged with live *P. gingivalis* on the previous day. This indicates that their first encounter with *P. gingivalis* had made the fibroblasts less responsive.

The lower responsiveness after a pre-challenge with live *P. gingivalis* contrasts with reports that gingival fibroblasts do not develop LPS-tolerance (Ara *et al*., 2009; Zaric *et al*., 2010). For instance, Ara and co-workers showed that 24 h of pre-treatment with various concentrations of *P. gingivalis* LPS had no effect on the IL-6 and IL-8 responses of human gingival fibroblasts to a new challenge with *P. gingivalis* LPS (Ara *et al*., 2009). Such contrasting observations may result from the fact that live *P. gingivalis,* with various virulence factors including its polysaccharide capsule and proteolytic gingipains, can induce different inflammatory pathways to those induced by its purified components (Zhou & Amar, 2007; Yu *et al*., 2010).

Porphyromonas gingivalis W83 represents the virulent, encapsulated K1 serotype (Laine & van Winkelhoff, 1998). While its capsule is known to reduce the host response (Singh *et al*., 2011), the K1 capsular polysaccharide has been shown to induce chemokine production in macrophages (d'Empaire *et al*., 2006). In the present study, we observed no clear differences between capsule-less mutant *epsC* and wild-type W83. Moreover, pre-challenge with the non-encapsulated *P. gingivalis* strain ATCC33277 caused reduced fibroblast responses to a re-challenge, as W83 did. We therefore presume that the capsule is not actively involved in the reduction of fibroblast sensitivity to a second challenge with *P. gingivalis*.

Porphyromonas gingivalis can also effectively manipulate the normal host response with its proteolytic lys-gingipain Kgp, and arg-gingipains RgpA and RgpB (Guo *et al*., 2010). Especially Kgp has been indicated as a major virulence factor in interacting with the host (Yongqing *et al*., 2011). Interestingly, we found that a *Δkgp*-mutant and an *ΔrgpA/rgpB*-mutant were less able to induce an inflammatory response in gingival fibroblasts than the wild-type, which seems in line with lower virulence of gingipain mutants in various experimental models (Guo *et al*., 2010). This might be the result of an impaired ability to adhere to, and stimulate, the fibroblasts (Pathirana *et al*., 2008).

In spite of this, the absence of either Kgp, or RgpA and RgpB, did not abolish the reducing effect of a *P. gingivalis* pre-challenge on fibroblast responses to a re-challenge, although for both mutants the effect did manifest less clearly than with the wild-type. The gingipains may therefore be involved in the reduced fibroblast responses to a *P. gingivalis* re-challenge, but they are not the only, or most important factor. How the gingipains are involved remains unclear. Altered expression of gingipains could affect the expression of other virulence factors of *P. gingivalis*. It can also be envisaged that degradation of cellular receptor molecules, or degradation of cytokines that might otherwise stimulate and enhance the inflammatory response, play a role.

Cytokines like IL-1β, IL-6 and IL-8 can be degraded quite efficiently by *P. gingivalis*-proteases (Fletcher *et al*., 1998; Stathopoulou *et al*., 2009). As a result of this cytokine proteolysis, the analysis of cytokine protein levels could lead to biased results, making it difficult to determine whether lower expression levels were caused by reduced fibroblast responses or by proteolysis. However, in spite of this, we could find elevated IL-8 protein levels in the supernatants of fibroblasts that had received a *P. gingivalis* challenge. Moreover, less IL-8 was present when fibroblasts were pre-challenged with *P. gingivalis*, than when they were pre-challenged with medium. Although these protein concentrations were probably underestimations because of proteolysis, they do indicate that gene expression and protein expression levels correspond. Gene expression therefore probably reflects the actual sensitivity of the fibroblasts to the bacterial challenges well.

Another difference between viable *P. gingivalis* or its LPS alone, is that viable *P. gingivalis* might actively invade fibroblasts and influence their inflammatory responses through transcriptional regulation. Darveau and co-workers reported that an 18-h challenge with live *P. gingivalis* inhibited mRNA-expression of IL-8 in human gingival epithelial cells. For this survival strategy of *P. gingivalis*, named local chemokine paralysis, invasion was necessary (Darveau *et al*., 1998). Although a 6-h pre-challenge in our study induced rather than inhibited chemokine expression, a number of *P. gingivalis* may have invaded the fibroblasts and survived intracellularly (Dorn *et al*., 2000; Amornchat *et al*., 2003). *Porphyromoans gingivalis* W83 is an invasive strain

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(Dolgilevich *et al.*, 2011), and it was recently demonstrated that mutant *epsC* is also invasive, even more than the wild-type (Irshad *et al.*, 2012), although we cannot state that mutant *epsC* caused even more reduced responses than the wild-type.

In this regard, reduced fibroblast responses after a *P. gingivalis* pre-challenge seem to be consistent with local chemokine paralysis by *P. gingivalis*, and furthermore it is probably not caused by a single virulence factor, but rather by active *P. gingivalis* and its array of virulence factors as a whole.

In contrast to two shorter and strong *P. gingivalis* pre-challenges and re-challenges, we found that fibroblast responsiveness towards a re-challenge was not affected when gingival fibroblasts were preincubated with 2×10^6 CFU ml^{-1} W83, or mutant *epsC*, and re-challenged with 2×10^8 CFU ml^{-1}, which resembles the work of Ara and co-workers in experimental set-up (Ara *et al.*, 2009). To reduce fibroblast responsiveness, a stronger stimulus with live bacteria appeared necessary. As a result of the higher concentration used during the 6-h pre-challenge, more *P. gingivalis* may have invaded the fibroblasts than during the 24-h preincubation. Furthermore, fibroblasts had also initiated a clear inflammatory response to the 6-h pre-challenge, whereas a response to the 24-h preincubation was not as strong. It is possible that reduced fibroblast responses to a re-challenge resulted from increased expression of mediators since the first response; however, it should be noted that the cytokines analysed were expressed at basal levels at the time-point before re-challenge.

Interestingly, a 24-h pre-challenge induced expression of proinflammatory IL-6, IL-8 and MCP-1 compared with non-challenged cells, but not of anti-inflammatory IL-1ra. This is striking, because in fibroblasts from a few donors the lower responses after a pre-challenge with *P. gingivalis* appeared to be accompanied by a higher expression of IL-1ra (donors B,C,E,G). Curiously, when fibroblasts from three donors had been pre-challenged with W83, the expression of IL-1ra was lower, not higher; yet when fibroblasts from two of these donors had been pre-challenged with mutant *epsC*, their IL-1ra expression was higher. In contrast, we observed no relation between the gene expression of anti-inflammatory TGF-β and reduced fibroblast responsiveness.

Although only in a few donors, increased IL-1ra expression might play a role in reduced IL-8 and MCP-1 responses: IL-1ra is an inhibitor of IL-1α and IL-1β signaling (Dinarello, 1994), and IL-1β can induce the expression of IL-8 and of MCP-1 (Yadav *et al.*, 2010). Eskan and co-workers showed that in gingival epithelial cells, IL-1β expression was quickly induced by *P. gingivalis*; this in turn regulated the expression of IL-8 and IL-6. Inhibition of the IL-1β-receptor reduced the IL-6 and IL-8 production (Eskan *et al.*, 2008).

Remarkably, IL-6 responses of fibroblasts reduced by *P. gingivalis* pre-challenge in fibroblasts from only one donor. It is possible that IL-6 is regulated differently from the other mediators. If so, this might have implications for its role in the response against *P. gingivalis*. However, as in our own earlier work, Eskan and co-workers demonstrated a strong correlation between IL-1β, IL-6 and IL-8 responses of gingival cells to *P. gingivalis* (Eskan *et al.*, 2008; Scheres *et al.*, 2010). This suggests another possibility: that we did not observe reduced IL-6 responses using the current experimental set-up because IL-6 responses were less pronounced, or occurred somewhat later than other responses, such as those of IL-8.

Overall, the responsiveness of gingival fibroblasts to live *P. gingivalis* was affected by a previous encounter if that encounter had represented a potent bacterial challenge, but the fibroblasts remained responsive in the presence of lower concentrations of *P. gingivalis.* Lower levels of *P. gingivalis* are generally found in non-periodontitis *P. gingivalis*-carriers or in less severe disease, whereas higher numbers of *P. gingivalis* are generally associated with more severe disease symptoms (Socransky *et al.*, 1991, 1998; Wolff *et al.*, 1993; Hyvarinen *et al.*, 2009). Gingival fibroblasts can provide a line of defense against oral microorganisms by attracting and regulating inflammatory cells. We speculate that fibroblasts remaining responsive in the presence of lower numbers of *P. gingivalis* might be beneficial, because they can initiate a proper response in the event of a new bacterial threat. In the presence of higher numbers of *P. gingivalis*, however, reduced chemokine responses by fibroblasts could lead to incorrect recruitment of an inflammatory cell infiltrate. This, in turn, could impair the clearance not only of *P. gingivalis*, but also of other microorganisms in the oral biofilm, causing persistent and exaggerated inflammation. A mouse study showed very elegantly that the presence of *P. gingivalis* caused an

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increased overall bacterial load in the oral cavity of mice, and increased inflammation and bone loss, through components of the host complement system, and by inhibiting chemokine production (Hajishengallis *et al*., 2011). Not only did this study show that impaired chemotaxis of leukocytes can indeed make an important contribution to periodontal inflammation, it also demonstrated that the tactics used by *P. gingivalis* to subvert host responses and survive can accelerate the inflammation and bone loss that are normally induced by the commensal oral flora (Hajishengallis *et al*., 2011).

Although our study included fibroblasts from periodontitis patients as well as from non-periodontitis donors, there were no obvious differences between these fibroblasts with regard to lower responsiveness after a previous *P. gingivalis* challenge. This suggests that reduced fibroblast responses can occur after a potent *P. gingivalis* challenge regardless of any existing inflammation, although it should be noted that the number of non-periodontitis donors was relatively low for a proper comparison.

In conclusion, our results suggest that although gingival fibroblasts are known not to develop tolerance against the LPS of *P. gingivalis*, their responsiveness towards the next bacterial stimulus can be affected by an encounter with this oral pathogen. Gingival fibroblasts that had previously encountered live *P. gingivalis* responded less strongly to a new bacterial challenge. It can be envisaged that such reduced responsiveness contributes to inadequate inflammatory signaling by fibroblasts. As fibroblasts can play a significant regulatory role in inflammation, this might importantly contribute to severe inflammation and tissue damage in periodontitis. A relevant topic for future research is whether fibroblast responses to other oral microorganisms or communities are also influenced by a previous encounter with *P. gingivalis*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Interleukin-1 receptor agonist (IL-1ra) gene expression in gingival fibroblasts per donor, after re-challenge with *Porphyromonas gingivalis* W83 and *epsC*. (A) mRNA expression of IL-1ra per donor in gingival fibroblasts that were pre-challenged for 6 h with live *P. gingivalis* W83 (grey bars) or medium alone (black bars); and re-challenged the next day for 6 h with live *P. gingivalis* W83. Bars represent the mean gene-expression level $\pm$ SD per donor in quadruplicate measurements. Two experiments were performed using gingival fibroblasts from eight donors in total. (B) Expression of IL-1ra per donor in gingival fibroblasts that were pre-challenged for 6 h with live *P. gingivalis epsC* (grey bars) or medium alone (black bars); and re-challenged with live *P. gingivalis epsC*. Fibroblasts from four donors were assayed in quadruplicate; bars represent the mean gene-expression level $\pm$ SD per donor. $*P < 0.05$. Results were tested with Student's *t*-test. In the event of unequal variances, Welch's correction was applied. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$, $\#P < 0.1$ (trend; A $P = 0.059$, B $P = 0.0504$), ns: not significant. Pg: *P. gingivalis.*

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