Title: Antimicrobial activity and regulation of CXCL9 and CXCL10 in oral keratinocytes.

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Running head: CXC ELR- chemokines in oral mucosa

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Abstract

It is well established that the CXCL9 and CXCL10, two chemokines that belong to the CXC family, are dysregulated in oral inflammatory conditions. It has not been established if these chemokines target the micro-organisms that form the oral biofilm. The aim of this study was thus to investigate the antimicrobial activity of CXCL9 and CXCL10 on oral microflora and their expression profile in oral keratinocytes following inflammatory and infectious stimuli. *Streptococcus sanguinis* was used as a model and *Escherichia coli* as a positive control. The antimicrobial effect of CXCL9/CXCL10 was tested using radial diffusion assay. mRNA transcripts were isolated from LPS-treated and untreated (control) oral keratinocyte cell lines at 2, 4, 6 and 8 h. The CXCL9/10 expression profile in the presence or absence of IFN-γ was assessed using semi-quantitative PCR. Both chemokines demonstrated antimicrobial activity. CXCL9 was the most effective against both *Strep. sanguinis*/*Ecoli*. CXCL10 mRNA was expressed in control cells and its production was enhanced by LPS stimulation at all time-points. Conversely, CXCL9 mRNA was not expressed in control or LPS-stimulated cells. Finally, IFN-γ stimulation enhanced basal expression of both CXCL9 and CXCL10 in oral keratinocytes. Oral epithelial derived chemokines demonstrate antimicrobial properties, particularly CXCL9. Bacterial and inflammatory stimulated up-regulation of CXC9/10, with the potential to prevent adherence and invasion of oral epithelial

cells, could represent a key element in oral bacterial colonization homeostasis and host defense mechanisms.

Keywords: Chemokines; CXCL10; CCL28; XCL9; Streptococci sanguis.

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Introduction

Bacteria colonize all surfaces of humans, but are particularly dense in lower gastrointestinal tract and in the oral cavity where Streptococci represent a large proportion of the resident microflora.

It has previously been shown that bacteria can adhere and invade host oral epithelial cells (1) and furthermore, there are bacterial receptors present in saliva that can be absorbed onto oral mucosal surfaces (2). Among them, the toll like receptors (TLR) expressed on host cells are involved in the recognition of conserved bacterial patterns, for example the cell wall component of gram negative bacteria, lipopolysaccharide (LPS) (3).

In order to counter bacterially-mediated tissue damage, keratinocytes produce a wide number of molecules encompassing antimicrobial peptides (AMPs), that are differentially regulated together with pro-inflammatory cytokines across epidermal layers following bacterial stimuli (4).

The chemokines monokine induced by IFN-γ CXCL9, and IFN-γ-induced protein-10 CXCL10 are two chemokines belonging to the CXC family, both binding the same CXCR3 receptor (5). Chemokines play an important role in directing the migration of specific immune cell populations and for some a direct anti-bacterial and/or -fungal activities has been demonstrated (6-8). As oral epithelial cells are known to produce chemokines (9-11) these may be playing a direct role in microbial defence.

It is known that the CXC ELR- chemokines can be induced by LPS in some cell types (12), and that these chemokines are expressed during bacterial infections (13). It is also known that LPS is capable of inducing a range of cytokines and chemokines from epithelial cells predominately by signalling through TLR-4 (14).

The induction of these chemokines may be triggered in response to an alteration in the microbial flora, which could in turn cause an ensuing immune cell infiltration. We recently found that CXCL9/10 are dysregulated in oral inflammatory disease (personal communication), but nothing is known about their antibacterial activity. Furthermore, while the induction of several cytokines in oral epithelial cells after LPSs stimulation was previously demonstrated (11,14-17), the role of CXCL9/10 in the infection and immunity of the oral cavity has never been investigated.

Therefore the aim of this paper was to investigate the potential of CXC ELR-chemokines to mediate microbicidal activity on the gram positive *Strep. sanguinis*, one of the most prevalent residents of the oral microflora, and the chemokines expression in oral keratinocytes after exposure to infectious and inflammatory stimuli.

The CCR10 ligands CCL27 (CTACK) and CCL28 (MEC) are two C-C chemokines that bind the CCR10 receptor found to exert a potent antimicrobial activity against Candida albicans, Gram-negative bacteria, and Gram-positive bacteria (7). We used these two chemokines as positive controls.

Material and Methods

Cell culture techniques

Normal Human Oral keratinocytes (NHOK) cell culture.

All normal oral mucosa was obtained from healthy patients attending the Oral Surgery Clinic, Eastman Dental Institute for routine third molar extraction. Three different NHOK strains (NHOK1, NHOK2, NHOK3) were isolated from the excised normal tissue. The samples were cut into approximately $1mm^3$ pieces and culturing at 37ºC /5% CO_2 in keratinocyte basal medium-2 containing the recommended growth supplements (Biowittaker, Wokingham, UK). The epithelial cells were then detached using 0.25% trypsin - 1 mM EDTA. The viability of the keratinocytes was confirmed by trypan blue exclusion. All of the cell lines/strains were derived prior to 2001 and therefore were not subject to ethical committee approval in the UK (18). The study was approved by the internal research committee at Eastman Dental Institute, University Collage London.

H357 cell culture

The oral squamous cell carcinoma cell line, H357, was established by PRIME *et al* (19). This cell line was grown in the same medium as described for the NHOK.

Bacterial cell culture and antimicrobial assessment

All bacterial stocks were maintained frozen at -70°C in trypticase soy broth (TSB) (Becton Dickinson) supplemented with 0.6% yeast extract (YE) (Oxoid, Basingstoke,UK) and 10% glycerol (BDH). Cultures were checked weekly both

visually and by Gram-staining for contamination with other bacteria. Stocks of *E. coli* NCTC JM22 and *Strep. sanguinis* NCTC 10904 (provided by Dr.Rod McNab at the Eastman Dental Institute, University College London, UK) bacterium were plated upon an agar plates containing 3% TSB. They were grown for 48 h at 37°C / 5% CO_2 and maintained by twice weekly subculture on TSB agar plates.

Each *E. coli* colony was taken and resuspended into 50 ml of 3% TSB. For the oral streptococci species 3 streaks of a cultured plate were resuspended into 10 ml of 3% TSB. The cultures were shaken at 250 rpm at 37^{o}C for 15-18 h. 50 ml of the *E. coli* culture or 2 ml of the oral streptococci species culture was transferred into 50 ml or 10 ml of 3% TSB respectively. This was shaken at 250 rpm for 3.5 h at 37°C. After this time the culture was adjusted to an OD of 1 at 620 nm.

To prepare the underlay, 50 ml of 100 mM sodium phosphate buffer, 5 g agarose, low EEO (Sigma, Poole, UK) and 5 ml 3% trypticase soy broth (TSB) were added to 1 litre of distilled water. Then the pH was then adjusted to 7.4, the agarose was dissolved by heating the solution in the microwave. Then the solution was dispensed into in 50 ml aliquots and autoclaved. The underlay aliquots were then stored at room temperature until use in the radial diffusion assays at which point they were heated in a microwave until fluid and then stored in a 60^{o}C water bath.

8 ml of *E. coli* or 16 ml of the streptococcal species was added to 5 ml of molten underlay and dispensed into a Petri dish, using a leveling tray. This underlay was allowed to set, then 3 mm holes were punched in the gel, using 10 ml pipettes (Starstedt). 5 µl of test solution diluted in 0.01% acetic acid was added to the wells.

10 g agarose, low EEO (Sigma, Poole, UK) was added to 6% TSB, aliquoted into 50 ml and autoclaved. The overlay aliquots were then stored at room temperature prior the use in the radial diffusion assays.

149 This plate was incubated for 3 h at 37°C, before 5 ml of overlay was added to the plate
150 and incubated at 37°C overnight.
151 *Radial diffusion assays* were then performed, adding 5 µl of either recombinant human
152 CXCL9/CXCL10/CCL27/CCL28 (all Peprotech EC, London, UK) or 0.01% acetic
153 acid to the wells before incubating the plates. 100 µM tetracycline was used as a
154 positive control for the assay.
155 Images of the plates were taken using Alphaimager software and the zones around the
156 cultures measured from 3 different points from the end of the well.
157
158 ***IFN-γ cell treatment assay***
159 In a modification of the method utilised by ALTENBURG *et al* (20), the H357 cells were
160 seeded at $8x10^4$ cells / well in a Falcon 6 well plate (Becton Dickinson, Oxford, UK)
161 with 3 ml of KBM-2 medium containing no hydrocortisone. The cells were incubated
162 for at least 3-5 days until cell culture was 60-80% confluent. Medium containing 1000
163 U ml^{-1} IFN-γ was added to 3 wells and control cell culture medium only was added to
164 the remaining 3 wells. The cells were incubated for 48 h. The supernatant was extracted,
165 centrifuged and stored at –70°C. The adherent cells were washed with PBS (Gibco Life
166 Technologies, Paisley, UK) before 0.5 ml of Trireagent (Sigma, Poole, UK) were
167 added. The suspension was then removed and stored at –70°C. The RNA was isolated
168 as described below.
169

CXCL9 and CXCL10 mRNA transcripts in oral epithelial cells in response to lipopolysaccharide (LPS) and IFN-γ: mRNA isolation and semi-quantitative RT-PCR

mRNA transcripts for 18S, CXCL9 or CXCL10 were isolated from H357 cells without (CON) or treated with LPS (LPS) for 2, 4, 6 or 8 h. The RNA was extracted using TRI Reagent® (Sigma, Poole , UK) and 2 ml Pellet Paint Co-precipitant (Novagen, Nottingham, UK) to visualise the RNA pellet.

Single strand cDNA synthesis was performed. 2 ml of RNA was added to 4 ml deoxynucleotides (dNTPs) (2.5 mM) (Sigma), 2 ml of random hexamers (50 mm) (Ambion, Texas, USA) and 9.5 ml dH2O. Then 1 ml of RNAaseIN (Ambion, Austin, Texas, USA), 2 ml 10x MuLVRT buffer and 0.5 ml M-MuLVRT (200 U ml^{-1}) (Boehringer-Mannheim, Germany) was added and incubated at 42^{o}C for 1 h.

RT-PCR for 18S, CXCL9 and CXCL10: Magnesium concentration was optimised for each primer as follows; 1 ml of cDNA was added to 4 ml dNTP (2.5 mM), 5 ml 10x buffer, 0.225 ml AmpliTaq (5.0 U ml^{-1}) (Perkin Elmer), 4 ml of each specific primer (5 mM), 1.5 mM, 3.0 mM or 4.5 mM MgCl in each reaction and dH_2O added to give a final volume of 50 ml. The products were separated on a 2% agarose (GibcoBRL Life Technologies, Paisley, UK) gel and visualised by staining with ethidium bromide (Sigma, Poole, UK), specific bands were visualised by ultra-violet trans-illumination in a MultiImage Light Cabinet (AlphaInnotech Corp., Cannock, UK) and digital images acquired and stored using AlphaImager Software (AlphaInnotech Corp., Cannock, UK).

CXCL9 (5'-ccaacaccccacagaagtgc–3', 5'-gccagcacctgctctgagac-3'), CXCL10 (5'-gccaattttgtccacgtgttg-3', 5'-aaagaatttgggccccttgg-3'), and 18S ribosomal RNA (5'-tttcggaactgaggccatga-3', 5'- gcatgccagagtctcgttcg -3') primers were generated for use

in this study (Genosys-Sigma, Poole, UK). The thermocycler (Techne Genius; Cambridge, UK) parameters utilised were at 94^{o}C for 45 s, 57^{o}C for 45 s, 72^{o}C for 45 s.

For each primer the linear range was determined by repeating the above reaction with optimised magnesium concentration for each primer and stopping the reaction at 15, 17, 19, 21, 23, 25, 27, 29, 31, 33 and 35 cycles. The mid-point of each linear range was determined by using intensity analysis of the bands with AlphaImager software, and this cycle length was utilised for each primer in subsequent reactions. 18S primer and 18S Competitor primers (Ambion, Texas, USA) were combined to ratios 1:9, 2:8 and 3:7 respectively. For each of the primers CXCL9/10, 4 ml of the 18S primer: competitor mix was added to the RT-PCR reaction. The band intensity of the 18S and specific primer was quantified for each primer in each sample with Phoretix 1D software (Phoretix, Newcastle, UK).

Unless otherwise specified all the experiments were performed at least in triplicate.

Results

CXCL9 and CXCL10 mRNA transcripts in an oral epithelial cell line in response to IFN-γ

The production of CXCL9/10 chemokines in oral mucosal keratinocytes was first assessed over time in preliminary experiments using the keratinocyte cell line H357 (Supplementary Figure 1). CXCL10 mRNA levels in H357 cells were detected as early as 3 h after IFN-γ treatment and appeared to peak at 24 h. In contrast, the control cells showed virtually undetectable mRNA levels over the same time period. CXCL9 mRNA transcripts demonstrated a similar pattern to CXCL10, with a biphasic pattern showing

a rapid induction of mRNA in the stimulated cells followed by a second peak at 24/48 h. Thus, the expression of CXCL9/10 in H357 cells can be significantly enhanced by IFN-γ in a time-dependent manner, with a peak after 48 h (Figure 1). Similar results were obtained in primary normal oral keratinocytes (NHOK) (Figure 2). These data were confirmed at the protein level by ELISA (data not shown). These results show that the expression of CXCL9/10 in oral epithelial cells are significantly enhanced by IFN-γ in a time-dependent manner.

CXCL9 and CXCL10 mRNA transcripts in an oral epithelial cell line in response to LPS

CXCL10 mRNA was expressed in control cells (without LPS exposure), but was enhanced in response to LPS stimulation after 2, 4 and 6 h incubation. This expression subsequently decreased after 8 h of stimulation (Figure 3). In contrast, CXCL9 mRNA was not expressed in either control or LPS-stimulated cells at any of the time-points tested.

Antimicrobial effect of CXCL9, CXCL10, CCL27 or CCL28 upon Strep. sanguinis and E. coli

CCL27 and CCL28 were used as positive controls. The clear zones of bacterial growth depletion that were produced after antimicrobial activity of the chemokines are indicated in Figure 4 and Figure 5. All of the chemokines tested demonstrated a level of antimicrobial activity at the tested concentration (Table 1). CXCL9 was the most effective of the tested chemokines against both *Strep. sanguinis* and *E. coli*. CCL27 and CXCL10 had a less effective antimicrobial action against *Strep. sanguinis* compared to

E.coli. CCL28 had the greatest antimicrobial action against *E. coli*. These results clearly demonstrate that the epithelial-derived chemokines CXCL9/10 exert antimicrobial activity.

Discussion

Chemokines are known to have antimicrobial effects but little is known of the action of chemokines derived from the oral mucosal epithelium. The present study is the first to examine the expression of CXC ELR- chemokines by oral epithelial cells when stimulated by bacteria-derived products such as LPS. The present study has established that a cell line of oral origin is capable of expressing mRNA of CXCL9/CXCL10 when stimulated with LPS. The production of these chemokines was also enhanced by inflammatory stimuli such as IFN-γ stimulation.

CXCL10 mRNA expression was increased in H357 cells after LPS stimulation, whereas CXCL9 mRNA expression was not induced over the same time-period. Previously we have shown that CXCL10 can act as a potent chemoattractant of lymphocytes, hence this local production of CXCL10 by oral epithelial cells in response to LPS could have important effects upon oral inflammation, perhaps crucially in the initial stages of inflammation.

Previous studies have also shown that LPS treatment alone can induce or enhance CXCL10 mRNA expression in several different cell types (12,21-23). However, in contrast to the present findings, LPS stimulation did not induce CXCL10 from cultured skin keratinocytes (24), suggesting perhaps that oral keratinocytes are more responsive to LPS stimulation than cutaneous keratinocytes. This difference in expression may reflect the high bacterial load in the oral cavity.

The rapid expression of mRNA of CXCL10 observed in the present study accords with that of murine macrophages (17,25) although LPS stimulation may be more transient than IFN-γ in CXCL10 production (10). This short-term effect may be essential to avoid over-stimulation of CXCL10 in response to resident bacteria in proximity to the epithelium. IL-10 is known to be able to down-regulate production of LPS-induced CXCL10 in macrophages (22). This cytokine is present within oral lichen planus (OLP) lesions and is increased in serum and saliva from patients with oral lichen planus (26), and thus may act to down-regulate expression of CXCL10 in oral inflammation.

There are few reports of LPS-induced production of CXCL9. The present study revealed that CXCL9 mRNA was not expressed by H357 cells when stimulated with LPS. In contrast to the present study, CXCL9 mRNA was found to be expressed in LPS-stimulated murine dendritic cells (12), however, in another study, this chemokine was not induced in the same murine cell line by LPS, despite induction of CXCL9 by IFN-γ (25).

It is possible that CXCL9 displays a delayed response in comparison to CXCL10, as CXCL9 mRNA induction in lung tissue of intravenously LPS-treated mice displays a later induction to CXCL10, and is never expressed to the same levels as CXCL10 (25). Many studies report that LPS and IFN-γ act synergistically to induce the production of high levels of CXCL10 mRNA, for example, in breast carcinoma cells (21,22). It is then possible that CXCL10 levels could be enhanced in oral inflammation where there is a presence of both LPS and IFN-γ, perhaps through the enhancement of specific TLRs (27).

Gram-positive bacteria, such as *Strep. sanguinis* contain components other than LPS that are known to stimulate chemokine release from various cell types and it would be interestingly to determine whether these are also capable of stimulating the production

of the CXC ELR- chemokines in oral epithelium. Many gram-positive bacterial components act on a different toll like receptor, TLR-2, which is functionally expressed upon keratinocytes (28). However, TLR-2 agonists do not induce CXCL 10 production in macrophages (29) or dendritic cells (30) in vitro. This suggests that TLR-2 agonistic bacterial products would also not induce CXCL10 in epithelial cells. Therefore, only products bound by TLR-4 would be influential in upregulating CXCL10 production in epithelial cells. In addition, as LPS-mediated CXCL10 production is TLR-4 dependant, it strongly suggests that oral keratinocytes bear functional TLR-4, therefore, LPS-stimulation of epidermal cells is not due to TLR-2 agonist contaminants in LPS preparations, as previously suggested (28).

Chemokine modulation in oral cells by bacterial products is thus complex and many different factors, including T cell contact, may play a factor in chemokine induction during an immune response.

The present studies suggest that in certain circumstances, bacterial products could stimulate oral epithelial cells to produce an inflammatory response through TLR-4 agonists (perhaps after continuous TLR-2 stimulation) and this may induce epithelially-derived CXCL10-mediated inflammation. This inflammation would presumably be characterised by activated memory T cell infiltration localised under the basal epithelium reminiscent of the pathology of OLP.

CXCL9 was shown to be a potent antimicrobial agent against both *Strep. sanguinis* and *E. coli*, as did CXCL10 but to a lesser degree, confirming the study by COLE et al (6). Although only one oral bacterial species was tested in the present study, the data hints that antimicrobial properties of these chemokines may assist in countering bacterial growth in the oral cavity.

318 The choice of using S. sanguinis as a model has been driven by the delicate role that
319 this bacterium plays in the maintenance of the oral flora balance. It is commonly found
320 in healthy tissues as pioneer colonizer as well as it is implicated in modulating the
321 virulence of bacterial biofilms (31). Furthermore, significant inhibitory effects of the
322 intracellular proteins produced by S. sanguinis on the growth and the morphology of
323 many other components of the oral flora, such as P. intermedia, P. gingivalis, C.
324 albicans, C. tropicalis and their biofilms have been demonstrated (32,33). This allow
325 us to conjecture and expect a series of chain effects if the CXCL9/10 antimicrobial
326 activity is transferred in an *in vivo* model. As previously showed for the two
327 chemokines CCL27 and CCL28, for which a wide spectrum of antimicrobial activity is
328 well established, the action of CXCL9/10 expected against other micro-organisms of
329 the oral cavity should follow a similar pattern (34).
330 The low production of CXCL9 by oral epithelial cells following IFN-γ or LPS
331 stimulation could potentially be a means of avoiding an over-active anti-microbial
332 response due to the potent antimicrobial activity of CXCL9.
333 In our study both CCL27/CCL28 showed to exert antimicrobial properties against *E.*
334 *coli* and *Strep. sanguinis*. CCL28 has been shown to have a microbicidal activity
335 against a wide range of bacteria (both Gram-negative and Gram-positive) and yeasts
336 (7, 35), and our findings confirmed that this chemokine was also effective against the
337 oral commensal *Strep. sanguinis*. This is only the second report of the antimicrobial
338 effect of CCL28 upon the enterobacterial *E. coli* after the study reported by BERRI et al
339 in 2014 (35).
340 The production of CXCL10 by oral epithelial cells, in inflammatory conditions such as
341 OLP, may be induced by resident bacteria that contain TLR-4 agonists. If this
342 inflammation resulted from constant TLR-2 stimulation, this may cause a CXCL10

based inflammation and a resultant influx of memory Th1 CD4+ cells. Furthermore, CXCL10 is antimicrobial at high concentrations. It may be upregulated by the presence of bacteria, thus playing a role in defense of oral epithelial cells. The antimicrobial activity of the chemokines tested is likely to be an important mechanism in the homeostasis of oral bacterial colonisation. Any IFN-γ in the oral epithelial area (which could presumably be produced by the infiltrating Th1 cells) may synergise with TLR-4 agonists to cause an increased inflammatory state. Further studies are still warranted to confirm these novel findings.

References

1) MEYER DH, SREENIVASAN PK, RIVES-TAYLOR PM. Evidence for invasion of a human oral cell line by Actinobacillus actinomycetemcomitans. *Infect Immun* 1991; **59**: 2719-2726.

2) HENDERSON B, POOLE S, WILSON M. Bacteria-Cytokine Interactions: In Health and Disease. Portland Press, London 1998.

3) ERRIDGE C, BENNETT-GUERRERO E, POXTON IR. Structure and function of lipopolysaccharides. *Microbes Infect* 2002; **4**: 837-851.

4) PERCOCO G, MERLE C, JAOUEN T, RAMDANI Y, BÉNARD M, HILLION M, MIJOUIN L, LATI E, FEUILLOLEY M, LEFEUVRE L, DRIOUICH A, FOLLET-GUEYE ML. Antimicrobial peptides and pro-inflammatory cytokines are differentially regulated across epidermal layers following bacterial stimuli. *Exp Dermatol* 2013; **22:** 800-6.

5) COX MA, JENH CH, GONSIOREK W, FINE J, NARULA SK, ZAVODNY PJ, HIPKIN RW. Human interferon-inducible 10kDa protein and human interferon-inducible T cell

368 αlphaa chemoattractant are allotropic ligands for human CXCR3: differential binding
369 to receptor states. *Mol Pharmacol* 2001; **59:** 707-715.
370 6) COLE AM, GANZ T, LIESE AM, BURDICK MD, LIU L, STRIETER RM. Cutting edge:
371 IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J*
372 *Immunol* 2001; **167:** 623-7.
373 7) HIESHIMA K, OHTANI H, SHIBANO M, IZAWA D, NAKAYMA T, KAWASAKI Y, SHIBA
374 F, SHIOTA M, KATOU F, SAITO T, YOSHIE O. CCL28 has dual roles in mucosal immunity
375 as a chemokine with broad-spectrum antimicrobial activity. *J Immunol* 2003; **170**:
376 1452-1461.
377 8) TANG YQ, YEAMAN MR, SELSTED ME. Antimicrobial peptides from human
378 platelets. *Infect Immun* 2002; **70:** 6524-33.
379 9) ICHIMURA M, HIRATSUKA K, OGURA N, UTSUNOMIYA T, SAKAMAKI H, KONDOH T,
380 ABIKO Y, OTAKE S, YAMAMOTO M. Expression profile of chemokines and chemokine
381 receptors in epithelial cell layers of oral lichen planus. *J Oral Pathol* 2013; **35:** 167-74.
382 10) OHTA K, SHIGEISHI H, TAKI M, NISHI H, HIGASHIKAWA K, TAKECHI M, KAMATA
383 N. Regulation of CXCL9/10/11 in oral keratinocytes and fibroblasts. *J Dent Res* 2008;
384 **87:** 1160-5.
385 11) KOCGOZLU L, ELKAIM R, TENENBAUM H, WERNER S. Variable cell responses to P.
386 gingivalis lipopolysaccharide. *J Dent Res* 2009; **88:** 741-5.
387 12) MEYER M, HENSBERGEN PJ, VAN DER RAAIJ-HELMER EM, BRANDACHER G,
388 MARGREITER R, HEUFLER C, KOCH F, NARUMI S, WERNER ER, COLVIN R, LUSTER AD,
389 TENSEN CP, WERNER-FELMAYER G. Cross reactivity of three T cell attracting murine
390 chemokines stimulating the CXC chemokine receptor CXCR3 and their induction in
391 cultured cells and during allograft rejection. *Eur J Immunol* 2001; **31:** 2521-7.
392 13) LAUW FN, SIMPSON AJH, PRINS JM, VAN DEVENTER SJH, CHAOWAGUL W, WHITE

393 NJ, VAN DER POLL T. The CXC chemokines gamma interferon (IFN-γ)-inducible
394 protein 10 and monokine induced by IFN-γ are released during severe melioidosis.
395 *Infect Immun* 2000; **68:** 3888-3893.
396 14) ROUABHIA M, ROSS G, PAGÈ N, CHAKIR J. Interleukin-18 and gamma interferon
397 production by oral epithelial cells in response to exposure to Candida albicans or
398 lipopolysaccharide stimulation. *Infect Immun* 2002; **70:** 7073-80.
399 15) RYU OH, CHOI SJ, LINARES AM, SONG IS, KIM YJ, JANG KT, HART T. Gingival
400 epithelial cell expression of macrophage inflammatory protein-1alpha induced by
401 interleukin-1beta and lipopolysaccharide. *J Periodontol* 2007; **78:** 1627-34.
402 16) SUGA T, MITANI A, MOGI M, KIKUCHI T, FUJIMURA T, TAKEDA H, HISHIKAWA T,
403 YAMAMOTO G, HAYASHI J, ISHIHARA Y, NOGUCHI T. Aggregatibacter
404 actinomycetemcomitans lipopolysaccharide stimulated epithelial cells produce
405 interleukin-15 that regulates T cell activation. *Arch Oral Biol* 2013; **58:** 1541-8.
406 17) YIN L, CHINO T, HORST OV, HACKER BM, CLARK EA, DALE BA, CHUNG WO.
407 Differential and coordinated expression of defensins and cytokines by gingival
408 epithelial cells and dendritic cells in response to oral bacteria. *BMC Immunol* 2010; 11:
409 37.
410 18) HASSONA Y, CIRILLO N, LIM KP, HERMAN A, MELLONE M, THOMAS GJ, PITIYAGE
411 GN, PARKINSON EK, PRIME SS. Progression of genotype-specific oral cancer leads to
412 senescence of cancer-associated fibroblasts and is mediated by oxidative stress and
413 TGF-β. *Carcinogenesis* 2013; **34:** 1286-95.
414 19) PRIME SS, NIXON SV, CRANE IJ, STONE A, MATTHEWS JB, MAITLAND NJ,
415 REMNANT L, POWELL SK, GAME SM, SCULLY C. The behaviour of human oral
416 squamous cell carcinoma in cell culture. *J Pathol* 1990; **160:** 259-69.
417 20) ALTENBURG A, BALDUS SE, SMOLA H, PFISTER H, HESS S. CD40 ligand-CD40

418 interaction induces chemokines in cervical carcinoma cells in synergism with IFN-
419 gamma. *J Immunol* 1999; **162:** 4140-7.
420 21) SUN H, KUNDU N, DORSEY R, JACKSON MJ, FULTON AM. Expression of the
421 chemokines IP-10 and MIG in IL-10 transduced tumors. *J Immunother* 2001; **24:** 138-
422 43.
423 22) KOPYDLOWSKI KM, SALKOWSKI CA, CODY MJ, VAN ROOIJEN N, MAJOR J,
424 HAMILTON TA, VOGEL SN. Regulation of macrophage chemokine expression by
425 lipopolysaccharide in vitro and in vivo. *J Immunol* 1999; **163:** 1537-1544.
426 23) ESKAN MA, ROSE BG, BENAKANAKERE MR, LEE MJ, KINANE DF. Sphingosine 1
427 phosphate 1 and TLR4 mediate IFN-beta expression in human gingival epithelial cells.
428 *J Immunol* 2008; **180:**1818-25.
429 24) BOORSMA DM, FLIER J, SAMPAT S, OTTEVANGER C, DE HAAN P, HOOFT L,
430 WILLEMZE R, TENSEN CP, STOOF TJ. Chemokine IP-10 expression in cultured human
431 keratinocytes. *Arch Dermatol Res* 1998; **290:** 335-41.
432 25) WIDNEY DP, XIA Y-R, LUSIS AJ, SMITH JB. The murine chemokine CXCL11 (IFN-
433 inducible T cell α attractant is an IFN-γ and lipopolysaccharide-inducible
434 glucocorticoid-attenuated response gene expressed in lung and other tissues during
435 endotoxemia. *J Immunol* 2000; **164:** 6322-6331.
436 26) DAN H, LIU W, WANG J, WANG Z, WU R, CHEN Q, ZENG X, ZHOU Y. Elevated IL-
437 10 concentrations in serum and saliva from patients with oral lichen planus.
438 *Quintessence Int* 2011; **42:**157-63.
439 27) UEHARA A, SUGAWARA S, TAKADA H. Priming of human oral epithelail cells by
440 interferon-γ to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids
441 and peptidoglycans. *J Med Microbiol* 2002; **51:** 626-635.
442 28) KAWAI K, SHIMURA H, MINAGAWA M, ITO A, TOMIYAMA K, ITO M. Expression of

functional toll-like receptor 2 on human epidermal keratinocytes. *J Dermatol Sci* 2002; **30:** 185-194.

29) TOSHCHAKOV V, JONES BW, PERERA PY, THOMAS K, CODY MJ, ZHANG S, WILLIAMS BR, MAJOR J, HAMILTON TA, FENTON MJ, VOGEL SN. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat Immunol* 2002; **3:** 392-8

30) RE F, STROMINGER JL. Toll-like receptor (TLR2) and TLR4 differentially activate human dendritic cells. *J Biol Chem* 2001; **276:** 37692-37699.

31) LEE SH. Antagonistic effect of peptidoglycan of Streptococcus sanguinis on lipopolysaccharide of major periodontal pathogens. *J Microbiol* 2015; **53**: 553-60.

32) MA S, LI H, YAN C, WANG D, LI H, XIA X, DONG X, ZHAO Y, SUN T, HU P, GUAN W. Antagonistic effect of protein extracts from Streptococcus sanguinis on pathogenic bacteria and fungi of the oral cavity. *Exp Ther Med* 2014; **7**: 1486-1494.

33) PEYRET-LACOMBE A, BRUNEL G, WATTS M, CHARVERON M, DUPLAN H. TLR2 sensing of F. nucleatum and S. sanguinis distinctly triggered gingival innate response. *Cytokine* 2009; **46**: 201-10.

34) HIESHIMA K, OHTANI H, SHIBANO M, IZAWA D, NAKAYMA T, KAWASAKI Y, SHIBA F, SHIOTA M, KATOU F, SAITO T, YOSHIE O. CCL28 has dual roles in mucosal immunity as a chemokine with broad-spectrum antimicrobial activity. *J Immunol* 2003; **170**: 1452-1461.

35) BERRI M, VIRLOGEUX-PAYANT I, CHEVALEYRE C, MELO S, ZANELLO G, SALMON H, MEURENS F. CCL28 involvement in mucosal tissues protection as a chemokine and as an antibacterial peptide. *Dev Comp Immunol* 2014; **44:** 286-90.

Figure legends:

Figure 1: 18S, CXCL9 and CXCL10 mRNA expression in the H357 cell line either interferon-gamma treated for 48 h (IFN) or control cells that were left untreated (CON) over the same time period. The left column "m.w." indicates the molecular weight (marker column).

Figure 2: CXCL9 and CXCL10 mRNA expression in 3 different normal human oral keratinocytes (NHOK1, NHOK2, NHOK3) either interferon-gamma treated for 48 h (IFN) or control cells that were left untreated for 48 h (CON). The values were normalized against 18S mRNA expression used as house keeping.

Figure 3: a) CXCL9 and CXCL10 expression by H357 cells with or without LPS stimulation: mRNA transcripts for 18S, CXCL9 or CXCL10 in H357 cells without (CON) or treated with LPS (LPS) for 2, 4, 6 or 8 h. b) Densitometric analysis of CXCL10 mRNA relative to 18S expression with (grey) and without (blue) LPS treatment.

Figure 4: Radial diffusion gels demonstrating the antimicrobial activity of 100 μg ml^{-1} human recombinant CXCL9, CXCL10, CCL27 and CCL28 upon *Strep. sanguinis* and *E. coli* bacterium. The negative control (-ve) contains 0.01% of acetic acid only. The diameter of the cleared zone around the well containing chemokine represents the antimicrobial properties of the chemokine.

Figure 5: The antimicrobial activity of CXCL9 on *E. coli* JM22 bacterium at 50, 10, and 1 μM. 0 μM contains 0.01% of acetic acid only.

Supplementary Figure 1: 18S, CXCL9 and CXCL10 mRNA expression in the H357 cell line. (ifn)= cells that were treated with interferon-gamma for 3, 6, 9, 24, 48 or 72 h; (con)= control cells that were left untreated over the same time period.