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ORIGINAL ARTICLE

Sterile-filtered saliva is a strong inducer of IL-6 and IL-8 in oral fibroblasts

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Abstract
Objectives Saliva has been implicated to support oral wound healing, a process that requires a transient inflammatory reaction. However, definitive proof that saliva can provoke an inflammatory response remained elusive.
Materials and methods We investigated the ability of freshly harvested and sterile-filtered saliva to cause an inflammatory response of oral fibroblasts and epithelial cells. The expression of cytokines and chemokines was assessed by microarray, RT-PCR, immunoassays, and Luminex technology. The involvement of signaling pathways was determined by Western blot analysis and pharmacologic inhibitors.
Results We report that sterile-filtered whole saliva was a potent inducer of IL-6 and IL-8 in fibroblasts from the gingiva, the palate, and the periodontal ligament, but not of oral epithelial cells. This strong inflammatory response requires nuclear factor-kappa B and mitogen-activated protein kinase signaling. The pro-inflammatory capacity is heat stable and has a molecular weight of <40 kDa. Genome-wide microarrays and Luminex technology further revealed that saliva substantially increased expression of other inflammatory genes and various chemokines. To preclude that the observed pro-inflammatory activity is the result of oral bacteria, sterile-filtered parotid saliva, collected under almost aseptic conditions, was used and also increased IL-6 and IL-8 expression in gingiva fibroblasts. The inflammatory response was, furthermore, independent of MYD88, an adapter protein of the Toll-like receptor signaling pathway.
Conclusions We conclude that saliva can provoke a robust inflammatory response in oral fibroblasts involving the classical nuclear factor-kappa B and mitogen-activated protein kinase signaling pathway.
Clinical relevance Since fibroblasts but not epithelial cells show a strong inflammatory response, saliva may support the innate immunity of defect sites exposing the oral connective tissue.

Keywords Saliva · Inflammation · Fibroblasts · Cytokines · NFκB · Microarray · Luminex technology

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Introduction

Saliva being produced by the submandibular and the parotid glands carries physiologic molecules including electrolytes, mucus, antibacterial compounds, enzymes, and growth factors [1]. Besides its function in lubrication and initiating food digestion, saliva proteins form the pellicle layer on enamel that supports mineralization [2] and biofilm formation [3]. Research has advanced in the biochemical characterization of pellicle proteins [4] and the adhesion of various types of bacteria [5]. Moreover, saliva is used for diagnostic purposes. The physiologic role of saliva becomes obvious at disease state, for example, in patients with Sjögren syndrome and patients

receiving head and neck radiotherapy. Saliva clearly holds a myriad of functions, and revealing its multiple tasks remains a challenge.

Saliva has been implicated to support oral wound healing. Desalivated rodents showed impaired healing of extraction sites or palatal wounds [6, 7]. Also, crude extracts of salivary glands change cell viability in vitro [8]. Whole saliva lowers the viability of the osteogenic MC3T3-E1 cell line; however, the cells failed to release significant amounts of inflammatory cytokines in response to saliva, e.g., interleukin-1 (IL-1) or tumor necrosis factor alpha. This missing significance was assumed by the authors as a consequence of interindividual release upon using saliva from different donors [9]. On the other hand, truncated lactoferrin isolated of whole saliva possesses inflammatory properties as shown by the increased IL-6 and IL-8 production of the epithelial human squamous carcinoma (HSC)-2 cells [10]. Thus, the data are somehow heterogeneous, which might be based on the use of different types of cells, from hard and soft tissue, and due to the fact that the cells were stimulated for varying times and with salivary components or whole saliva, respectively. Also, other components from saliva such as epidermal growth factor (EGF) [11], transforming growth factor (TGF)-beta [12], histatin [13], and cystatin [14] can provoke a cellular reaction. Saliva can thus cause a complex cellular response, including inflammation on the oral tissue.

Inflammation is a part of the innate immunity and ultimately linked with wound healing. The cytokines IL-6 and IL-8, which are highly expressed at wound site, attract and activate immune cells [15]. IL-6 is mostly related to inflammation, host defense, and tissue injury [16], while IL-8 is known to be a very important pro-inflammatory mediator and chemoattractant [17]. In the oral cavity, gingiva and periodontal ligament fibroblasts are capable to produce IL-6 and IL-8 upon incubation with pro-inflammatory agents such as bacteria [18] and activation of pattern recognition receptors [19]. Saliva IL-6 and IL-8, which are like other inflammatory cytokines mostly derived from gingival crevicular fluid or tissue exudates are increased in caries patients [20] and during gingival inflammation [21, 22], even though the link to periodontitis [23] and periimplantitis [24] is vague. These data support the association of saliva and the production of IL-6 and IL-8 in the oral cavity.

Surprisingly, and considering that many of the single components of saliva have been tested for their capacity to cause a cellular reaction [25], in vitro research with whole saliva is rare. Research with whole saliva, however, is particularly important because single components only partially reflect the complexity of the physiologic molecular cocktail. The purpose of the present study was therefore to test the hypothesis that freshly harvested sterile-filtered whole saliva is able to provoke an inflammatory response of oral cells in vitro.

Material and methods

Cells and stimulation of cells with saliva

Cells, which may come in contact with saliva in the oral cavity as well a monocytic cell line and a carcinoma cell line, were used to figure out possible different responses of the different cell types to saliva. For experiments with primary cells, cell pools made from cells of three different donors each were used. Human gingiva, palatal, and periodontal ligament fibroblasts were prepared from tissue grafts after approval (Kantonale Ethikkommission Bern) and informed consent by the donor were obtained. The donors were selected on the basis of the absence of previous history of inflammation. To isolate fibroblasts, tissue explants were cultivated in Dulbecco's modified Eagle medium (DMEM, Invitrogen Corporation, Carlsbad, CA) supplemented with 10 % fetal calf serum (FCS; PAA Laboratories, Linz, Austria) and antibiotics (Invitrogen) at 37 °C, 5 % CO_2, and 95 % humidity. Fibroblasts that grew out from the explants and had not undergone more than five passages were used. U937, a human leukemic monocyte lymphoma cell line, and oral squamous cell carcinoma, HSC-2, were kindly provided by the Medical University of Vienna, Austria. Primary human gingival epithelial cells were obtained from CELLnTEC Advanced Cell Systems AG (Bern, Switzerland). Human gingiva, palatal, and periodontal ligament fibroblasts as well as U937, HSC-2, and primary human gingival epithelial cells were used in indicated experiments to investigate the response of various cell types to saliva. All other experiments were performed with a pool of human gingiva fibroblasts. Cells were plated in growth medium at 30,000 cells/cm^2 into culture dishes. The following day, cells were incubated in serum-free medium containing freshly prepared sterile-filtered whole saliva immediately after saliva collection. Cell incubation showed that 2-fold but not 10-fold dilution visually changes cell morphology (Fig. 1c). Thus, if not otherwise indicated, cells were exposed to saliva at the 10-fold dilution and incubated for 6 h for expression analysis and 24 h for immunoassays.

Saliva sampling and treatment

Whole saliva was collected from donors, who were non-smokers and had no oral inflammation, while chewing

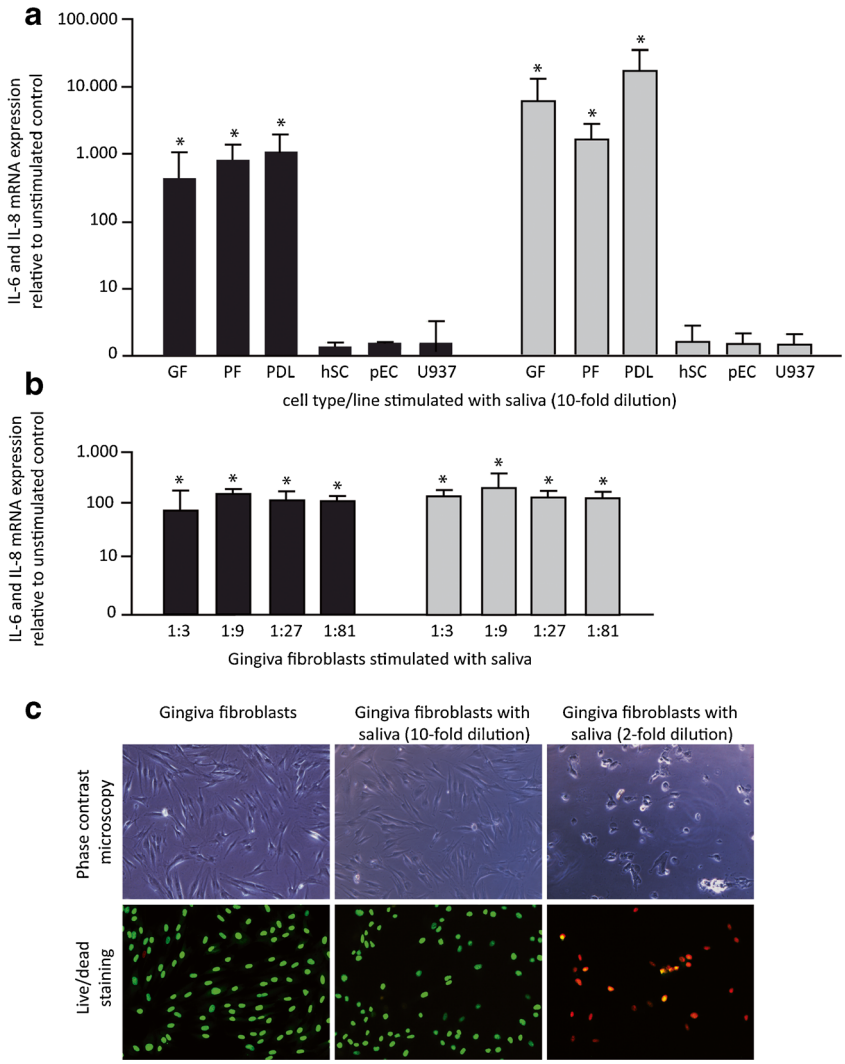

Fig. 1 Saliva stimulates IL-6 and IL-8 in various cell types of the oral cavity. Cells were incubated in serum containing medium at a density of 30,000 cells/cm^2. The following day, cells were incubated in serum-free medium containing saliva. Fibroblasts from the human gingiva (*GF*), palatal (*PF*), and periodontal ligament fibroblasts (*PDL*) as well as oral squamous cell carcinoma (*HSC-2*) and primary human gingival epithelial cells (*pEC*) and a human leukemic monocyte lymphoma cell line (*U937*) were exposed to saliva. The fibroblastic cells showed a strong response to saliva as indicated by the >10-fold increase in the expression of IL-6 (*black bars*) and IL-8 (*gray bars*). The monocytic cell line and both epithelial cells, however, showed a rather moderate response with an increase below 10-fold (**a**). Dose-response curves indicate that the maximal stimulation of the cytokines was reached at a dilution factor of approximately 10-fold, with less expression at the 3-fold dilution (**b**). Saliva at a higher concentration caused severe damages, while saliva at the 1:10 dilution used in the present study provides no harm to the cells (**c**). A significant difference to unstimulated control was defined to be $p<0.05$ ($*p<0.05$)

paraffin wax (Ivoclar Vivadent AG, Schaan, Liechtenstein). Donors provided their written informed consent to participate in this study. Saliva was collected between 09:00 and 11:00 a.m., and donors were abstained from

Table 1 Primer sequence of RT-PCR

Gene	Forward primer	Reverse primer	Reference
IL-6	gaaaggagacatgtaacaagagt	gattttcaccaggcaagtct	[35]
IL-8	aacttctccacaaccctctg	ttggcagccttcctgatttc	[36]
ICAM-1	ccttcctcaccgtgtactgg	agcgtagggtaaggttcttgc	
CXCL1	tcctgcatcccccatagtta	cttcaggaacagccaccagt	
CXCL2	cccatggttaagaaaatcatcg	cttcaggaacagccaccaat	
CXCL3	aaatcatcgaaaagatactgaacaag	ggtaagggcagggaccac	
CXCL6	gtccttcgggctccttgt	cagcacagcagagacaggac	
CCL7	gaaagcctctgcagcacttc	aatctgtagcagcaggtagttgaa	
CCL20	gctgctttgatgtcagtgct	gcagtcaaagttgcttgctg	
PTGS2	cttcacgcatcagtttttcaag	tcaccgtaaatatgatttaagtccac	
BCL2A1	caggagaatggataaggcaaa	ccagccagatttaggttcaaa	

eating and drinking for 1 h prior to collection. The local ethical board of the University of Bern approved sampling. Immediately after collection, saliva was centrifuged at 4,000× *g* for 5 min. Saliva was filtered sterile (1 ml per 0.22 μm PES syringe filter, TPP AG, Trasadingen, Switzerland) to remove viable microorganisms [26]. Samples were immediately used to stimulate cells.

Parotid saliva sampling and microbiological cultures

For indicated experiments, stimulated parotid saliva was harvested. After mouth rinse with 0.12 % chlorhexidine gluconate, the parotid duct was wiped with a cotton swab with hydrogen peroxide. A lemon drop was sucked on the tongue, and after rejecting the first five drops, parotid saliva was collected with a sterile syringe. Undiluted parotid saliva was subjected to a blood agar and a lysogeny broth agar plate at a volume of 200 μl (dilution series was performed in preliminary tests). The number of colonies was determined after incubation at 37 °C for 48 h. The limit of detection per plate was 1 colony-forming unit (CFU), which means 5 CFU/ml. Sterile-filtered parotid saliva at a 10-fold dilution was also used to stimulate human gingiva fibroblasts for 6 h.

Blocking of MYD88 and TLRs

To further exclude that the inflammatory reaction induced by saliva is caused by microorganism and their products, myeloid differentiation primary response (MYD)88, an adapter protein of the Toll-like receptor signaling pathway, and the Toll-like receptors 2 and 4 themselves were blocked. Human gingiva fibroblasts were preincubated with MYD88 inhibitory peptide at 100 μM for 24 h and Toll-like receptors (TLR)2 and TLR4 inhibitory peptide at 50 μM for 1 hour, respectively. Afterwards sterile-filtered whole saliva in a 10-fold dilution was used to stimulate the cells for 6 h, and expression of inflammatory genes was performed.

Saliva treatment

In order to understand more what components of saliva are responsible for the pro-inflammatory reaction of human gingiva fibroblasts, different saliva treatment methods were performed with sterile-filtered whole saliva

Table 2 Saliva stimulates IL-6, IL-8, and cytokine protein expression (pg/ml) in various cell types of the oral cavity. All fibroblastic cells, independent of their origin, showed a robust response to saliva as indicated by the >10-fold increase in the expression of IL-6 and IL-8, detected by ELISA technology. Luminex analysis (ProcartaPlex™ Multiplex Immunoassay, eBioscience Vienna, Austria) for human IL-6, human IL-8, human GM-CSF, human GRO-alpha, human MCP-3, and human MIP-3alpha showed statistically significant differences between untreated fibroblasts (w/o) and fibroblasts treated with saliva (saliva)

ELISA (*n*=1)						
	IL-6 w/o	IL-6 saliva	IL-8 w/o	IL-8 saliva		
GF	247	7,586	536	10,486		
PF	386	15,834	455	18,823		
PDL	234	5,069	132	7,473		
hSC	199	230	805	982		
pEC	182	760	727	750		
U937	182	173	291	555		
Luminex (*n*=3)						
	IL-6 w/o	IL-6 saliva	IL-8 w/o	IL-8 saliva	MCP-3 w/o	MCP-3 saliva
GF	1,010 (±1,542)	21,101 (±14,341)	n.d.	3,364 (±3960)	n.d.	190 (±245)
PF	158 (±335)	4,207 (±1,101)	n.d.	680 (±660)	n.d.	n.d.
PDL	198 (±34)	11,821 (±14,419)	n.d.	1,536 (±2,009)	n.d.	n.d.
	GRO-a w/o	GRO-a saliva	MIP-3a w/o	MIP-3a saliva	GM-CSF w/o	GM-CSF saliva
GF	62 (±54)	704 (±521)	n.d.	31 (±53)	n.d.	284 (±407)
PF	156 (±130)	456 (±131)	n.d.	n.d.	n.d.	175 (±7)
PDL	n.d.	140 (±83)	n.d.	n.d.	n.d.	n.d.

Data represent the mean plus standard deviation of three independent experiments

n.d. not detectable

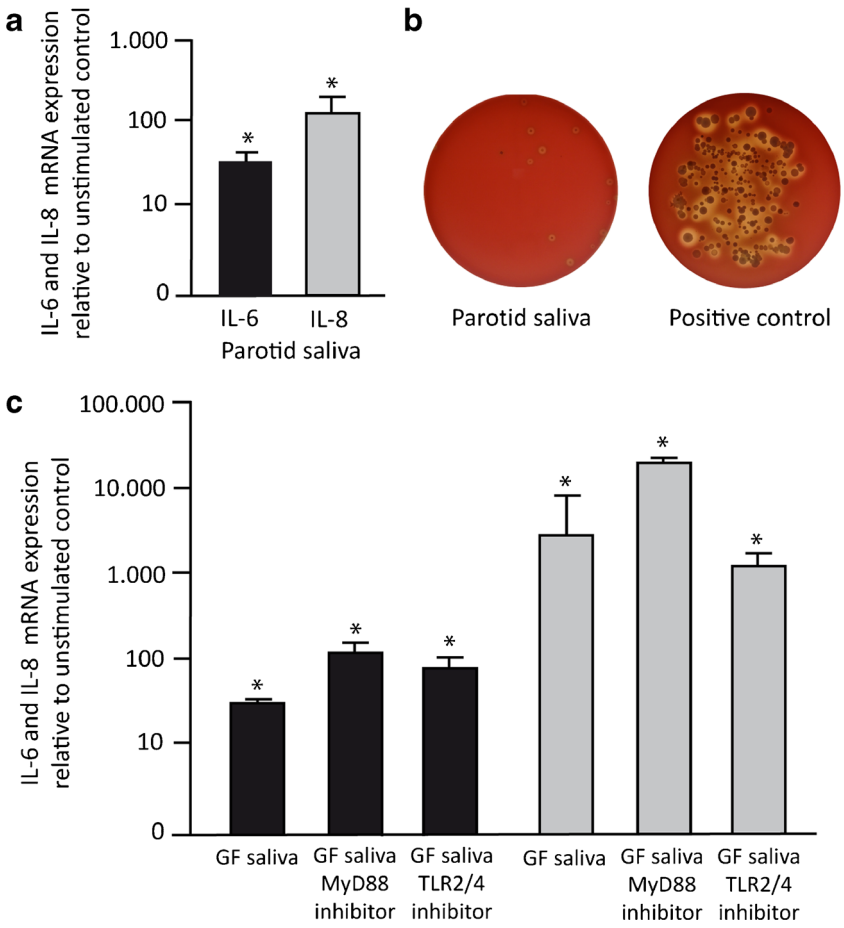

Fig. 2 Sterile-prepared parotid saliva stimulates IL-6 and IL-8. Parotid saliva was prepared under aseptic conditions. This sterile-filtered parotid saliva increased IL-6 (*black bars*) and IL-8 (*gray bars*) expression in human gingiva fibroblasts (>10-fold) (**a**). The number of colonies was less than 10^2/ml (**b**). Blocking MYD88 and toll-like receptors 2 and 4, essential receptors in the signaling of lipoteichoic acid and lipopolysaccharides, had no effect on the strong pro-inflammatory activity of sterile-filtered saliva in gingiva fibroblasts (**c**). A significant difference to unstimulated control was defined to be $p<0.05$ ($*p<0.05$)

immediately after collection. For size exclusion chromatography of sterile-filtered whole saliva, Micro Bio-Spin 30 Columns (Bio-Rad Laboratories, Hercules, CA) were used. Heating of sterile-filtered whole saliva for 5 min at 96 °C was performed in a thermomixer (Eppendorf AG, Hamburg, Germany). Freezing of sterile-filtered whole

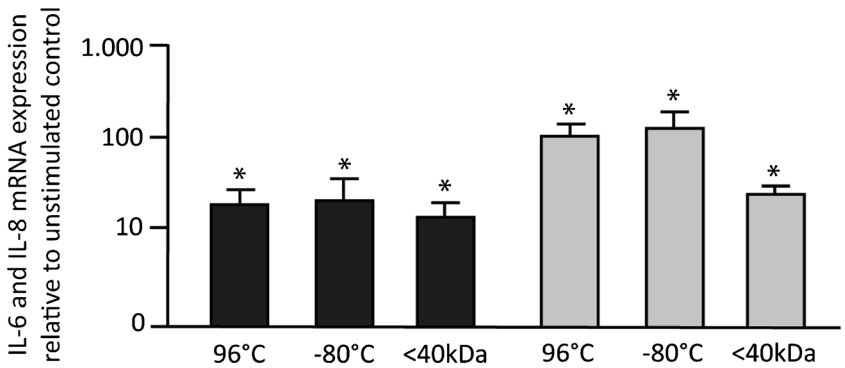

Fig. 3 Saliva after heating at 96 °C maintains the ability to provoke IL-6 and IL-8 expression. Different saliva treatment methods were performed in order to restrict the pro-inflammatory component. Sterile-filtered whole saliva, however, maintained the capacity to provoke IL-6 (*black bars*) and IL-8 (*gray bars*) expression by human gingiva fibroblasts when heated to 96 °C. Freezing as well as a molecular weight fraction obtained with a cutoff of 40 kDa increased IL-6 and IL-8 expression in oral fibroblasts. A significant difference to unstimulated control was defined to be $p<0.05$ ($*p<0.05$)

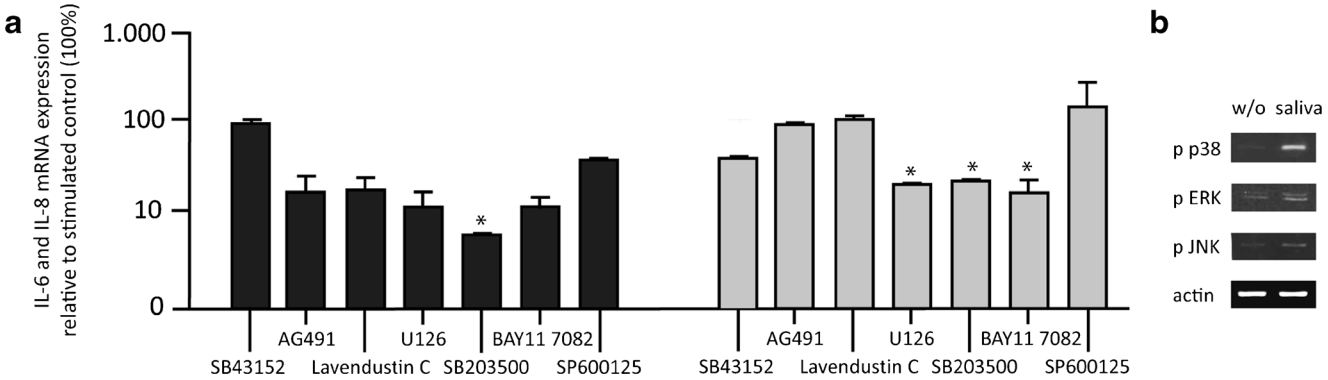

Fig. 4 Saliva-induced IL-6 and IL-8 require MAPK, but not TGF-β receptor I kinase or EGF receptor signaling. SB431542, the TGF-β receptor I kinase inhibitor, AG-494 and Lavendustin C, both inhibitors of EGF receptor kinase, did not significantly change the expression pattern of IL-6 (*black bars*) and IL-8 (*gray bars*) in human gingiva fibroblasts. Inhibitors of the MAPK family and NFκB like U0126, BAY 11-7082, and SB203580 reduced the expression of IL-6 and IL-8 in the presence of saliva. Interestingly, SP600125 enhanced the expression of IL-6 and IL-8 (**a**). Moreover, saliva increased phosphorylation of ERK, p38, and JNK in gingiva fibroblasts as shown in Western blot analysis (**b**). A significant difference to stimulated control was defined to be $p<0.05$ ($*p<0.05$)

saliva was performed at -80 °C for 24 h. Cells were stimulated at a 10-fold dilution immediately after preparation of the saliva.

Stimulation of cells with saliva and pathway inhibitors

Human gingiva fibroblasts were also exposed to inhibitors of mitogen-activated protein kinase (MAPK) signaling, e.g., U0126, SB203580, and SP600125, all at 10 μM (Santa Cruz Biotechnology, SCBT; Santa Cruz, CA) in serum-free medium. Cyclooxygenase (COX) 1 and 2 were blocked with indomethacin at 1 μM (Sigma). BAY 11-7082 was used to block nuclear factor-kappaB (NFκB) signaling at 10 μM. Inhibitors of epidermal growth factor (EGF) receptor kinase were AG-494 at 10 μM and lavendustin C at 10 μM (all Enzo Life Sciences, Inc., Farmingdale, NY). SB431542, a TGF-β receptor I kinase inhibitor was used at 10 μM (SCBT).

Gene chip analysis

Total cellular RNA from human gingiva fibroblasts was isolated with the High Pure RNA Isolation Kit (Roche F. Hoffmann-La Roche, Basel Switzerland). Quality control of the RNAs, as well as labeling, hybridization, and scanning of the hybridized arrays, was performed by arrows biomedical Deutschland GmbH (Münster, Germany) using the Human GE 4x44K V2 Microarray Kit with SurePrint Technology (Agilent, Santa Clara, CA, USA).

Gene expression analysis

Reverse transcription (RT) was performed with Transcriptor Universal cDNA Master (Roche), and polymerase chain reaction (PCR) was done with the FastStart Universal SYBR Green Master (Roche) on a 7500 Real-Time PCR System (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA). Primers were designed in the online Universal ProbeLibrary Assay Design Center (Roche), and sequence is provided in Table 1. The messenger RNA (mRNA) levels were calculated by normalizing to GAPDH using the ΔCt method. The immunoassay for human IL-6 and IL-8 was obtained from Enzo Life Sciences and PeproTech GmbH (Hamburg, Germany), respectively.

Protein expression analysis by Luminex and immunoassay

Luminex analysis was performed by ProcartaPlex™ Multiplex Immunoassay (eBioscience Vienna, Austria) for human IL-6, human IL-8, human granulocyte-macrophage colony-stimulating factor (GM-CSF), human growth regulated oncogene (GRO)-alpha, human monocyte chemotactic protein (MCP)-3, and human macrophage inflammatory protein (MIP)-3alpha. The immunoassay for human IL-6 and IL-8 was obtained from Enzo Life Sciences and PeproTech GmbH (Hamburg, Germany), respectively.

Western blot analysis

Human gingiva fibroblasts were serum-starved for overnight and then treated with freshly prepared sterile-filtered whole saliva immediately after saliva collection at the 10-fold

Table 3 Saliva induced substantial changes in other inflammatory genes as shown in a microarray. Experiment 1: gingiva fibroblasts with and without sterile-filtered whole saliva (1:10), 6 h

Probeset ID	Gene name	Log intensity (GF wo)	Log intensity (GF saliva)	Ratio	Absolute fold change
A_32_P87013	IL8	2.58899	15.2936	0.000149807	6675.27
A_23_P7144	CXCL1	5.72941	17.4016	0.000306423	3263.47
A_23_P92107	SLC15A2	13.2984	3.51625	880.482	880.482
A_23_P71037	IL6	7.48191	17.1568	0.0012234	817.395
A_23_P17065	CCL20	2.31199	11.9564	0.00124952	800.307
A_33_P3395422	XM_001720080	2.49852	11.5931	0.00182919	546.69
A_24_P250922	PTGS2	4.24932	13.2267	0.00198399	504.035
A_23_P152002	BCL2A1	3.87836	12.8247	0.00202714	493.306
A_33_P3330264	CXCL1	5.78246	14.7101	0.00205358	486.953
A_23_P340019	NLRC3	12.062	3.44555	392.473	392.473
A_23_P153320	ICAM1	5.39503	13.9985	0.00257097	388.958
A_23_P315364	CXCL2	6.32403	14.8663	0.00268238	372.803
A_23_P55632	SERPINB3	2.97668	11.3418	0.00303283	329.725
A_24_P257416	CXCL2	6.14868	14.2923	0.00353611	282.796
A_24_P183150	CXCL3	4.72527	12.8097	0.00368421	271.429
A_23_P133408	CSF2	2.59006	10.6121	0.00384703	259.941
A_33_P3278279	TXNDC8	2.83172	10.6824	0.00433221	230.829
A_33_P3243230	ENST00000401931	2.70622	10.5018	0.00450087	222.179
A_33_P3447304	OXR1	15.9867	8.2525	212.925	212.925
A_23_P78037	CCL7	2.96843	10.5768	0.00512451	195.141
A_33_P3334798	ANTXRL	2.83134	10.3628	0.00540511	185.01
A_24_P303091	CXCL10	2.50982	9.94392	0.00578246	172.937
A_23_P121676	CXXC4	2.68255	9.9863	0.00632925	157.997
A_33_P3718269	ENST00000517927	2.45629	9.71179	0.00654451	152.8
A_33_P3319791	NR4A1	2.59948	9.82877	0.00666449	150.049
A_23_P398566	NR4A3	6.37319	13.5613	0.00685746	145.827
A_33_P3343175	CXCL10	2.62246	9.80532	0.00688246	145.297
A_32_P8546	C6orf176	2.86401	9.98688	0.00717468	139.379
A_33_P3394559	PRAMEF6	2.69292	9.7646	0.00743382	134.52
A_23_P104819	TREH	3.07481	10.0711	0.00783261	127.671
A_33_P3327106	LOC100129900	2.74187	9.6895	0.0081013	123.437
A_23_P26024	C15orf48	6.05466	12.9827	0.00821206	121.772
A_23_P79518	IL1B	3.16344	10.0609	0.00838799	119.218
A_24_P98006	MCHR2	3.5985	10.493	0.00840521	118.974
A_23_P314755	STC1	4.64903	11.5271	0.00850148	117.627
A_33_P3407013	ENST00000420258	2.79436	9.53102	0.00937698	106.644
A_23_P216118	UNC5D	2.70118	9.27273	0.010514	95.1116
A_24_P63380	BMPR1B	3.16638	9.68382	0.0109158	91.6104
A_24_P237270	ADORA2A	2.50244	9.00375	0.0110385	90.5919
A_23_P155786	SULT1E1	2.85388	9.3288	0.0112423	88.9499
A_23_P25155	GPR84	2.44536	8.79923	0.0122263	81.791
A_33_P3265129	ACSM2A	2.8229	9.13686	0.0125692	79.5594
A_23_P89431	CCL2	10.8917	17.1389	0.0131645	75.9617
A_23_P155755	CXCL6	2.54966	8.77347	0.0133797	74.7401
A_23_P86470	CH25H	6.81034	12.9794	0.0138972	71.9568

Table 3 (continued)

Probeset ID	Gene name	Log intensity (GF wo)	Log intensity (GF saliva)	Ratio	Absolute fold change
A_33_P3313899	LOC728228	3.0997	9.21134	0.0144615	69.1492
A_23_P420863	NOD2	2.77057	8.87364	0.0145477	68.7396
A_23_P64828	OAS1	3.52295	9.58639	0.0149528	66.8771
A_33_P3216758	CEACAM16	3.84972	9.89933	0.0150968	66.2391
A_23_P353048	SSPO	2.566	8.57327	0.0155465	64.3233

This table shows the top 50 regulated genes, sorted by their absolute fold change. A fold change of, e.g., 2 indicates that the gene is 2-fold upregulated in the GF wo vs GF saliva sample

dilution for 30 min. Cell extracts containing sodium dodecyl sulfate (SDS) buffer and protease inhibitors (SCBT) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Whatman, GE Healthcare, General Electric Company, Fairfield, CT). Membranes were blocked in a supplied buffer (LI-COR Biosciences; Lincoln, NE). Binding of the antibody raised against phospho-p38, phospho-c-Jun N-terminal kinase (JNK) (both from Cell Signaling Technology, Danvers, MA), phospho-extracellular signal-regulated kinase (ERK), and β-actin (both SCBT). Primary antibodies were detected with the appropriate secondary antibody directly labeled with near-infrared dyes and detected with the appropriate imaging system (LI-COR Biosciences; Lincoln, NE).

Statistical analysis

Data represent the mean plus standard deviation of three independent experiments, each performed in triplicates. Differences in mRNA expression of target genes between stimulated and unstimulated cells were tested using a nonparametric Mann-Whitney *U* test for paired samples (SPSS version 19.0, SPSS Inc., Chicago, IL, USA). Alpha of 5 % was considered significant.

Results

Sterile-filtered whole saliva stimulates IL-6 and IL-8 in various cell types of the oral cavity

As shown in Fig. 1a, all fibroblastic cells, independent of their origin, showed a significant response to saliva as indicated by the >10-fold increase in the expression of IL-6 and IL-8 ($p<0.05$). The monocytic cell line and both epithelial cells, however, showed a rather moderate response with an increase in the two cytokines below 10-fold (Fig. 1a) ($p>0.05$). The response of the fibroblasts to saliva translates into protein as shown with ELISA (Table 2) and Luminex technology using the culture supernatants (Table 2).

Freshly harvested sterile-filtered whole saliva increases mRNA of IL-6 and IL-8

Sterile-filtered whole saliva considerably (>10-fold) increased the mRNA level of the IL-6 and IL-8 ($p<0.05$). Dose-response curves indicate that the maximal stimulation was reached at a dilution factor of 10-fold (Fig. 1b). Time-response curves showed a substantial expression after 6 h (data not shown). Cell morphology was maintained in diluted saliva (Fig. 1c). Further experiments were thus performed with sterile-filtered whole saliva at the 10-fold dilution and exposure time of 6 h.

Bacteria and microorganism do not cause the inflammatory reaction

To preclude that the observed pro-inflammatory activity is the result of oral bacteria, we used sterile-filtered parotid saliva collected under almost aseptic conditions instead of sterile-filtered whole saliva. This sterile-filtered parotid saliva increased IL-6 and IL-8 expression in gingiva fibroblasts (>10-fold; Fig. 2a) ($p<0.05$). The number of colonies was less than 10^2/ml (from approximately 5 to 75 CFU/ml) (Fig. 2b), and a second blood agar plate and the lysogeny broth agar plates showed one single colony or no colonies, respectively (data not shown). Bacterial toxins are unlikely to be responsible for the inflammatory reaction under these conditions.

Sterile-filtered whole saliva also significantly ($p<0.05$) increased the mRNA level of the IL-6 and IL-8 in the case when MYD88 and Toll-like receptors 2 and 4, respectively, were blocked, which is a supplementary proof that the effect is

Table 4 Saliva induced substantial changes in other inflammatory genes as shown in a microarray. Experiment 2: gingiva fibroblasts with and without sterile-filtered whole saliva (1:10), 6 h

Probeset ID	Gene name	Log intensity (GF wo)	Log intensity (GF saliva)	Ratio	Absolute fold change
A_23_P7144	CXCL1	3.14235	16.267	0.00011197	8931.27
A_32_P87013	IL8	3.21679	14.8875	0.00030674	3260.12
A_23_P133408	CSF2	3.19471	12.1627	0.00199694	500.765
A_23_P315364	CXCL2	5.01458	13.7903	0.00228163	438.284
A_24_P183150	CXCL3	3.43033	12.0855	0.00248047	403.149
A_23_P71037	IL6	6.70614	15.2084	0.00275781	362.606
A_23_P153320	ICAM1	5.73492	13.6422	0.00416554	240.065
A_23_P152002	BCL2A1	4.56323	12.0878	0.00543099	184.129
A_24_P250922	PTGS2	4.06508	11.5464	0.00559627	178.691
A_33_P3243230	ENST00000401931	3.31141	10.3956	0.00736964	135.692
A_23_P17065	CCL20	3.02568	9.96244	0.00816258	122.51
A_32_P83270	LOC727982	5.47545	12.4016	0.00822283	121.613
A_33_P3329133	A_33_P3329133	10.3814	3.54916	113.949	113.949
A_33_P3330264	CXCL1	6.82458	13.3723	0.0106891	93.5535
A_33_P3232692	IL24	4.0788	10.5369	0.0113741	87.9188
A_23_P144843	ESM1	3.34632	9.67079	0.012478	80.1411
A_33_P3308142	GPCRLTM7	9.66042	3.34008	79.912	79.912
A_24_P257416	CXCL2	6.9368	13.1254	0.0137103	72.938
A_23_P89431	CCL2	8.9014	15.0877	0.0137321	72.8219
A_23_P79518	IL1B	4.01301	10.1022	0.0146883	68.0814
A_23_P314755	STC1	4.43322	10.4882	0.0150407	66.4861
A_33_P3718269	ENST00000517927	3.12341	9.00892	0.0169155	59.1174
A_33_P3353372	ENST00000430825	3.40559	9.14634	0.0187009	53.4734
A_24_P247536	FAM133B	3.51247	9.16346	0.0199014	50.2478
A_33_P3374987	C7orf52	9.10426	3.53162	47.5918	47.5918
A_33_P3381821	CAPN14	9.8493	4.30557	46.6476	46.6476
A_33_P3341269	A_33_P3341269	9.70211	4.22435	44.5626	44.5626
A_33_P3362826	ANKRD31	8.92233	3.48238	43.4098	43.4098
A_33_P3219095	LOC728606	9.83125	4.61898	37.0723	37.0723
A_23_P904	BEND5	8.82691	3.62997	36.6805	36.6805
A_24_P237270	ADORA2A	3.15832	8.31359	0.0280614	35.6362
A_24_P558750	LOC100132963	8.6176	3.49169	34.9183	34.9183
A_24_P122137	LIF	8.44265	13.5675	0.0286594	34.8926
A_32_P182299	C1orf168	8.45535	3.38041	33.7062	33.7062
A_23_P164100	C17orf64	9.85009	4.81162	32.8648	32.8648
A_33_P3261862	OR2A1	11.9851	6.96219	32.5122	32.5122
A_23_P26024	C15orf48	6.60795	11.5287	0.0330147	30.2896
A_33_P3363355	ICAM4	5.24671	10.0793	0.035095	28.4941
A_23_P10206	HAS2	5.58408	10.3984	0.0355423	28.1355
A_23_P78037	CCL7	4.81524	9.616	0.0358779	27.8723
A_33_P3387971	ENST00000457336	9.48331	4.76869	26.2568	26.2568
A_33_P3293456	GATA4	10.3526	5.65175	26.0074	26.0074
A_23_P30163	FLJ13197	9.90088	5.2655	24.8535	24.8535
A_23_P398566	NR4A3	7.38279	11.965	0.0417462	23.9543
A_24_P362595	PAX3	8.22594	3.66421	23.6166	23.6166

Table 4 (continued)

Probeset ID	Gene name	Log intensity (GF wo)	Log intensity (GF saliva)	Ratio	Absolute fold change
A_33_P3312519	KPRP	4.89513	9.44004	0.0428397	23.3429
A_33_P3237150	BMP2	6.51219	10.9824	0.0451162	22.165
A_33_P3309456	DNAH8	8.07288	3.61994	21.9012	21.9012
A_33_P3319791	NR4A1	3.23374	7.68543	0.0456991	21.8823
A_23_P13753	NFE2	7.95151	3.52037	21.5728	21.5728

This table shows the top 50 regulated genes, sorted by their absolute fold change. A fold change of, e.g., 2 indicates that the gene is 2-fold upregulated in the GF wo vs GF saliva sample

caused by the saliva and not by microorganism or their products (Fig. 2c).

Saliva after heating at 96 °C maintains the ability to provoke IL-6 and IL-8 expression

Saliva when heated to 96 °C maintained the capacity to provoke IL-6 and IL-8 expression by gingiva fibroblasts. Freezing preserved the pro-inflammatory activity of saliva. A molecular weight fraction obtained with a cutoff of 40 kDa increased IL-6 and IL-8 expression (Fig. 3). All saliva treatment methods indicated the same results—an increase of IL-6 and IL-8 expression in oral fibroblasts ($p<0.05$).

Saliva-induced IL-6 and IL-8 require NFκB and MAPK signaling

U0126, BAY 11-7082, and SB203580 reduced the expression of IL-6 and IL-8 in the presence of saliva (Fig. 4a). Interestingly, SP600125 enhanced the expression of IL-6 and IL-8 in this setting. SB431542, the TGF-β receptor I kinase inhibitor, AG-494, and Lavendustin C, both inhibitors of EGF receptor kinase, did not significantly influence the expression pattern of IL-6 and IL-8 in gingiva fibroblasts (Fig. 4a). In line with these data, saliva increased phosphorylation of ERK, JNK, and p38 in gingiva fibroblasts (Fig. 4b). As the ERK involvement is lacking in LPS-stimulated IL-6 in fibroblasts [27], this is a further indication that microorganisms are not involved.

Saliva induced substantial changes in other inflammatory genes and proteins

To rule out that the observed changes are restricted to the two inflammatory cytokines, a screening approach with a microarray (Tables 3, 4, 5, and 6) and a Luminex analysis were performed. RT-PCR confirmed that saliva caused a substantial increase of the selected genes, e.g., ICAM-1, CXCL1, CXCL2, CXCL3, CXCL6, CCL7, CCL20, CSF 2, SERPIN B3, PTGS2, and BCL2A1 (Fig. 5). Cytokines examined using the Luminex technology also showed an increase in fibroblasts treated with saliva (Table 2).

Discussion

Saliva has beneficial effects on wound healing in preclinical studies [6, 7], and wound healing requires an early inflammatory response [15]. It was thus reasonable to suggest that saliva causes its beneficial effect on wound healing by supporting the inflammatory reaction. However, the in vitro data linking saliva, its components, and the inflammatory response in vitro are not entirely consistent [9, 10]. We now report that saliva initiates a massive pro-inflammatory reaction of oral fibroblasts, but not in epithelial cells. These results are indicated by a strong increase in the expression of the "indicator cytokines" IL-6, IL-8, and a panel of other inflammatory mediators. Moreover, we provide insights into the cellular mechanisms involving NFκB and MAPK signaling and show that saliva can be heated up to 96 °C and size fractionated but still maintaining the pro-inflammatory activity. These data add another piece of knowledge to the large spectrum of biologic function of saliva. Even though the clinical relevance remains a matter of speculation, there is good reason to assume that saliva can provoke an inflammatory response in the oral connective tissue, when contact occurs through an injury of the epithelial layer.

If we relate our findings to those of others, two studies are particularly relevant: those of Proksch et al. [9] and Komine et al. [10]. In the first study [9], murine osteogenic MC3T3-E1 cells showed a slight but no significant inflammatory response to saliva, which is in contrast to the sharp increase of IL-6 and IL-8 in our in vitro system. These discrepancies can have multiple reasons including the species differences, the lyophilization process, the use of different types of cells, and different stimulation times

Table 5 Saliva induced substantial changes in other inflammatory genes as shown in a microarray. GO enrichment analysis: experiment 1

Function	Type	Enrichment score	Enrichment *p* value	% Genes in group that are present	# Genes in list, in group	# Genes not in list, in group	GO ID
Inflammatory response	Biological process	37.583	4.76E−17	32.526	94	195	6,954
Extracellular space	Cellular component	33.5596	2.66E−15	23.3173	194	638	5,615
Response to virus	Biological process	31.1282	3.03E−14	42.4779	48	65	9,615
Cell-cell signaling	Biological process	25.6075	7.57E−12	30.2905	73	168	7,267
Positive regulation of transcription from RNA polymerase II promoter	Biological process	25.5894	7.70E−12	22.7994	158	535	45,944
Plasma membrane	Cellular component	24.1036	3.40E−11	16.9154	612	3,006	5,886
Chemokine activity	Molecular function	23.6638	5.28E−11	53.0612	26	23	8,009
Signal transduction	Biological process	23.327	7.40E−11	20.412	218	850	7,165
Response to lipopolysaccharide	Biological process	20.7765	9.48E−10	34.3511	45	86	32,496
Cytokine-mediated signaling pathway	Biological process	20.0092	2.04E−09	28.7671	63	156	19,221
Cytokine activity	Molecular function	19.3653	3.89E−09	31.0976	51	113	5,125
Type I interferon-mediated signaling pathway	Biological process	19.1956	4.61E−09	43.0769	28	37	60,337
Growth factor activity	Molecular function	18.906	6.15E−09	30.7229	51	115	8,083
Defense response to virus	Biological process	17.5171	2.47E−08	31.6547	44	95	51,607
Integral to plasma membrane	Cellular component	17.3154	3.02E−08	19.4015	201	835	5,887
Angiogenesis	Biological process	16.1778	9.42E−08	27.6382	55	144	1,525
Positive regulation of endothelial cell proliferation	Biological process	15.7807	1.40E−07	42.5926	23	31	1,938
Positive regulation of epithelial cell proliferation	Biological process	15.7807	1.40E−07	42.5926	23	31	50,679
Negative regulation of viral genome replication	Biological process	15.0232	2.99E−07	55.5556	15	12	45,071
Positive regulation of smooth muscle cell proliferation	Biological process	14.8828	3.44E−07	46.3415	19	22	48,661
Chemotaxis	Biological process	14.796	3.75E−07	31.8584	36	77	6,935
Positive regulation of ERK1 and ERK2 cascade	Biological process	14.4698	5.20E−07	34.4828	30	57	70,374
Extracellular region	Cellular component	13.55	1.30E−06	17.3835	291	1,383	5,576
Positive regulation of interleukin-6 production	Biological process	13.5017	1.37E−06	48.4848	16	17	32,755
Positive regulation of cell proliferation	Biological process	13.2704	1.72E−06	22	88	312	8,284
Inner ear morphogenesis	Biological process	13.1364	1.97E−06	38.5965	22	35	42,472
Lung alveolus development	Biological process	12.9994	2.26E−06	47.0588	16	18	48,286
Negative regulation of apoptotic process	Biological process	12.9011	2.50E−06	21.2719	97	359	43,066
Response to exogenous dsRNA	Biological process	12.6915	3.08E−06	66.6667	10	5	43,330
Toll-like receptor 4 signaling pathway	Biological process	12.4329	3.99E−06	33.3333	27	54	34,142

The table shows the top 40 GO functional groups sorted by their enrichment score after GO enrichment analysis with the 4,255 regulated genes. Cutoff: FC > 2.0 or FC ≤ 2

with salivary components or whole saliva, respectively. The second study [10] was based on saliva of periodontitis patients where lactoferrin was cleaved by protease-3, an enzyme typically released from neutrophil granulocytes. They also include saliva of the parotid gland. The truncated lactoferrin has a potent inflammatory activity for HSC-2 cell increasing IL-6 and IL-8—which is in line with the present study. However, in our hands, whole saliva from healthy donors failed to cause a substantial increase of IL-6 and IL-8 in HSC-2 cells, suggesting that truncated lactoferrin can only partially explain the findings of the present study.

In our attempt to understand the signaling cascade that accounts responsible for the strong expression of IL-6 and IL-8, we have performed a traditional approach with pharmacologic inhibitors and detection of

Table 6 Saliva induced substantial changes in other inflammatory genes as shown in a microarray. GO enrichment analysis: experiment 2

Function	Type	Enrichment score	Enrichment *p* value	% Genes in group that are present	# Genes in list, in group	# Genes not in list, in group	GO ID
Plasma membrane	Cellular component	26.2944	3.81E−12	13.8474	501	3,117	5,886
Inflammatory response	Biological process	25.6263	7.42E−12	24.5675	71	218	6,954
Integral to plasma membrane	Cellular component	23.8173	4.53E−11	17.0849	177	859	5,887
Sequence-specific DNA binding	Molecular function	22.8939	1.14E−10	19.7417	107	435	43,565
Extracellular space	Cellular component	22.314	2.04E−10	17.6683	147	685	5,615
Negative regulation of transcription from RNA polymerase II promoter	Biological process	18.7649	7.09E−09	19.1837	94	396	122
Positive regulation of transcription from RNA polymerase II promoter	Biological process	18.4929	9.30E−09	17.6046	122	571	45,944
Cytokine activity	Molecular function	15.9752	1.15E−07	25	41	123	5,125
Angiogenesis	Biological process	15.2185	2.46E−07	23.1156	46	153	1,525
Extracellular region	Cellular component	14.947	3.23E−07	14.3369	240	1,434	5,576
Multicellular organismal development	Biological process	14.8683	3.49E−07	18.0698	88	399	7,275
Growth factor activity	Molecular function	14.5784	4.66E−07	24.0964	40	126	8,083
Cell-cell signaling	Biological process	13.7973	1.02E−06	21.1618	51	190	7,267
Signal transduction	Biological process	13.6454	1.19E−06	15.1685	162	906	7,165
Sequence-specific DNA binding transcription factor activity	Molecular function	12.9735	2.32E−06	15.3439	145	800	3,700
Chemotaxis	Biological process	12.3066	4.52E−06	25.6637	29	84	6,935
Negative regulation of growth	Biological process	11.9435	6.50E−06	52.6316	10	9	45,926
Positive regulation of apoptotic process	Biological process	11.1806	1.39E−05	20.7921	42	160	43,065
Positive regulation of gene expression	Biological process	10.7139	2.22E−05	23.7705	29	93	10,628
RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription	Molecular function	10.6509	2.37E−05	30.5085	18	41	1,077
Chemokine activity	Molecular function	10.5564	2.60E−05	32.6531	16	33	8,009
Steroid hormone receptor activity	Molecular function	10.2735	3.45E−05	32	16	34	3,707
Integral to membrane	Cellular component	10.0453	4.34E−05	12.223	535	3,842	16,021
Positive regulation of smooth muscle cell proliferation	Biological process	9.96277	4.71E−05	34.1463	14	27	48,661
G-protein coupled receptor signaling pathway	Biological process	9.92099	4.91E−05	17.9245	57	261	7,186
Postsynaptic membrane	Cellular component	9.73556	5.91E−05	20.3209	38	149	45,211
Transcription regulatory region DNA binding	Molecular function	9.66103	6.37E−05	21.118	34	127	44,212
Macrophage chemotaxis	Biological process	9.53532	7.23E−05	58.3333	7	5	48,246
Negative regulation of apoptotic process	Biological process	9.53232	7.25E−05	16.4474	75	381	43,066
Palate development	Biological process	9.45095	7.86E−05	28.125	18	46	60,021
Positive regulation of leukocyte migration	Biological process	9.33212	8.85E−05	66.6667	6	3	2,687
Artery morphogenesis	Biological process	9.2838	9.29E−05	45	9	11	48,844
Negative regulation of ERK1 and ERK2 cascade	Biological process	9.2838	9.29E−05	45	9	11	70,373
RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in negative regulation of transcription	Molecular function	9.18031	0.00010305	37.931	11	18	1,078
Termination of G-protein coupled receptor signaling pathway	Biological process	9.06133	0.00011607	33.3333	13	26	38,032
Protein dimerization activity	Molecular function	8.94231	0.00013074	20.625	33	127	46,983
Cellular response to zinc ion	Biological process	8.85821	0.00014221	53.8462	7	6	71,294

Table 6 (continued)

Function	Type	Enrichment score	Enrichment *p* value	% Genes in group that are present	# Genes in list, in group	# Genes not in list, in group	GO ID
Suckling behavior	Biological process	8.85821	0.00014221	53.8462	7	6	1,967
Intracellular receptor mediated signaling pathway	Biological process	8.8236	0.00014722	36.6667	11	19	30,522
Positive regulation of protein kinase activity	Biological process	8.76945	0.00015541	32.5	13	27	45,860

The table shows the top 40 GO functional groups sorted by their enrichment score after GO enrichment analysis with the 3,569 regulated genes. Cutoff: FC > 2.0 or FC ≤ 2

phosphorylation signals of MAPK. Our findings that NFκB, p38, and ERK signal pathways mediate the cellular response to increase IL-6 and IL-8 expression are well documented for other in vitro settings. Interestingly, blocking of the TGF-β receptor I kinase or EGF receptor kinase did not change the pro-inflammatory response to saliva. TGF-β is present in saliva and can stimulate IL-6 and IL-8 expression in vitro—however, the impact of recombinant TGF-β is substantially less pronounced than we have observed with saliva. Even though TGF-β and EGF are present in saliva and the receptor can mediate IL-6 and IL-8 expression [28], in our study, blocking of TGF-β receptor I kinase or EGF receptor kinase had no such an effect. Moreover, indomethacin failed to change IL-6 and IL-8 expression, indicating that cyclooxygenase-dependent lipid metabolites are not required for this cellular reaction. Together, the data suggest that the classical NFκB, p38, and ERK signal pathways mediate the pro-inflammatory reaction of oral fibroblasts to saliva in vitro.

The question arises if salivary bacteria and their products make the stimulation of inflammatory cytokines by fibroblasts and a reduced induction in epithelial cells expected. We therefore harvested saliva from the stimulated parotid gland under aseptic conditions. This freshly prepared parotid saliva also enhanced the IL-6 and IL-8 expression in gingiva fibroblasts and, importantly, only showed low bacterial counts when grown on blood and LB agar, in contrast to whole saliva which contains a high bacterial load. However, we need to specify that the expression of IL-6 and IL-8 in gingiva fibroblasts was lower when stimulated with parotid saliva than with sterile-filtered whole saliva. This, together with the fact that sterile-filtered whole saliva heated up to 96 °C maintains the strong pro-inflammatory effect, does not exclude the participation of microorganisms and their products. Otherwise parotid saliva still showed a more than 10-fold significant increase in inflammatory genes, and further experiments with sterile-filtered whole saliva showed that the inflammatory response was independent of MYD88, and Toll-like receptors 4 and 2, respectively, which are central to the signaling of lipoteichoic acid and lipopolysaccharides [29]. Therefore, we assume that microorganisms did not cause the strong pro-inflammatory effects in this in vitro study. However, saliva might still

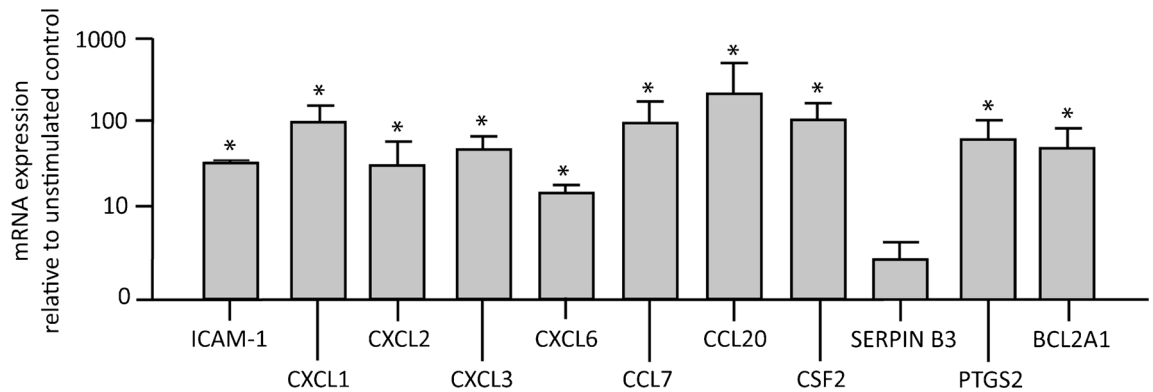

Fig. 5 Saliva induced substantial changes in other inflammatory genes and proteins. The whole genome array revealed genes that were strongly expressed when human gingiva fibroblasts were exposed to saliva. RT-PCR partially confirmed these data; the most striking was the increase of the selected genes, e.g., ICAM-1, CXCL1, CXCL2, CXCL3, CXCL6, CCL7, CCL20, CSF 2, SERPIN B3, PTGS2, and BCL2A1. A significant difference to unstimulated control was defined to be $p<0.05$ ($*p<0.05$).

contain microorganisms and bacterial components below the detection limit for the period of investigation used in our study.

We have also tried to understand what components of saliva are responsible for the pro-inflammatory reaction of oral fibroblasts. For example, boiling was performed on histatin 5 to reveal the impact of proteases in saliva [30]. Importantly, whole saliva contains a unique mixture of proteolytic enzymes that are even resistant to protease inhibitor cocktails [30]. Thus, in line with our protocol, freshly prepared saliva should be preferred over frozen samples for in vitro research. In the present study, saliva heat-treated for 95 °C for 5 min [31] maintained the capacity to boost IL-6 and IL-8 expression in the oral fibroblasts. Heat-stable preparations from bacteria that activate pattern recognition receptors can cause a pro-inflammatory cell response [32]; however, the bacterial load of freshly prepared saliva immediately filtered sterile is improbable. Size fractionation showed data that a pro-inflammatory activity is present within the <40-kDa fraction. This is, however, only a first step for characterization, as it is known that more than 60 % of the saliva proteins have a molecular weight less than 60 kDa [33, 34]. Our data are in line with the findings that saliva lactoferrin cleaved peptides, all showing less than 60 kDa, possesses inflammatory properties [10]. However, whole saliva is complex, and it requires further studies to understand if our finding can be attributed to saliva lactoferrin cleaved peptides alone.

The present study has opened the door for future research. At the saliva level, we would like to further characterize what component(s) in saliva are responsible for the strong inflammatory response of oral fibroblasts. At the level of oral fibroblasts, we will further advance the preliminary data obtained with the whole genome-wide microarray. By this screening approach, the full complexity of the strong cellular response to saliva becomes obvious. Moreover, fibroblast from other sources such as the dental pulp or cells involved in inflammation or tissue repair should be tested for their response to saliva. The key question, however, remains open, namely the clinical relevance of our findings—does saliva indeed cause a local inflammatory response upon contact with fibroblasts when epithelial layer is interrupted on wound site? If so, does this reaction help the innate immune system to support wound healing? We close the discussion with these challenging questions.

In summary, we conclude that freshly harvested sterile-filtered saliva can provoke a strong inflammatory response of oral fibroblasts, but not in epithelial cells in vitro. These in vitro findings may serve as a base for further in vitro investigations and following preclinical and clinical studies aiming at inflammatory response and even regeneration of oral fibroblasts in regenerative dentistry.

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Conflict of interest The authors declare that they have no conflict of interest.

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