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Gingival and periodontal ligament fibroblasts differ in their inflammatory response to viable *Porphyromonas gingivalis*

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Background and Objective: Porphyromonas gingivalis is an oral pathogen strongly associated with destruction of the tooth-supporting tissues in human periodontitis. Gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) are functionally different cell types in the periodontium that can participate in the host immune response in periodontitis. This study aimed to investigate the effects of viable *P. gingivalis* on the expression of genes associated with inflammation and bone degradation by these fibroblast subsets.

Material and Methods: Primary human GF and PDLF from six healthy donors were challenged *in vitro* with viable *P. gingivalis* W83 for 6 h. Gene expression of inflammatory cytokines in GF and PDLF was analyzed using real-time PCR, and protein expression was analyzed using ELISA.

Results: Viable *P. gingivalis* induced a strong *in vitro* inflammatory response in both GF and PDLF. We found increased gene expression of interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α, monocyte chemotactic protein-1 and regulated upon activation, normal T-cell expressed and secreted (RANTES). Macrophage colony-stimulating factor was induced and the expression of osteoprotegerin was decreased in GF, but not in PDLF. In nonchallenged cells, a higher level of expression of IL-6 was observed in GF than in PDLF. Between individual donors there was large heterogeneity in responsiveness to *P. gingivalis*. Also, in each individual, either GF or PDLF was more responsive to *P. gingivalis*.

Conclusion: Considerable heterogeneity in responsiveness to *P. gingivalis* exists both between GF and PDLF and between individuals, which may be crucial determinants for the susceptibility to develop periodontitis.

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Periodontitis is a complex chronic infectious disease that leads to destruction of the tooth- supporting tissues, including alveolar bone, and may eventually result in tooth loss. The disease develops as a result of the host-mediated inflammatory response to a pathogenic microflora residing in periodontal pockets (1,2).

Bacterial species that have been associated with periodontitis include *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans*. *P. gingivalis* is recognized as a major etiologic agent (3–6); it is a gram-negative anaerobic rod that possesses a number of virulence factors, including

lipopolysaccharide, fimbriae, capsule polysaccharide and cysteine proteases (7). Besides the presence of *P. gingivalis* or other periodontopathic bacteria, also other factors play a role in the onset and progression of periodontitis; these include genetic susceptibility, systemic diseases and environmental factors such as smoking and stress (8).

Among the different cell types in the periodontium that may be involved in the host immune-response in periodontitis are gingival fibroblasts (GF), and periodontal ligament fibroblasts (PDLF). Gingival fibroblasts are connective-tissue cells that are located in the gingiva apical to the gingival epithelium. Periodontal ligament fibroblasts are present in the periodontal ligament, the tissue that anchors the teeth to the alveolar bone. They play an important role in the homeostasis of the periodontal ligament and in alveolar bone remodelling (9–11). In periodontitis, GF and PDLF may be involved in the regulation of osteoclast formation and activity by producing osteoclast-stimulating and osteoclast-inhibiting cytokines (12–15).

Although GF and PDLF are spatially located not far apart and have a similar spindle-shaped phenotype *in vitro*, they belong to different periodontal tissues and have distinct functional characteristics. As shown by Fujita and co-workers (16), the two cell types have different gene expressions of apolipoprotein D, and major histocompatibility complex-DR-α and -β *in vitro*. Furthermore, PDLF, but not GF, are known to express high levels of the enzyme tissue nonspecific alkaline phosphatase (ALP), an indicator for the osteoblast-like nature of PDLF (17). Differences have also been shown at the transcriptional level by microarray analyses, which revealed different gene-expression patterns in GF and PDLF (18).

Also, GF and PDLF have been suggested to play different roles in the regulation of osteoclast formation. We have previously shown (19) that GF are more able to inhibit osteoclast formation than PDLF, possibly through the production of osteoprotegerin (OPG). This correlated well with a study in which stimulation with interleukin (IL)-1α caused a stronger increase in OPG gene expression in GF than in PDLF, and suggested that this response was regulated by different protein kinases in the two cell types (20). In a study by Yashiro and co-workers (21), GF expressed more IL-11, a stimulator of osteoclast formation *in vitro* (22), than PDLF when stimulated with transforming growth factor-β.

As GF and PDLF have different functional characteristics, their role in the inflammatory process in periodontitis may also be different. Both cell types respond to *P. gingivalis* virulence factors by increasing the expression of IL-6, IL-1β, IL-8, RANKL, OPG, tumor necrosis factor-α (TNF-α), prostaglandin E_2 and several matrix metalloproteinases (23–29). Moreover, GF can alter the expression of Toll-like receptors on their cell surface in response to *P. gingivalis* lipopolysaccharide (30). *P. gingivalis* can also influence the viability of GF and induce both pro-apoptotic and anti-apoptotic genes in GF (31,32).

Although virulence factors of *P. gingivalis* have been extensively studied, the effect of viable *P. gingivalis* cells on GF and PDLF remains relatively unknown. In the *in vivo* situation, *P. gingivalis* express a whole subset of virulence factors that may interact with and stimulate host cells in a different way than a single virulence factor.

The present study therefore aimed to compare cytokine responses by GF and PDLF upon challenge by viable *P. gingivalis*. We hypothesized that viable *P. gingivalis* are potent stimulators of host cells. We also hypothesized that GF and PDLF would respond to *P. gingivalis* by initiating an inflammatory response that might be different between GF and PDLF. We focussed on alterations in the gene expression and in the production of several cytokines involved in different aspects of the regulation of bone metabolism, namely: the pro-inflammatory cytokines IL-6, IL-1β, TNF-α, RANKL and macrophage colony-stimulating factor (M-CSF) that activate quiescent osteoclasts or induce osteoclast differentiation; the chemokines IL-8, monocyte chemotactic protein-1(MCP-1) and regulated upon activation, normal T-cell expressed and secreted (RANTES) that can attract osteoclast precursor cells; and OPG, an inhibitor of osteoclast formation (15). In the present paper we show that there are functional differences between GF and PDLF in their response to viable *P. gingivalis*.

Material and methods

Bacterial strains and culture

P. gingivalis W83 was cultured anaerobically (80% N_2, 10% H_2, 10% CO_2), until log growth-phase was reached, in brain–heart infusion broth enriched with hemin (5 mg/L) and menadione (1 mg/L). Bacterial cultures were checked for purity by Gram staining. Viable *P. gingivalis* were harvested from liquid culture by centrifugation. Bacterial pellets were washed twice in sterile phosphate-buffered saline and resuspended in antibiotic-free Dulbecco's minimal essential medium (DMEM; Gibco BRL, Paisley, UK) containing 10% fetal calf serum (FCS) (HyClone, Logan, UT, USA). The optical density was measured at 690 nm to establish the number of colony-forming units. To obtain dead bacteria, liquid cultures were heat inactivated at 60°C for 1 h before harvesting the bacteria. Heat-killed bacteria did not grow on blood-agar plates, but remained intact, as shown with Gram staining.

Fibroblasts

The GF and PDLF were obtained from erupted third molars extracted from six healthy donors (one man, five women, 16–31 years of age, mean age 22.5 years). One donor was a current smoker and five donors had never smoked. Donors had given written informed consent, and the study was approved by the VUmc Medical Ethical committee. Donors were periodontally healthy and no alveolar bone loss was observed on X-rays. Donors did not suffer from systemic diseases and female donors were not pregnant.

The GF were recovered by collecting free gingiva-remains from the tooth using a scalpel. The PDLF were recovered by scraping exclusively the middle third of the root using a scalpel. Tissue samples were washed twice in DMEM supplemented with 10% FCS and 2% antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin and 250 ng/mL of amphotericin B; Antibiotic antimycotic solution; Sigma, St Louis, MO, USA), and cultured in DMEM containing 10% FCS and 2% antibiotics, in a humidified atmosphere with 5% CO_2 at 37°C. Cells were stored frozen in liquid nitrogen at passage 4. Experiments were performed with cells from passages 5–7.

As PDLF are known to express higher levels of ALP than GF (17), we analyzed the ALP expression in nonchallenged GF and PDLF from all donors; in all donors the ALP levels were higher in PDLF than in GF ($p = 0.0313$).

Bacterial challenge

The GF and the PDLF were seeded into 24-well plates and subsequently grown until subconfluent in 0.5 mL of antibiotic-free DMEM containing 10% FCS. Bacteria were added to fibroblast cultures at concentrations of 1×10^6/mL (multiplicity of infection 50 : 1), 2×10^6/mL (100 : 1), 6×10^6/mL (300 : 1), 1×10^7/mL (500 : 1), 2×10^7/mL (1000 : 1) and 2×10^8/mL (10,000 : 1). Only DMEM with 10% FCS was added to control GF and PDLF (nonchallenged).

PCR analysis with primers specific for *P. gingivalis* (33), modified for Lightcycler® 480 (Roche Diagnostics, Indianapolis, IN, USA), showed that at concentrations of 2×10^7 and 2×10^8 bacteria/mL, about 10% of bacteria were in contact with fibroblasts while the rest remained in the supernatant. Infected GF and PDLF cultures were incubated for 6 h in a humidified aerobic atmosphere with 5% CO_2 at 37°C. Hereafter, fibroblast morphology was checked for abnormalities or cell detachment using phase-contrast microscopy (Olympus CK2; Olympus, Tokyo, Japan) (Fig. S1A). Viability of fibroblasts was tested using the Trypan Blue exclusion test of cell viability. The bacterial challenge did not affect the viability of the fibroblasts. Viability of bacteria had not decreased after 6 h of anaerobic culture (Fig. S1B).

After bacterial challenge, fibroblast cultures were placed on ice and the supernatant (conditioned medium) was harvested for protein assays and stored at −20°C. Subsequently, fibroblasts were immediately washed twice with cold (4°C) Hank's buffered saline solution (Gibco BRL) and lysis buffer, as supplied with the RNeasy Mini Kit for RNA extraction (Buffer RLT; Qiagen, Hilden, Germany), supplemented with β-mercaptoethanol, was added to the fibroblasts. Then, RNA was isolated. Experiments were performed in quadruplicate.

mRNA expression

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

Real-time PCR primers for IL-1β, IL-6, IL-8, TNF-α, MCP-1, RANTES, M-CSF, OPG, RANKL and the housekeeping genes β2-microglobulin and porphobilinogen deaminase, were designed using PRIMER EXPRESS software, version 2.0 (Applied Biosystems, Foster City, CA, USA) (Table 1) (19), or ordered from Applied Biosystems (RANKL; assay ID Hs00243522-m1). To avoid amplification of genomic DNA, each amplicon spanned at least one intron. The external standard curve used in the PCR reactions was a mixture of bone extract cDNA, peripheral blood mononuclear cell cDNA, GF and PDLF cDNA, and the quantitative PCR human reference total RNA (Stratagene, La Jolla, CA, USA). Real-time PCR was performed

Table 1. Real-time PCR primer sequences

Gene primer sequences	Gene ID ensembl	Primer sequences	
		5′–3′ Forward	5′–3′ Reverse
IL-1β	00000125538	CTTTGAAGCTGATGGCCCTAAA	AGTGGTGGTCGGAGATTCGT
IL-6	00000136244	GGCACTGGCAGAAAACAACC	GGCAAGTCTCCTCATTGAATCC
IL-8	00000169429	GGCAGCCTTCCTGATTTCTG	CTGACATCTAAGTTCTTTAGCACTCCTT
TNF-α	00000206439	CCCAGGGACCTCTCTCTAATCA	GCTTGAGGGTTTGCTACAACATG
MCP-1	00000108691	CAGCCAGATGCAATCAATGC	TGCTGCTGGTGATTCTTCTATAGCT
RANTES	00000161570	CATCTGCCTCCCCATATTCCT	TGCCACTGGTGTAGAAATACTCCTT
M-CSF	00000184371	CCGAGGAGGTGTCGGAGTAC	AATTTGGCACGAGGTCTCCAT
OPG	00000164761	CTGCGCGCTCGTGTTTC	ACAGCTGATGAGAGGTTTCTTCGT
ALP	00000162551	GCTTCAAACCGAGATACAAGCA	GCTCGAAGAGACCCAATAGGTAGT
RANKL 1.	00000120659	CATCCCATCTGGTTCCCATAA	GCCCAACCCCGATCATG
β2 microglobulin	00000166710	AAGATTCAGGTTTACTCACGTC	TGATGCTGCTTACATGTCTCG
PBGD	00000149397	TGCAGTTTGAAATCATTGCTATGTC	AACAGGCTTTTCTCTCCAATCTTAGA

ALP, alkaline phosphatase; IL, interleukin; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage colony-stimulating factor; OPG, osteoprotegerin; PBGD, porphobilinogen deaminase; RANTES, regulated upon activation, normal T-cell expressed and secreted; TNF-α, tumor necrosis factor-α.

on the ABI PRISM 7000 (Applied Biosystems). Reactions were performed with 2 ng of cDNA in a total volume of 8 μL containing SYBR Green PCR Master Mix, consisting of SYBR Green I Dye, AmpliTaq Gold DNA polymerase, deoxyribonucleotide triphosphates with deoxyuridine triphosphate instead of deoxythymidine triphosphate, passive reference and buffer (Applied Biosystems) and 0.99 pM of each primer. After an activation step with the AmpliTaq Gold DNA polymerase for 10 min at 94°C, 40 cycles were run of a two-step PCR consisting of a denaturation step at 95°C for 30 s and an annealing and extension step at 60°C for 1 min. Subsequently, the PCR products were subjected to melting curve analysis to test if any nonspecific PCR products were generated. The PCR reactions of the different amplicons had equal efficiencies. Samples were normalized for the expression of the housekeeping genes β2-microglobulin or porphobilinogen deaminase, which were not affected by the experimental conditions, by calculating the ΔCt ($Ct_{\text{housekeeping gene}} - Ct_{\text{gene of interest}}$), and the expression of different genes was expressed as $2^{-(\Delta Ct)}$. Fold increase in gene expression (induction) was expressed as $2^{-(\Delta\ \Delta Ct)}$, wherein Δ ΔCt = $\Delta Ct_{\text{challenged}} - \text{average Ct-value}_{\text{nonchallenged}}$.

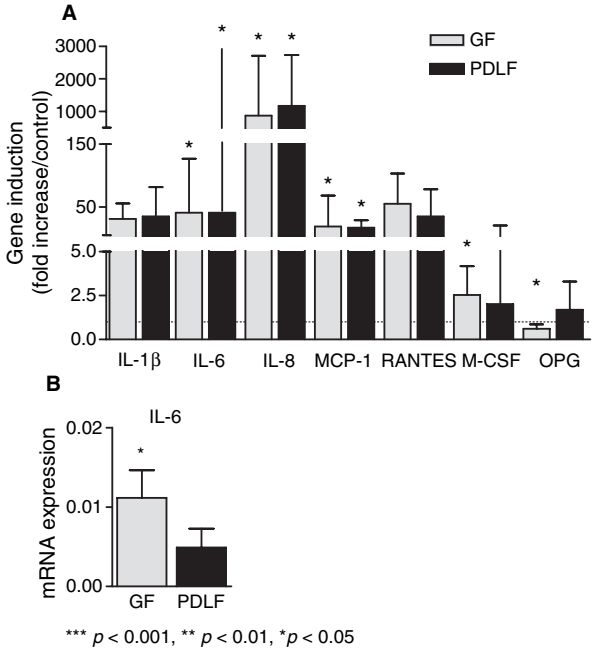

Fig. 1. (A) Gene induction of cytokines and chemokines in gingival fibroblasts (GF) (grey) and in periodontal ligament fibroblasts (PDLF) (black) by *Porphyromonas gingivalis* at 2×10^8 bacteria/mL after a 6-h challenge. Interleukin (IL)-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) were significantly induced in both GF ($p = 0.0156$ for all three genes) and PDLF ($p = 0.0156$ for all three genes). IL-1β and regulated upon activation, normal T-cell expressed and secreted (RANTES) appear to be induced in GF and PDLF in four donors, but induction was not significant, and in two donors a fold-increase could not be calculated. In GF ($n = 6$), macrophage colony-stimulating factor (M-CSF) was slightly induced ($p = 0.0313$) and osteoprotegerin (OPG) was down-regulated ($p = 0.0156$). Bars represent the medians + interquartile range of the average induction levels of six donors from two independent bacterial challenge experiments performed in duplicate. The asterisks indicate significant induction by *P. gingivalis*. (B) mRNA expression of IL-6 (relative to the housekeeping gene) in nonchallenged GF and PDLF. IL-6 is expressed more highly in nonchallenged GF than in nonchallenged PDLF ($p = 0.0313$). The bars represent the means of two independent experiments performed in duplicate.

ELISA

ELISAs to determine the protein levels of IL-6, IL-8, IL-1β and TNF-α were performed using PeliKine ELISA kits (Sanquin Blood Supply Foundation, Amsterdam, the Netherlands), according to the manufacturer's protocol. Before the ELISAs, conditioned medium from infected or control fibroblasts was harvested and centrifuged to pellet any bacterial remains present. Dilutions were prepared in dilution buffer supplied with the PeliKine kit. Experiments were performed in duplicate with undiluted and 10 × diluted supernatants.

Statistical analysis

Gene induction was tested using the Wilcoxon signed rank test (Fig. 1A). Heterogeneity between donors and *P. gingivalis* survival in aerobic culture (Fig. S1B) were tested using one-way analysis of variance. Differences between viable and dead *P. gingivalis,* and between GF and PDLF within a donor (Fig. 2), were tested using the Student's *t*-test; protein levels were tested using the paired Student's *t*-test (Fig. 3). Differences between nonchallenged GF and PDLF in the expression of IL-6 (Fig. 1B), OPG and ALP were tested using Wilcoxon's matched pairs test. Tests were performed using GRAPHPAD PRISM software (version 4, by MacKiev Software™). Correlations (Table 2), were calculated using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

Results

GF and PDLF respond to viable *P. gingivalis*

An initial set of experiments was performed to establish the optimal experimental conditions for using to challenge GF and PDLF with *P. gingivalis*. The GF and PDLF from two donors (A and B) were challenged for 6 h with different concentrations of viable *P. gingivalis*. *P. gingivalis* induced an increase in the expression of the IL-6 gene in GF and PDLF. Induction of IL-6 gene expression by *P. gingivalis* was dose-dependent, and the responses were statistically significant at 2×10^8 bacteria/mL in both GF (donor A, $p < 0.001$; donor B,

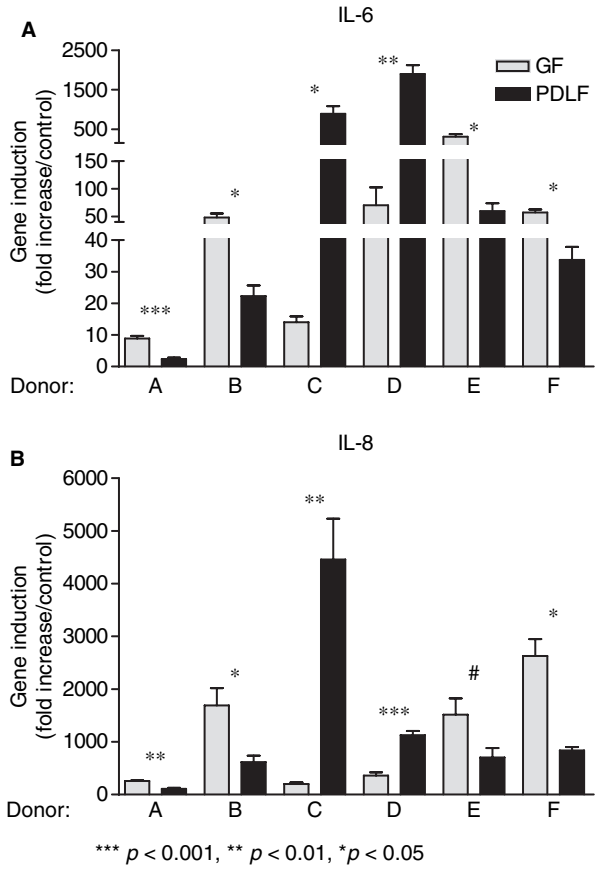

Fig. 2. (A) Interleukin (IL)-6 induction by *Porphyromonas gingivalis* (after a 6-h challenge) in gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) from all donors analyzed separately. (B) IL-8 induction by *P. gingivalis* (after a 6-h challenge) in GF and PDLF from all donors analyzed separately. In all donors the responses between GF and PDLF differed in strength; the asterisks indicate significant differences between GF and PDLF; # indicates a trend ($p = 0.0943$). Bars represent the mean + standard error of the mean of triplicate measurements from one of two independent experiments with similar results.

$p < 0.001$) and PDLF (donor A, $p < 0.05$; donor B, $p < 0.01$).

To investigate whether viable bacteria are more potent stimulators of fibroblasts than dead bacteria, GF and PDLF from the same two donors were challenged with viable or heat-killed *P. gingivalis*. Viable *P. gingivalis* appeared to induce higher responses than heat-killed *P. gingivalis* for IL-6 gene expression in GF (donor A: 8-fold vs. 4-fold, $p < 0.0035$; donor B: 45-fold vs.19-fold, $p = 0.056$), and for IL-8 gene expression in GF (donor A: 256-fold vs. 38-fold, $p < 0.0001$; donor B: 1700-fold vs. 400-fold, $p = 0.0135$) and in PDLF (donor B: 690-fold vs. 51-fold, $p < 0.0001$).

Gene expression in GF and PLDF

All GF and PDLF responded to *P. gingivalis* challenge by increasing the gene expression of most cytokines measured (Fig. 1A), but the scale of the responses varied greatly among the six donors. For example, the induction of IL-8 gene expression varied between 160-fold and 4000-fold in GF ($p < 0.0001$) and between 100-fold and 3100-fold in PDLF ($p = 0.0017$) (Fig. 2B). Despite this heterogeneity, several overall trends were observed.

Expression of the IL-6 gene was clearly induced by *P. gingivalis* in GF (40-fold, $p = 0.0156$) as well as in PDLF (40-fold, $p = 0.0156$) (Fig. 1A). Interestingly, expression of the IL-6 gene in nonchallenged fibroblasts was higher in GF than in PDLF ($p = 0.0313$) (Fig. 1B), whereas the other cytokines were expressed at similar levels in nonchallenged GF and PDLF.

Gene expression of IL-8 was strongly induced in GF (870-fold, $p = 0.0156$) and in PDLF (1170-fold, $p = 0.0156$), and MCP-1 was induced in GF (18-fold, $p = 0.0156$) and in PDLF (20-fold, $p = 0.0156$). IL-1β and RANTES appeared to be induced, but not significantly, in GF (18-fold and 55-fold respectively) and in PDLF (20-fold and 32-fold respectively) of four donors (Fig. 1A), and in two donors, RANTES and IL-1β mRNA could only be detected after challenge. The inductions were significant in each donor when analyzed separately. M-CSF was moderately induced in GF, but not in PDLF, by about two-fold ($p = 0.0313$). In both GF and PDLF, RANKL was not detectable either before or after challenge with *P. gingivalis*.

As IL-1β, IL-6 and IL-8 are induced via a common pathway, indicating activation of nuclear factor-kappaB pathway (34), we analysed whether their expression levels in challenged and nonchallenged GF and PDLF were correlated by calculating Pearson's correlation coefficient. The expression of all three genes was strongly correlated in GF and PDLF, with correlation coefficients close to 1 (Table 2). In both GF and PDLF, correlation was strongest between IL-6 and IL-8.

In nonchallenged fibroblasts, OPG tended to be expressed slightly more highly in GF than in PDLF ($p = 0.0625$). After bacterial challenge, OPG was down-regulated in GF, but in PDLF no clear responses could be measured (Fig. 1A). TNF-αexpression was detectable only after bacterial challenge in both GF and PDLF, and not in nonchallenged cells (data not shown).

GF and PDLF respond differently to viable *P. gingivalis*

As the cytokine responses to *P. gingivalis* challenge varied in strength

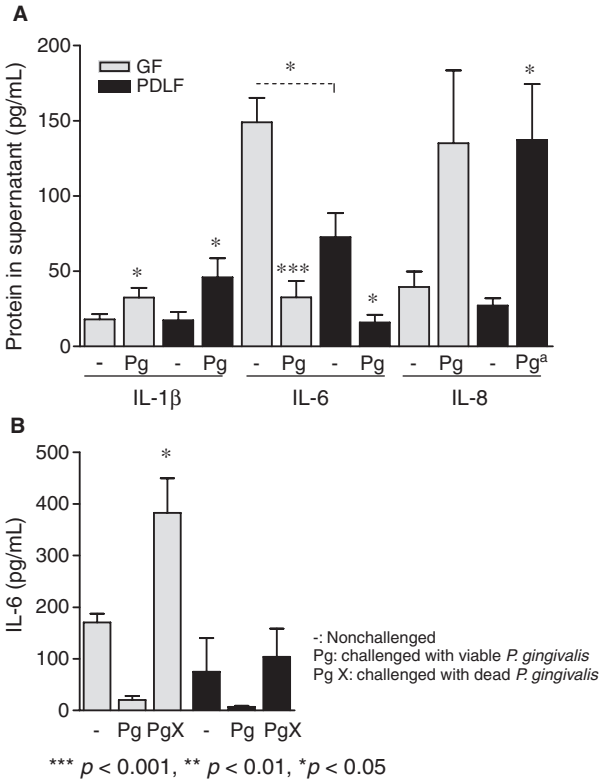

Fig. 3. (A) The levels of secreted interleukin (IL)-1β, IL-6 and IL-8 in culture supernatant from gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) challenged with *Porphyromonas gingivalis* (Pg) for 6 h or nonchallenged. The levels of IL-1β were elevated upon challenge with *P. gingivalis* in GF ($p = 0.0120$) and PDLF ($p = 0.0212$). The levels of IL-6 protein were decreased in challenged compared with nonchallenged GF ($p = 0.0003$) and PDLF ($p = 0.0111$). IL-8 was elevated in PDLF ($p = 0.0356$). Asterisks indicate significant differences between challenged and nonchallenged cells. Nonchallenged GF secrete more IL-6 than nonchallenged PDLF ($p = 0.0147$). Bars represent the means + standard error of the mean (SEM) of duplicate measurements in undiluted samples and 10 × diluted samples from GF and PDLF ($n = 6$) in two experiments. (B) The levels of secreted IL-6 protein from nonchallenged GF and PDLF, or from GF and PDLF challenged for 6 h with either viable *P. gingivalis* or dead *P. gingivalis*. IL-6 levels were elevated upon challenge with dead *P. gingivalis* in GF ($p = 0.0495$), and appeared to be slightly elevated in PDLF in comparison with nonchallenged cells. Bars show means + SEM of duplicate measurements from GF and PDLF from two donors in two experiments. Asterisks indicate significant differences.

Table 2. Correlation among interleukin (IL)-6, IL-8 and IL-1β gene expression between gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF)

Correlations		IL-6 + IL-8	IL-6 + IL-1β	IL-8 + IL-1β
GF[a]	Pearson's *r*	0.956	0.94	0.866
	p-value	< 0.001	< 0.001	< 0.001
PDLF[a]	Pearson's *r*	0.931	0.813	0.926
	p-value	0.001	< 0.001	< 0.001

[a]Correlations were calculated from the average gene-expression levels from nonchallenged and challenged fibroblasts from six donors in at least duplicate measurements from two independent experiments.

between fibroblasts from the six donors, we also compared the responses of fibroblasts from each donor separately. It appeared that in each donor, a substantial difference existed between the IL-6 and IL-8 gene-expression responses in GF and PDLF. Either GF or PDLF responded more strongly upon stimulation with *P. gingivalis* (Fig. 2A,B).

Protein secretion

To establish whether the altered gene expression of cytokines also resulted in increased protein secretion, culture supernatants of challenged and nonchallenged GF and PDLF were analyzed for the presence and level of IL-1β, IL-6, IL-8 and TNF-α. The IL-1β protein levels were elevated in *P. gingivalis*-challenged GF compared with nonchallenged GF ($p = 0.0120$) and PDLF ($p = 0.0212$) (Fig. 3A). Surprisingly, the IL-6 protein levels appeared to be decreased in the supernatant of challenged cells (Fig. 3B). To investigate if the decrease in IL-6 protein levels in supernatants from challenged fibroblasts was caused by degradation by viable *P. gingivalis*, we analyzed the supernatants of GF and PDLF challenged with heat-killed bacteria for the presence of IL-6. Dead *P. gingivalis* induce IL-6 gene expression in GF and PDLF, but bacteria-related release of proteases does not occur (28). The IL-6 protein levels were higher in supernatants collected from GF stimulated with dead *P. gingivalis* ($p = 0.0495$) and appeared to be slightly increased in PDLF challenged with dead *P. gingivalis*, indicating that IL-6 was indeed degraded by viable *P. gingivalis*.

The IL-8 protein levels were elevated in challenged PDLF ($p = 0.0356$) and also somewhat, although not significantly, in GF. Again, heterogeneity existed between donors. Donors who produced the strongest induction of the IL-8 gene in response to *P. gingivalis* also showed the highest elevations in protein levels; induction values in IL-8 gene expression and secreted protein levels strongly correlated in both GF (Spearman's rho 0.761, $p = 0.004$) and PDLF (rho = 0.802, $p = 0.002$). Corresponding with gene expression, IL-6 protein levels were higher in nonchallenged GF than in PDLF ($p = 0.0147$, Fig. 3B). TNF-α could not be detected in challenged and nonchallenged fibroblasts using ELISA.

Discussion

The aim of this study was to investigate responses of GF and PDLF to *P. gingivalis*. To study these responses,

we used an *in vitro* infection model in which primary human GF and PDLF were challenged with *P. gingivalis* W83, which represents the virulent K1 serotype (35–37).

This study, and earlier work by Zhou and co-workers (23), showed that the scale of the responses of GF and PDLF to *P. gingivalis* varies strongly between individuals. Because it is possible that heterogeneity is caused by the host genetic background of the cells, we compared GF and PDLF that were collected from the same donor.

To stimulate GF and PDLF we used viable bacteria, hypothesizing that viable bacteria are stronger stimulators of host cells than dead bacteria. Moreover, viable bacteria contain a complete subset of virulence factors. Previously it has been shown that viable *P. gingivalis* induce cytokine responses in monocytes and macrophages that are different from those induced by purified lipopolysaccharide or by the major fimbrial protein FimA (38–40). In the present study, viable *P. gingivalis* indeed induced stronger IL-6 and IL-8 gene-expression responses in GF and PDLF than dead bacteria. But even with viable *P. gingivalis*, a relatively high concentration of bacteria was needed to elicit clear responses in GF and PDLF. This may be a result of the fact that only about 10% of bacteria were able to come into contact with the fibroblasts in the experimental set up used. Also, *P. gingivalis* might not be a very potent immunogen; *A. actinomycetemcomitans*, for example, was recently shown to be a stronger stimulator of dendritic cells than *P. gingivalis* (41). Moreover, GF and PDLF are not antigen-recognizing cells such as macrophages or dendritic cells.

A main clinical symptom of periodontitis is resorption of the alveolar bone. Therefore, we analyzed a series of cytokines known to be involved in osteoclastogenesis and bone resorption. In both GF and PDLF, gene expression of IL-6, IL-8, TNF-α and MCP-1 increased in response to *P. gingivalis*, whereas OPG gene expression decreased in GF. RANTES and IL-1β gene expression also appeared to be induced in all six donors, but statistical significance was not reached. These findings are in line with previous data: not only have *P. gingivalis* virulence factors been shown to induce the expression of these cytokines (25–30,42,43), but Yamamoto and co-workers (24) also showed that viable *P. gingivalis* can induce IL-1β, IL-6, IL-8 and TNF-α in PDLF.

The induction of chemokines (IL-8, RANTES and MCP-1) and osteoclast-stimulating cytokines (IL-1β, IL-6 and TNF-α), and the decrease in OPG expression in GF, strongly suggest that this response to *P. gingivalis* can stimulate osteoclast formation and activity. However, we did not detect RANKL gene expression in challenged or non-challenged GF and PDLF, although Belibasakis and co-workers (28) showed that the culture supernatant of *P. gingivalis* induced RANKL in both GF and PDLF after 6 h of challenge. Again, viable *P. gingivalis* may have a different effect on host cells than their excreted proteins alone.

The increase in gene expression of IL-1β and IL-8 was also expressed by the elevated secreted protein levels. However, IL-6 was degraded, probably by proteases produced by the viable bacteria. It is likely that TNF-α was also degraded, and that the protein levels of IL-1β and IL-8 were underestimations, as these are also known to be susceptible to degradation by *P. gingivalis* (44–48). Similarly, Steffen and co-workers (49) described lower protein levels of IL-1β, IL-6 and IL-8 in supernatants from GF challenged with viable bacteria in comparison to dead *P. gingivalis*, and demonstrated that this was the result of proteolytic breakdown. Although recent research shows that IL-8 can be degraded very rapidly by viable *P. gingivalis* (50), we found increased IL-8 protein levels from challenged fibroblasts. However, in that study (50), gingival epithelial cells were challenged with *P. gingivalis,* and subsequently the medium was removed and viable *P. gingivalis* were added to this medium, allowing for proteolytic breakdown. In our set up we measured IL-8 production after 6 h of infection during which both IL-8 production by fibroblasts and IL-8 degradation by proteases can take place at the same time. As the gene expression of IL-8 was induced very strongly by *P. gingivalis*, the production of IL-8 protein was probably high enough to result in increased protein levels. Also, the extent of proteolytic breakdown can be influenced by the strain of *P. gingivalis* used, as noted by Bodet *et al.* (47).

We not only observed differences between individuals, but also differences between GF and PDLF. Non-challenged GF expressed more IL-6 than PDLF at both mRNA and protein levels. This may indicate a more activated state of GF than of PDLF, as GF are more likely to encounter oral pathogens.

Nonchallenged GF also expressed slightly more OPG than PDLF, a finding in line with earlier studies (19,20). We previously showed that, possibly by producing more OPG, GF were better inhibitors of osteoclast formation than PDLF (19). The OPG down-regulation we found in GF could mean that upon bacterial challenge, GF can lose the ability to inhibit osteoclast formation and bone resorption.

When donors were analyzed separately, we found strong differences between responses in GF and PDLF in the gene expression of IL-6 and IL-8. In four donors, GF were more responsive than PDLF to *P. gingivalis*; and in two donors, PDLF were more responsive than GF. Whether this difference in response has any implications for a person's susceptibility to periodontitis remains unclear. As GF are more likely to encounter *P. gingivalis* or other periodontal bacteria than PDLF, it seems of biological significance that GF would be more responsive. By attracting monocytes and granulocytes through producing RANTES and IL-8, they can initiate a first line of defence. Because PDLF are located more closely to the alveolar bone and have an osteoblast-like nature, their inflammatory response might affect the regulation of osteoclast formation and activation more explicitly.

Our finding, that viable *P. gingivalis* W83 induces inflammatory responses in both GF and PDLF, suggests that these cells can be involved in

inflammation and stimulation of osteoclast formation in periodontitis. It is now widely accepted that fibroblasts can play important roles in chronic infections leading to tissue damage (51,52). Our results show that considerable heterogeneity exists both between GF and PDLF and between individuals, which may be crucial determinants for their susceptibility to develop periodontitis.

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Supporting information

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

Figure S1. A: Fibroblast morphology of GF and PDLF from two donors, as seen with inverted phase-contrast microscopy, before challenge with *P. gingivalis* (left panels; $t = 0$ h), after 6 h challenge with *P. gingivalis* (middle panels; $t = 6$ h pg), or 6 h DMEM/non-challenged (right panels, $t = 6$ h control). No differences in fibroblast morphology are observed between challenged and non-challenged fibroblasts after 6 h, or between $t = 0$ h and $t = 6$ h. No cell rounding or floating of cells was observed before or after challenge with *P. gingivalis*. GF and PDLF from the two donors shown (donor C and donor D) are representative for GF and PDLF from all donors. B: Survival of *P. gingivalis* after aerobic incubation in DMEM with 10% FCS. The number of viable *P. gingivalis* (CFUs/mL) does not decrease after 3 h or 6 h of aerobic incubation in the same set-up as fibroblast challenge experiments. Dots represent the average CFUs/mL of two independent experiments performed in duplicate.

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References

1. Darveau RP, Tanner A, Page RC. The microbial challenge in periodontitis. *Periodontol 2000* 1997;**14:**12–32.
2. Feng Z, Weinberg A. Role of bacteria in health and disease of periodontal tissues. *Periodontol 2000* 2006;**40:**50–76.
3. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25:**134–144.
4. Socransky SS, Haffajee AD. Implications of periodontal microbiology for the treatment of periodontal infections. *Compend Suppl* 1994;**685:**688–693.
5. Slots J, Ting M. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. *Periodontol 2000* 1999;**20:**82–121.
6. Van Winkelhoff AJ, Loos BG, Van der Reijden WA, Van der Velden U. *Porphyromonas gingivalis*, *Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. *J Clin Periodontol* 2002;**29:**1023–1028.
7. O'Brien-Simpson NM, Veith PD, Dashper SG, Reynolds EC. Antigens of bacteria associated with periodontitis. *Periodontol 2000* 2004;**35:**101–134.
8. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet* 2005; **366:**1809–1820.
9. Oshiro T, Shiotani A, Shibasaki Y, Sasaki T. Osteoclast induction in periodontal tissue during experimental movement of incisors in osteoprotegerin-deficient mice. *Anat Rec* 2002;**266:**218–225.
10. Berkovitz BK. Periodontal ligament: structural and clinical correlates. *Dent Update* 2004;**52:**54.
11. Beertsen W, McCulloch CA, Sodek J. The periodontal ligament: a unique, multifunctional connective tissue. *Periodontol 2000* 1997;**13:**20–40.
12. Sakata M, Shiba H, Komatsuzawa H *et al*. Expression of osteoprotegerin (osteoclastogenesis inhibitory factor) in cultures of human dental mesenchymal cells and epithelial cells. *J Bone Miner Res* 1999;**14:**1486–1492.
13. Nagasawa T, Kobayashi H, Kiji M *et al*. LPS-stimulated human gingival fibroblasts inhibit the differentiation of monocytes into osteoclasts through the production of osteoprotegerin. *Clin Exp Immunol* 2002;**130:**338–344.
14. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. *J Dent Res* 2001;**80:**887–891.
15. Datta HK, Ng WF, Walker JA, Tuck SP, Varanasi SS. The cell biology of bone metabolism. *J Clin Pathol* 2008;**61:**577–587.
16. Fujita T, Iwata T, Shiba H *et al*. Identification of marker genes distinguishing human periodontal ligament cells from human mesenchymal stem cells and human gingival fibroblasts. *J Periodont Res* 2007;**42:**283–286.
17. Groeneveld MC, Everts V, Beertsen W. Alkaline phosphatase activity in the periodontal ligament and gingiva of the rat molar: its relation to cementum formation. *J Dent Res* 1995;**74:**1374–1381.
18. Han X, Amar S. Identification of genes differentially expressed in cultured human periodontal ligament fibroblasts vs. human gingival fibroblasts by DNA microarray analysis. *J Dent Res* 2002;**81:** 399–405.
19. de Vries TJ, Schoenmaker T, Wattanaroonwong N *et al*. Gingival fibroblasts are better at inhibiting osteoclast formation than periodontal ligament fibroblasts. *J Cell Biochem* 2006;**98:**370–382.
20. Hormdee D, Nagasawa T, Kiji M *et al*. Protein kinase-A-dependent osteoprotegerin production on interleukin-1 stimulation in human gingival fibroblasts is distinct from periodontal ligament fibroblasts. *Clin Exp Immunol* 2005;**142:**490–497.
21. Yashiro R, Nagasawa T, Kiji M *et al*. Transforming growth factor-beta stimulates interleukin-11 production by human periodontal ligament and gingival fibroblasts. *J Clin Periodontol* 2006;**33:**165–171.
22. Girasole G, Passeri G, Jilka RL, Manolagas SC. Interleukin-11: a new cytokine critical for osteoclast development. *J Clin Invest* 1994;**93:**1516–1524.
23. Zhou J, Windsor LJ. Heterogeneity in the collagen-degrading ability of *Porphyromonas gingivalis*-stimulated human gingival fibroblasts. *J Periodont Res* 2007;**42:** 77–84.
24. Yamamoto T, Kita M, Oseko F, Nakamura T, Imanishi J, Kanamura N. Cytokine production in human periodontal ligament cells stimulated with *Porphyromonas gingivalis*. *J Periodont Res* 2006;**41:**554–559.
25. Wang PL, Ohura K. *Porphyromonas gingivalis* lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. *Crit Rev Oral Biol Med* 2002;**13:**132–142.
26. Wang PL, Azuma Y, Shinohara M, Ohura K. Toll-like receptor 4-mediated signal pathway induced by *Porphyromonas*

gingivalis lipopolysaccharide in human gingival fibroblasts. *Biochem Biophys Res Commun* 2000;**273:**1161–1167.
27. Takeshita A, Imai K, Hanazawa S. CpG motifs in *Porphyromonas gingivalis* DNA stimulate interleukin-6 expression in human gingival fibroblasts. *Infect Immun* 1999;**67:**4340–4345.
28. Belibasakis GN, Bostanci N, Hashim A *et al*. Regulation of RANKL and OPG gene expression in human gingival fibroblasts and periodontal ligament cells by *Porphyromonas gingivalis*: a putative role of the Arg-gingipains. *Microb Pathog* 2007;**43:**46–53.
29. Tabeta K, Yamazaki K, Akashi S *et al*. Toll-like receptors confer responsiveness to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts. *Infect Immun* 2000;**68:** 3731–3735.
30. Wang PL, Oido-Mori M, Fujii T *et al*. Heterogeneous expression of Toll-like receptor 4 and downregulation of Toll-like receptor 4 expression on human gingival fibroblasts by *Porphyromonas gingivalis* lipopolysaccharide. *Biochem Biophys Res Commun* 2001;**288:**863–867.
31. Urnowey S, Ansai T, Bitko V, Nakayama K, Takehara T, Barik S. Temporal activation of anti- and pro-apoptotic factors in human gingival fibroblasts infected with the periodontal pathogen, *Porphyromonas gingivalis*: potential role of bacterial proteases in host signalling. *BMC Microbiol* 2006;**6:**26.
32. Morioka M, Hinode D, Nagata A *et al*. Cytotoxicity of *Porphyromonas gingivalis* toward cultured human gingival fibroblasts. *Oral Microbiol Immunol* 1993; **8:**203–207.
33. Boutaga K, van Winkelhoff AJ, Vandenbroucke-Grauls CM, Savelkoul PH. Comparison of real-time PCR and culture for detection of *Porphyromonas gingivalis* in subgingival plaque samples. *J Clin Microbiol* 2003;**41:**4950–4954.
34. Kumar A, Takada Y, Boriek AM, Aggarwal BB. Nuclear factor-kappaB: its role in health and disease. *J Mol Med* 2004;**82:**434–448.
35. van Winkelhoff AJ, Appelmelk BJ, Kippuw N, de Graaff J. K-antigens in *Porphyromonas gingivalis* are associated with virulence. *Oral Microbiol Immunol* 1993; **8:**259–265.
36. Davey ME, Duncan MJ. Enhanced biofilm formation and loss of capsule synthesis: deletion of a putative glycosyltransferase in *Porphyromonas gingivalis*. *J Bacteriol* 2006;**188:**5510–5523.
37. Laine ML, van Winkelhoff AJ. Virulence of six capsular serotypes of *Porphyromonas gingivalis* in a mouse model. *Oral Microbiol Immunol* 1998;**13:**322–325.
38. Zhou Q, Desta T, Fenton M, Graves DT, Amar S. Cytokine profiling of macrophages exposed to *Porphyromonas gingivalis*, its lipopolysaccharide, or its FimA protein. *Infect Immun* 2005;**73:**935–943.
39. Zhou Q, Amar S. Identification of signaling pathways in macrophage exposed to *Porphyromonas gingivalis* or to its purified cell wall components. *J Immunol* 2007;**179:**7777–7790.
40. Zhou Q, Amar S. Identification of proteins differentially expressed in human monocytes exposed to *Porphyromonas gingivalis* and its purified components by high-throughput immunoblotting. *Infect Immun* 2006;**74:**1204–1214.
41. Vernal R, Leon R, Herrera D, Garcia-Sanz JA, Silva A, Sanz M. Variability in the response of human dendritic cells stimulated with *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*. *J Periodont Res* 2008;**43:**689–697.
42. Kusumoto Y, Hirano H, Saitoh K *et al*. Human gingival epithelial cells produce chemotactic factors interleukin-8 and monocyte chemoattractant protein-1 after stimulation with *Porphyromonas gingivalis* via toll-like receptor 2. *J Periodontol* 2004;**75:**370–379.
43. Wada N, Maeda H, Yoshimine Y, Akamine A. Lipopolysaccharide stimulates expression of osteoprotegerin and receptor activator of NF-kappa B ligand in periodontal ligament fibroblasts through the induction of interleukin-1 beta and tumor necrosis factor-alpha. *Bone* 2004;**35:**629–635.
44. Mikolajczyk-Pawlinska J, Travis J, Potempa J. Modulation of interleukin-8 activity by gingipains from *Porphyromonas gingivalis*: implications for pathogenicity of periodontal disease. *FEBS Lett* 1998;**440:**282–286.
45. Fletcher J, Reddi K, Poole S *et al*. Interactions between periodontopathogenic bacteria and cytokines. *J Periodont Res* 1997;**32:**200–205.
46. Fletcher J, Nair S, Poole S, Henderson B, Wilson M. Cytokine degradation by biofilms of *Porphyromonas gingivalis*. *Curr Microbiol* 1998;**36:**216–219.
47. Bodet C, Chandad F, Grenier D. Modulation of cytokine production by *Porphyromonas gingivalis* in a macrophage and epithelial cell co-culture model. *Microbes Infect* 2005;**7:**448–456.
48. Calkins CC, Platt K, Potempa J, Travis J. Inactivation of tumor necrosis factor-alpha by proteinases (gingipains) from the periodontal pathogen, *Porphyromonas gingivalis*. Implications of immune evasion. *J Biol Chem* 1998;**273:**6611–6614.
49. Steffen MJ, Holt SC, Ebersole JL. *Porphyromonas gingivalis* induction of mediator and cytokine secretion by human gingival fibroblasts. *Oral Microbiol Immunol* 2000;**15:**172–180.
50. Stathopoulou PG, Benakanakere MR, Galicia JC, Kinane DF. The host cytokine response to *Porphyromonas gingivalis* is modified by gingipains. *Oral Microbiol Immunol* 2009;**24:**11–17.
51. Flavell SJ, Hou TZ, Lax S, Filer AD, Salmon M, Buckley CD. Fibroblasts as novel therapeutic targets in chronic inflammation. *Br J Pharmacol* 2008; **153**(suppl 1)**:**S241–S246.
52. Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D, Salmon M. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol* 2001;**22:**199–204.