RESEARCH ARTICLE

Dual action of bacteriocin PLNC8 $\alpha\beta$ through inhibition of Porphyromonas gingivalis infection and promotion of cell proliferation

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One sentence summary: The two-peptide bacteriocin PLNC8 $\alpha\beta$ lyses the periodontal pathogen Porphyromonas gingivalis, and suppresses P. gingivalis-mediated cytotoxicity and accumulation of inflammatory mediators from gingival fibroblasts.

Editor: Richard T. Marconi

ABSTRACT

Periodontitis is a chronic inflammatory disease that is characterised by accumulation of pathogenic bacteria, including Porphyromonas gingivalis, in periodontal pockets. The lack of effective treatments has emphasised in an intense search for alternative methods to prevent bacterial colonisation and disease progression. Bacteriocins are bacterially produced antimicrobial peptides gaining increased consideration as alternatives to traditional antibiotics. We show rapid permeabilisation and aggregation of P. gingivalis by the two-peptide bacteriocin PLNC8 $\alpha\beta$. In a cell culture model, P. gingivalis was cytotoxic against gingival fibroblasts. The proteome profile of fibroblasts is severely affected by P. gingivalis, including induction of the ubiquitin-proteasome pathway. PLNC8 $\alpha\beta$ enhanced the expression of growth factors and promoted cell proliferation, and suppressed proteins associated with apoptosis. PLNC8 $\alpha\beta$ efficiently counteracted P. gingivalis-mediated cytotoxicity, increased expression of a large number of proteins and restored the levels of inflammatory mediators. In conclusion, we show that bacteriocin PLNC8 $\alpha\beta$ displays dual effects by acting as a potent antimicrobial agent killing P. gingivalis and as a stimulatory factor promoting cell proliferation. We suggest preventive and therapeutical applications of PLNC8 $\alpha\beta$ in periodontitis to supplement the host immune defence against P. gingivalis infection and support wound healing processes.

Keywords: Porphyromonas gingivalis; periodontitis; cell proliferation; proteomics; bacteriocin; PLNC8

INTRODUCTION

Periodontitis is a gradually progressive disease and one of the most common infectious diseases in humans, which severely affects the life quality of patients. Lack of good predictive tests is a contributing factor to the difficulty of early diagnosis and prevention, which is based on visual and radiographic assessment (Pihlstrom, Michalowicz and Johnson 2005). Periodontitis is characterised by bacterial accumulation in dental pockets including Porphyromonas gingivalis that changes the composition of commensal bacteria in the oral cavity and...
disrupts host immune responses, ultimately leading to a destructive inflammatory condition (Darveau, Hajishengallis and Curtis 2012). This bacterium is considered a key pathogen in periodontitis and has been associated with systemic conditions, such as cardiovascular disease (Pihlstrom, Michalowicz and Johnson 2005; Pussinen et al. 2007). Manipulation of host cells and inflammatory responses is primarily associated with the ability of P. gingivalis to express an array of proteolytic enzymes, including collagenases and cysteine proteinases (Bostanci and Belibasakis 2012). Porphyromonas gingivalis has been shown to invade host cells, including gingival epithelial cells (Lamont et al. 1995) and endothelial cells (Deshpande, Khan and Genco 1998), which demonstrate a strategy to evade detection by the host immune system. This mechanism involves internalisation of the bacteria into phagosomes with subsequent activation of cellular autophagy and inhibition of lysosomal fusion (Belanger et al. 2006; Tan, Zhang and Zhou 2017), leading to persistent infection. Gingival fibroblasts constitute a cell type of the periodontium and provide a structural framework of the tissue and play a key role in mediating inflammatory responses. Several pathogen recognition receptors are expressed by gingival fibroblasts, including Toll-like and protease-activated receptors, indicating that these cells are well equipped upon encountering periodontal pathogens (Ara et al. 2009; Morandini et al. 2011). Porphyromonas gingivalis proteinases can severely damage the integrity of epithelial and gingival cells through apoptosis (Urnov et al. 2006), which could represent a route for the translocation of P. gingivalis to other sites, including vessel walls. Consequently, there is today an intense search for new antimicrobials that are able to restrict bacterial colonisation and pathogenesis, stimulate cell proliferation and maintain tissue integrity (Czaplewski et al. 2016).

Bacteriocins are a group of antimicrobial peptides, secreted by bacteria as part of their defence mechanism. This group of antimicrobial peptides is considered a promising alternative to traditional antibiotics against bacterial colonisation and subsequent pathogenesis (Cotter, Ross and Hill 2013). These amphipathic peptides have a net positive charge and can interact with negatively charged microbial membranes. The two-peptide bacteriocin PLNC8 αβ, from L. plantarum NC8, belongs to class II bacteriocins that display structural stability against heat and a wide range of pH. PLNC8 αβ has been suggested to kill microbes through formation of pores (Maldonado, Ruiz-Barba and Jimenez-Diaz 2003), scrambled-PLNC8 α (H2N-TWLKYGHGDAKLWSWSKPLNLFYQYVK-β-αβ(COOH)) and scrambled-PLNC8 β (H2N-LKLMNTYGFTSFRTYESVKEVHAKGISHVPPYK-αβ(COOH)) were synthesised using conventional Fmoc chemistry on a Quartet automated peptide synthesizer (Protein Technologies, Inc.) in a 100 μmol scale. Preloaded Fmoc-Phe/His/Lys Wang resins were used as solid support for PLNC8 α and PLNC8 β, respectively. Peptide elongation was performed using 4-fold excesses of amino acid (Iris biotech gmbh) and activator (TBTU, Iris biotech gmbh) and using 8-fold excesses of base (DIEPA). Fmoc removal was accomplished by treatment with piperidine (20% in DMF, v/v). All peptides were cleaved from their solid support using a mixture of TFA, triisoproylsilane and water (95:2:5:2.5, v/v/v) for 2 h before being filtered, concentrated and precipitated twice in cold diethylether. Crude peptides were purified on a C-18 reversed phase column (Kromatek HiQ-Sil C18HS) attached to a semipreparative HPLC system (Dionex) using an aqueous gradient of acetonitrile (10%–46%) containing 0.1% TFA. Mass identity of all peptides was confirmed by MALDI-ToF MS (Applied Biosystems) using α-cyano-4-hydroxycinnamic acid as matrix (Fig. S1, Supporting Information).

**Antimicrobial activity of bacteriocin PLNC8 αβ**

The antimicrobial effects of PLNC8 αβ on P. gingivalis were visualised by transmission electron microscopy (TEM). Briefly, viable P. gingivalis ATCC 33277 was centrifuged and the bacterial pellet washed with Krebs-Ringer Glucose buffer (KRG) (120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO4, 1.7 mM KH2PO4, 8.3 mM NaHPO4, and 10 mM glucose, pH 7.3). PLNC8 αβ was added to a final concentration of 2.5 μM (molar ratio of 1:1) for 2 min, followed by fixation in 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.3. Samples were washed in 0.1M phosphate buffer and postfixed in 2% osmium tetroxide in 0.1M phosphate buffer for 2 h and embedded into LX-112 (Ladd, Burlington, Vermont, USA). Ultrathin sections (~50–60 nm) were cut by a Leica ultracut UCT/Leica EM UC 6 (Leica, Wien, Austria). Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Hitachi HT 7700 (Tokyo, Japan). Images were captured using a Veleta camera (Olympus Soft Imaging Solutions, GmbH, Münster, Germany). The antimicrobial activity of PLNC8 αβ and scrambled-PLNC8 αβ was determined by using Sytox® Green, which can only penetrate damaged membranes and fluoresces upon binding to nucleic acids.

**EXPERIMENTAL PROCEDURES**

**Cell and bacterial culture conditions**

Primary human gingival fibroblasts (CRL-2014, American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Invitrogen Ltd, Paisley, UK) and incubated in a stable environment at 95% air, 5% CO2 and 37 °C. The cells were used at passages 3–7.

Porphyromonas gingivalis ATCC 33277 (ATCC, Manassas, VA) was grown in suspension at 37 °C in an anaerobic chamber (80% N2, 10% CO2 and 10% H2, Concept 400 Anaerobic Workstation; Ruskin Technology Ltd, Leeds, UK). The bacterial concentration was determined by viable count by culturing the bacteria on fastidious anaerobe agar (45.7 g/liter, pH 7.2, Acumedia, Neogen, Lansing, USA), supplemented with 5% defibrinated horse blood and was adjusted to correlate with ~10⁶ CFU/ml.

**Peptide synthesis**

All chemicals were bought from Sigma Aldrich unless otherwise noted and used without further purification. The peptides PLNC8 α (H2N-DLTTKILWSSWGYLGKARWNLLKHPYVQFCOOH), PLNC8 β (H2N-SVPTSVYTLGIKILWSAYKHRTIEKSFNKGFYHCOC), (Maldonado, Ruiz-Barba and Jimenez-Diaz 2003), scrambled-PLNC8 α (H2N-TWLKYGHGDAKLWSWSKPLNLFYQYVK-αβ(COOH)) and scrambled-PLNC8 β (H2N-LKLMNTYGFTSFRTYESVKEVHAKGISHVPPYK-αβ(COOH)) was determined by using Sytox® Green, which can only penetrate damaged membranes and fluoresces upon binding to nucleic acids.
Cytotoxicity

Lactate dehydrogenase activity (LDH, Cell Biolabs, Inc., San Diego, USA) was measured in culture supernatants retrieved from gingival fibroblasts that were treated to P. gingivalis and PLNC8 αβ. The absorbance was measured at 450 nm. Cytotoxic effects were calculated relative to the untreated cells that were set to 0.

Exposure of human gingival fibroblasts to Porphyromonas gingivalis and PLNC8 αβ

Human gingival fibroblasts were seeded in six-well plates (10⁵ cells/well) in DMEM, supplemented with 10% FBS and incubated for 24 h. The cells were then starved for 24 h in DMEM without FBS. Prior to exposure, the cells were washed twice with FBS and then pre-warmed DMEM supplemented with 1% FBS was added. Human gingival fibroblasts were exposed to P. gingivalis (MOI:100) and PLNC8 αβ (2.5 μM), individually and in combination, for 24 h. Images of the cells were captured using Olympus SC50 camera, connected to Olympus CKX41 microscope (magnification ×100), and the supernatants were collected and stored at −80°C until further use. The cells were washed with PBS and lysed in 200 μl lysis buffer (2% sodium dodecyl sulfate, 50 mM triethylammonium bicarbonate (TEAB)) and frozen at −80°C prior to proteomic analysis.

Sample preparation and digestion for proteomic analysis

The cell lysates (three biological replicates for each condition) were thawed and centrifuged, and total protein concentration was determined with Pierce 660 Protein assay (Thermo Scientific, Rockford, IL, USA). Same protein amounts from the three samples in the control group were pooled into a representative control sample. Aliquots containing 30 μg of each sample and the control sample were pooled into trypsin using the filter-aided sample preparation method (Wisniewski et al. 2009). Briefly, protein samples were reduced with 100 mM dithiothreitol at 60°C for 30 min, transferred to 30 kDa MWCO Pall Nanosep centrifugal filters (Sigma-Aldrich), washed with 8M urea repeatedly and alkylated with 10 mM methyl methanethiosulfonate. Digestion was performed in 50 mM TEAB and 1% sodium deoxycholate (SDC) buffer at 37°C by addition of Pierce MS grade Trypsin (Thermo Fisher Scientific) in a ratio of 1:100 relative to protein amount and incubated overnight. An additional portion of trypsin was added and incubated for another 2 h. Peptides were collected by centrifugation.

Digested peptides were labelled using TMT 10-plex isobaric mass tagging reagents (Thermo Scientific) according to the manufacturer’s instructions. The labelled samples were combined into one TMT-set, and SDC was removed by acidification with 10% TFA. An aliquot corresponding to 100 μg was fractionated into eight fractions using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Scientific), according to the manufacturer’s protocol. The fractions were dried in Speedvac and reconstituted in 20 μl of 3% acetonitrile and 0.1% formic acid for analysis.

LC-MS/MS analysis and database search

Each peptide fraction was analysed in an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) interfaced with EASY nanoLC 1000 liquid chromatography system. Peptides were separated on an in-house constructed analytical column (300 x 0.075 mm I.D.) packed with 3 μm Reprosil-Pur C18-AQ particles (Dr. Maisch, Germany), using the gradient from 5% to 25% B over 45 min and, from 25% to 80% B over 5 min, at a flow of 300 nL/min. Solvent A was 0.2% formic acid in water and solvent B was 0.2% formic acid in acetonitrile. Precursor ion mass spectra were acquired at 120 000 resolution and MS/MS analysis was performed in a data-dependent multinoitch mode, where CID spectra of the most intense precursor ions were recorded in ion trap at collision energy setting of 30 for 3 s (‘top speed’ setting). Charge states 2 to 7 were selected for fragmentation, and dynamic exclusion was set to 30 s. MS³ spectra for reporter ion quantitation were recorded at 60 000 resolution with HCD fragmentation at collision energy of 55 using the synchronous precursor selection.

The data files for the set were merged for identification and relative quantification using Proteome Discoverer version 1.4 (Thermo Fisher Scientific). The search was against the Human Swissprot Database version November 2014 (Swiss Institute of Bioinformatics, Switzerland) using Mascot 2.3.2.0 (Matrix Science) as a search engine with precursor mass tolerance of 5 ppm and fragment mass tolerance of 50 mmu. Methionine oxidation was set as a variable modification. Cysteine alkylation, TMT-label on peptide N-terminals and lysines were selected as fixed modifications. Trypsin was selected as enzyme in the searches and peptides were accepted with zero missed cleavage. The control sample was used as denominator and for calculation of the ratios. The detected peptide threshold in the software was set to a minimum quantification value of 5000 and a 1% false discovery rate by searching against a reversed database, and identified proteins were grouped by sharing the same sequences to minimise redundancy. Only peptides unique for a given protein were considered for identification of the proteins, excluding those common to other isoforms or proteins of the same family.

Detection of cytokines and growth factors

Enzyme-linked immunosorbent assay (ELISA) was performed on supernatants retrieved from gingival fibroblasts that were exposed to P. gingivalis and PLNC8 αβ. The levels of CXCL8 (Human IL-8 ELISA MAX Deluxe, Nordic Biosite, Sweden), TGF-β1 (BD OptEIA Set Human TGF-β1, BD Biosciences, USA) and IL-6 (Human IL-6 ELISA MAX Deluxe, Nordic Biosite, Sweden) were quantified according to the manufacturer’s instructions. The relative levels of growth factors were detected in the supernatants using Human Growth Factor Antibody Array C1 (RayBiotech, Sweden) according to the manufacturer’s instructions.

Statistical analysis

All data were analysed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). One-way ANOVA with Tukey’s multiple comparison test was used for the comparisons between the different treatments. P-values are referred to as *, #P < 0.05; **, ##P < 0.01; ###, ###P < 0.001. Statistical significance of the differentially expressed proteins (ATCC vs negative control, PLNC8 αβ vs negative control and ATCC+PLNC8 αβ vs negative control) from mass spectrometry was identified using the linear model from limma package (Ritchie et al. 2015).

RESULTS

PLNC8 αβ counteracts Porphyromonas gingivalis-mediated cytotoxicity

Exposure of human gingival fibroblasts with 2.5 μM PLNC8 αβ was not cytotoxic, but induced proliferation as the cell number
PLNC8 αβ counteracts the cytotoxic effects of *P. gingivalis* on human gingival fibroblasts. The cells were either left untreated or stimulated with 2.5 μM of PLNC8 αβ, *P. gingivalis* ATCC 33277 (MOI:100) or a combination of PLNC8 αβ and *P. gingivalis* for 24 h. (A) Representative images of four independent experiments of the cells (magnification ×100). (B) Cytotoxic effects were determined by measuring the activity of lactate dehydrogenase (LDH) in culture supernatants. ∗∗∗P < 0.001 (significance compared to the negative control that was set to 0). ##P < 0.01 (significance compared to *P. gingivalis*-treated cells).

### Table 1. Proteins from human gingival fibroblasts were identified using relative quantitative mass spectrometry with isobaric labeling (TMT).

| Condition                  | #Proteins | Recovery (%) | No. of sign. proteins |
|----------------------------|-----------|--------------|----------------------|
| PLNC8 αβ                   | 1847      | 85.5         | 719                  |
| *P. gingivalis*            | 794∗∗∗     | 36.8         | 314                  |
| *P. gingivalis*/PLNC8 αβ  | 1117∗∗     | 51.7         | 479                  |

*Calculated from the mean of total protein number detected in the untreated control samples (2159).

Increased compared to the control (Fig. 1A). However, fibroblast cell viability decreased markedly upon exposure to *P. gingivalis* that caused detachment of a large number of cells. The results were verified by a significant increase in LDH activity, used as a marker for cell toxicity (Fig. 1B). The presence of PLNC8 αβ efficiently counteracted the cytotoxic effects of *P. gingivalis*, and the morphology of the fibroblasts was similar to the unstimulated control.

**Functional proteomic analysis of human gingival fibroblasts**

The observed effects encouraged us to study the proteome profile of human gingival fibroblasts in response to PLNC8 αβ or *P. gingivalis*, or their combination. The total number of detected intracellular proteins from untreated cells was 2159, while cells stimulated with PLNC8 αβ, *P. gingivalis* and a combination of PLNC8 αβ and *P. gingivalis* resulted in detection of 1847, 794 and 1117 proteins, respectively (Table 1). Interestingly, incubation of *P. gingivalis*-infected fibroblasts with PLNC8 αβ enabled detection of a larger number of proteins, compared to cells treated with *P. gingivalis* alone. Further analyses were aimed at evaluating proteins with statistically significant altered levels compared to untreated cells (Table 1). The relatively low number of significant proteins detected in *P. gingivalis*-treated cells was elevated in the presence of PLNC8 αβ. Venn analysis diagram shows that treatment of gingival fibroblasts with PLNC8 αβ and *P. gingivalis* resulted in detection of more proteins compared to cells treated with *P. gingivalis* alone (Fig. 2A).

Stimulation of cells with PLNC8 αβ caused upregulation of proteins related to translation (ribosomal subunits) and the mitochondrial respiratory chain (cytochrome c and NADH dehydrogenases), as well as superoxide dismutase, EGFR, PDGFRα, TGFβ-induced protein and apolipoprotein E and B-100 (Table S1-A, Supporting Information). Downregulated proteins induced by PLNC8 αβ included caspase 3, diablo, TP53-regulating kinase, proteasomal subunits, ubiquitin-conjugating enzyme E2 and IGF2 mRNA-binding protein.

Among the 69 *P. gingivalis*-modulated proteins, the majority were downregulated (48 proteins) and associated with translation and protein localization, including eukaryotic translation elongation factor 2, ribosomal protein 8, 13, 10, 18, annexin 6A, transport protein Sec61, α-enolase and peroxiredoxin-1 (Table S1-B, Supporting Information). PLNC8 αβ successfully counteracted *P. gingivalis* and promoted cell growth and proliferation by inducing the expression of DEAH box poly peptide 9, importin S1, eukaryotic translation initiation factor 3E and 4A1, dynein, actin, synembryn-A, mitogen-activated protein kinase 3 and 14, interleukin enhancer binding factor 3, fibrillarin, lys-63-specific deubiquitinase BRCC3 and integrin β3 (Table S1-C, Supporting Information).

Analysis of protein expressions in a heat map shows opposite effects when comparing PLNC8 αβ with *P. gingivalis* (Fig. 2B). Proteins that were not detected in PLNC8 αβ-treated cells were found to be significantly altered by *P. gingivalis*, while the majority of proteins significantly changed by PLNC8 αβ did not appear in *P. gingivalis*-treated cells. More proteins were expressed in
cells stimulated with both PLNC8 αβ and P. gingivalis, compared to only P. gingivalis. This may involve two different mechanisms by PLNC8 αβ: (i) antibacterial action with inhibited proteinase-mediated degradation of proteins and (ii) direct cell-regulatory action including activation of specific intracellular pathways. PLNC8 αβ induced proteins that accumulated in the pathway of the mitochondrial respiratory chain (data not shown). Highly upregulated proteins by PLNC8 αβ include decorin, apolipoprotein B-100, metalloproteinase inhibitor 1 and caspase 14, as well as other proteins involved in cell junction, growth and proteinase inhibition (Table 2A). Among the downregulated proteins, we found primarily cytoskeletal matrix factors and other proteins that are associated with programmed cell death and apoptosis (data not shown).

Interestingly, the highest upregulated protein in P. gingivalis-infected cells was E3 ubiquitin-protein ligase (Table 2B) and other factors that were enriched in ubiquitin-mediated degradation and apoptosis. Downregulated proteins during P. gingivalis infection include ribosomal subunits and other proteins involved in mRNA localisation and translation. The overall effects of the combined treatment with PLNC8 αβ and P. gingivalis involved upregulation of proteins that associated with transcription, translation and metabolic processes, including arginase 1, phospholipid phosphatase 1, interleukin enhancer-binding factor 2 and leucine-rich repeat flightless-interacting protein 1 (Table 2C). Among the downregulated proteins in cells exposed to a combination of PLNC8 αβ and P. gingivalis, galectin-1, vimentin and lamin A/C were found and pathways including programmed cell death and apoptosis were also suppressed.

Reactome pathway analysis showed differential protein accumulation in pathways that are associated with ubiquitin-mediated degradations (Fig. 3A). Protein modification and degradation by the conserved ubiquitin-proteasome pathway is associated with many cellular processes, including cell cycle regulation, apoptosis and cell signaling in inflammation. All significantly altered proteins in the ubiquitin-proteasome pathway were further analysed with STRING. PLNC8 αβ significantly suppressed all proteins that were enriched in GO:00 70647 (protein modification by small protein conjugation or removal) (Fig. 3B). However, P. gingivalis treatment with and without PLNC8

Table 2. Proteins showing the largest differential expression patterns in human gingival fibroblasts.

| Uniprot | logFC | P value | adj.P.Val | Annotation |
|---------|-------|---------|-----------|------------|
| Q8NI35  | 3.51  | 6E-07   | 3E-04     | InaD-like prot. |
| P19823  | 3.16  | 6E-08   | 7E-05     | Inter-α-trypsin inhibitor chain H2 |
| P07585  | 2.97  | 6E-05   | 1E-03     | Decorin |
| P69090  | 2.23  | 6E-07   | 3E-04     | Haemoglobin subunit α |
| P02765  | 2.21  | 8E-06   | 5E-04     | α-2-HS-glycoprot. |
| P30711  | 2.10  | 5E-05   | 9E-04     | Glutathione S-transferase θ-1 |
| P01024  | 2.08  | 1E-03   | 7E-03     | Complement C3 |
| P02538  | 1.64  | 4E-03   | 1E-02     | Keratin, type II cytoskeletal 6A |
| P01008  | 1.44  | 5E-06   | 4E-04     | Antithrombin-III |
| P04114  | 1.43  | 7E-06   | 5E-04     | Apolipoprotein B-100 |
| Q14247  | −1.61 | 1E-04   | 1E-03     | Sр substrate cortactin |
| P07437  | −1.69 | 3E-03   | 1E-02     | Tubulin β-chain |
| P68371  | −1.87 | 4E-03   | 1E-02     | Tubulin β-4B chain |
| P02751  | −1.90 | 1E-04   | 1E-03     | Fibronectin |
| P07951  | −2.17 | 3E-05   | 7E-04     | Tropomyocin β-chain |
| Q13765  | −2.56 | 3E-03   | 1E-02     | Nascent polypeptide-associated complex subunit α |
| P02675  | −2.84 | 1E-03   | 6E-03     | Fibrinogen β-chain |
| P52565  | −3.35 | 1E-02   | 3E-02     | Rho GDP-dissociation inhibitor 1 |
| Q14847  | −3.42 | 1E-02   | 3E-02     | LIM and SH3 domain prot. 1 |
| P61353  | −4.71 | 3E-03   | 1E-02     | 60S ribosomal prot. L27 |

Figure 2. Proteome profiling of human gingival fibroblasts in response to P. gingivalis and PLNC8 αβ. (A) Venn diagram shows the number of differentially regulated proteins by P. gingivalis ATCC 33277 and PLNC8 αβ alone and in combination. (B) Heat map showing the relative protein expression levels in the different treatments (n = 3). The white colour indicates undetected/unaltered proteins in the different treatments.
### Table 2. (Continued)

| Uniprot | logFC | P value | adj.P.Val | Annotation |
|---------|-------|---------|-----------|------------|
| Q9UBS8  | 3.80  | 2E-05   | 5E-04     | E3 ubiquitin-protein ligase |
| Q32MZ4  | 3.59  | 1E-06   | 2E-04     | Leucine-rich repeat flightless-interacting prot. 1 |
| O14494  | 3.46  | 2E-05   | 5E-04     | Phospholipid phosphatase 1 |
| Q9UBB4  | 3.33  | 2E-02   | 2E-02     | Ataxin-10 |
| Q12905  | 3.31  | 4E-04   | 3E-03     | Interleukin enhancer-binding factor 2 |
| Q7LSN1  | 3.28  | 5E-06   | 3E-04     | COP9 signalosome complex subunit 6 |
| O14494  | 3.01  | 2E-05   | 5E-04     | Glutaminase kidney isoform, mitochondrial |
| P48449  | 2.70  | 2E-04   | 2E-03     | Lanosterol synthase |
| P20674  | 2.62  | 5E-04   | 3E-03     | Cytochrome C oxidase subunit 5A, mitochondrial |
| Q9H0U3  | 2.58  | 7E-04   | 4E-03     | Magnesium transporter prot. 1 |
| P62244  | –3.11 | 2E-06   | 5E-04     | 40S ribosomal protein S8 |
| Q01518  | –3.42 | 1E-05   | 3E-04     | Adenylyl cyclase-associated prot. 1 |
| Q09666  | –3.42 | 2E-05   | 3E-04     | Neuroblast differentiation-associated prot. AHNAK |
| Q6NZI2  | –3.77 | 2E-02   | 3E-04     | Gelsolin |
| P10599  | –3.84 | 1E-02   | 3E-02     | Protein S100-A11 |
| P10599  | –4.02 | 1E-02   | 3E-02     | Thioredoxin |
| P31949  | –4.09 | 1E-02   | 3E-02     | Peptidyl-prolyl cis-trans isomerase B |
| P6703   | –4.23 | 3E-03   | 3E-04     | Protein S100-A6 |
| P63104  | –4.47 | 1E-02   | 3E-02     | 14–3-3 prot. \(\zeta/\delta\) |
| P02545  | –4.61 | 1E-03   | 3E-03     | Prelamin-A/C |
| P09382  | –5.18 | 3E-04   | 1E-03     | Galectin-1 |
| P08670  | –5.42 | 5E-04   | 3E-04     | Vimentin |
| P05496  | –6.08 | 3E-05   | 3E-04     | ATP synthase F0 complex subunit C1 |

| Uniprot | logFC | P value | adj.P.Val | Annotation |
|---------|-------|---------|-----------|------------|
| O14494  | 3.37  | 1E-07   | 1E-04     | Phospholipid phosphatase 1 |
| Q12905  | 3.27  | 6E-03   | 1E-02     | Interleukin enhancer-binding factor 2 |
| Q32MZ4  | 2.99  | 3E-06   | 2E-04     | Leucine-rich repeat flightless-interacting prot. 1 |
| P05089  | 2.85  | 3E-03   | 5E-03     | Arginase-1 |
| Q968I3  | 2.59  | 5E-07   | 2E-04     | \(\gamma\)-Secretase subunit APH-1A |
| O14494  | 2.57  | 4E-06   | 2E-04     | Glutaminase kidney isoform, mitochondrial |
| Q7LSN1  | 2.32  | 1E-05   | 2E-04     | COP9 signalosome complex subunit 6 |
| P25311  | 2.29  | 3E-05   | 3E-04     | Zink-\(\alpha-2\)-glycoprot. |
| P7NFOQ8 | 2.23  | 3E-03   | 3E-04     | Synembryn-A |
| Q9H0U3  | 2.23  | 6E-05   | 4E-04     | Magnesium transporter prot. 1 |
| P63596  | –3.77 | 2E-02   | 3E-02     | Gelsolin |
| P31949  | –3.84 | 3E-06   | 2E-04     | Protein S100-A11 |
| P10599  | –4.02 | 1E-02   | 3E-02     | Thioredoxin |
| P32326  | –4.09 | 1E-02   | 3E-02     | Peptidyl-prolyl cis-trans isomerase B |
| P6703   | –4.23 | 3E-03   | 3E-04     | Protein S100-A6 |
| P63104  | –4.47 | 4E-05   | 3E-04     | 14–3-3 prot. \(\zeta/\delta\) |
| P02545  | –4.61 | 2E-03   | 6E-03     | Prelamin-A/C |
| P09382  | –5.18 | 3E-04   | 1E-03     | Galectin-1 |
| P08670  | –5.42 | 8E-05   | 5E-04     | Vimentin |
| P05496  | –6.08 | 3E-05   | 3E-04     | ATP synthase F0 complex subunit C1 |

\(\alpha\beta\) significantly induced proteins in the pathway of ubiquitin-dependent catabolic processes (GO:0006511) (Fig. 3C and D). Treatment with both PLNC8 \(\alpha\beta\) and \(P. ginvialis\) suppressed several protein of the proteasomal complex, including those that belong to the regulatory subunit 26S, such as PRS4, PSD11 and PSMD1. Furthermore, the deubiquitin enzyme BRCC3 was found to be significantly induced. These results associate well with the previously observed effects of PLNC8 \(\alpha\beta\)-induced cell proliferation and \(P. ginvialis\)-mediated cell death.

**Differential expression of inflammatory mediators**

The evident induction of ubiquitin-mediated degradation and catabolic effects by \(P. ginvialis\) on human gingival fibroblasts, leading to cell death, prompted us to quantify the accumulation of cytokines that are key players in inflammation. *Porphyromonas ginvialis* suppressed the accumulation of the chemokine CXCL8, while significantly induced release of the anti-inflammatory mediator TGF-\(\beta\)1 (Fig. 4A). These effects are most probably due to the potent activity of bacterial proteinases that have been documented previously (Bengtsson, Khalaf and Khalaf 2015). While PLNC8 \(\alpha\beta\) alone did not alter the release of these cytokines, interestingly, this bacteriocin was able to prevent \(P. ginvialis\)-mediated alteration of inflammatory mediators.

The effects of \(P. ginvialis\) and PLNC8 \(\alpha\beta\) on cell viability and TGF-\(\beta\)1 regulation motivated us to determine the relative expression levels of an array of different growth factors. PLNC8 \(\alpha\beta\) stimulation of fibroblasts resulted in moderate changes, including induction of HB EGF, IGF-2, IGF-1 and its soluble receptor, and suppression of M-CSF and its receptor (Fig. 4B). However, \(P. ginvialis\) caused extensive induction of a wide range of growth factors, including EGF, HGF and members that belong to the IGF and PDGF family of proteins. A combination of both PLNC8 \(\alpha\beta\) and \(P. ginvialis\) induced IGF-1 sR, and SCF and its receptor
Figure 3. Porphyromonas gingivalis induces catabolic processes in human gingival fibroblasts. (A) The corresponding Entrez IDs were retrieved based on the UniProt ID of the differentially expressed proteins, and were used for Reactome pathway analysis in the reactomePA package. Network analysis of ubiquitination and proteasome associated proteins using STRING, with a confidence interaction parameter of 0.4. (B) PLNC8 αβ significantly suppressed proteins enriched in the pathway of protein modification-dependent catabolic processes (blue nodes, FDR = 9.46e−14). (C) Porphyromonas gingivalis ATCC 33277 alone and (D) P. gingivalis in combination with PLNC8 αβ resulted in significant induction of proteins associated with ubiquitin-dependent protein catabolic processes (red nodes, FDR = 1.48e−14 and 7.07e−18, respectively).

compared to cells challenged with only P. gingivalis. These results are supported in the proteome data, where PLNC8 αβ was found to upregulate PDGFR α and β, EGFR and TGFBI, while P. gingivalis upregulated PDGFR β (Table S1, Supporting Information).

DISCUSSION

The overuse of antibiotics has increased the occurrence of complications in healthcare systems due to bacterial resistance (Blaser 2011). This has resulted in an intense search for new and effective antimicrobials with less possibility to induce antimicrobial resistance and with decreased cytotoxicity for host cells (Czaplewski et al. 2016). This study suggests that bacteriocin PLNC8 αβ could potentially be used as an alternative effective agent to traditional antibiotics in the prevention and treatment of infectious diseases, including periodontis.

We have recently shown that the two-peptide bacteriocin PLNC8 αβ binds to P. gingivalis, resulting in rapid and efficient lysis (Khalaf et al. 2016). We have verified our findings in this study by using TEM and show that P. gingivalis is efficiently and rapidly lysed by PLNC8 αβ (data not shown). Furthermore, the antimicrobial activity of PLNC8 αβ was shown to be specific, as scrambled peptides did not cause bacterial lysis (data not shown).

Porphyromonas gingivalis-mediated cytotoxic effects on human gingival fibroblasts are well described. These effects have primarily been associated with proteinases, as heat inactivation of culture media or crude extracts diminished their cytotoxic activity (Johansson, Bergenholtz and Holm 1996; Wang et al. 1999). Furthermore, we have previously shown that the cytotoxicity of P. gingivalis is associated with lysine-specific, but not arginine-specific, gingipains (Bengtsson, Khalaf and Khalaf 2015). Interestingly, PLNC8 αβ showed no toxicity towards gingival fibroblasts, but rather induced cell proliferation. Furthermore, this finding, in combination with the antimicrobial activity of PLNC8 αβ, significantly reduced P. gingivalis-mediated cytotoxicity. Safety is a key factor in order to develop bacteriocin-based applications for medicinal purposes. This is a feature that many bacteriocins share, including PLNC8 αβ (Cotter, Ross and Hill 2013).

The virulence of P. gingivalis extends beyond its ability to induce apoptosis, as shown in the proteome profile of gingival fibroblasts. The significantly reduced number of identified proteins in P. gingivalis-infected cells may be due to the potent
Figure 4. Porphyromonas gingivalis alters the release and accumulation of inflammatory mediators and growth factors. Human gingival fibroblasts were treated with PLNC8 αβ (2.5 μM), P. gingivalis ATCC 33277 (MOI:100) or a combination of both for 24 h. (A) Quantification of secreted CXCL8, IL-6 and TGF-β1 shows that PLNC8 αβ is able to partially restore the effects caused by P. gingivalis to normal levels. *,**,***P < 0.05; ***,***P < 0.01; ****P < 0.001 (* significance compared to untreated cells; # significance compared to P. gingivalis-treated cells). (B) The relative expression levels of 41 human growth factors and receptors were detected in the supernatants of cell exposed to PLNC8 αβ and/or P. gingivalis. While PLNC8 αβ caused moderate changes, P. gingivalis exposure resulted in extensive induction of an array of different growth factors, many of which belong to the IGF and PDGF family of proteins. PLNC8 αβ antagonised the effects of P. gingivalis.

The proteome profile of gingival fibroblasts revealed an interesting trend when analysing all the significant proteins in a heat map. Undetected proteins in PLNC8 αβ-treated cells were found to be significantly altered by P. gingivalis, while the majority of proteins that were significantly changed by PLNC8 αβ were not detected in P. gingivalis-treated cells. Although the relatively enzymatic activity of cysteine proteinases that have been reported to hydrolyse a broad spectrum of host substrates, including surface receptors (Kitamura et al. 2002; Belibasakis, Bostanci and Reddi 2010) and inflammatory mediators, such as IL-6 and CXCL8 (Palm, Khalaf and Bengtsson 2013; Khalaf, Lonn and Bengtsson 2014).
high bacterial concentration used in the cell infection assays, PLNC8 αβ efficiently antagonised P. gingivalis and promoted cell survival. These effects may be mediated by enhanced expression of proteins involved in intracellular membrane trafficking, including Ras-related proteins, general vesicular transport factors, cytoskeleton-associated and lysosomal-associated membrane proteins, actin, dynein, protein transport SEC and AP-3 complex subunits. These factors also promote fusion of the autophagosome with the lysosome, a mechanism that is avoided by P. gingivalis (Dorn, Dunn and Progulske-Fox 2001; Ham, Sreelatha and Orth 2011), by suppressing the lysosome-associated proteinases cathepsin D and Z. Alongside with the evasion strategy of P. gingivalis by residing in autophagosomes, nutrient acquisition is important for survival and proliferation, which could be provided by the autophagosome and the ubiquitin-proteasome pathway. All significantly altered proteins in the ubiquitin-proteasome pathway were found to be upregulated by P. gingivalis, including ubiquitin C. In correlation, a recent study by Zeidan-Chulia and Gursoy (2015) reported that ubiquitin C, together with Jun protooncogene and metalloproteinase-14, formed ideal biomarkers for early diagnosis of periodontitis. Furthermore, P. gingivalis has previously been shown to disarm innate immune responses through ubiquitin and proteasome-dependent degradation of MyD88 (Maekawa et al. 2014).

PLNC8 αβ was observed to promote cell proliferation and antagonise P. gingivalis-mediated cell death. Concomitantly, PLNC8 αβ induced the expression of IGF-1 and its soluble receptor. These effects could be enhanced via integrin β3, which was also significantly induced by PLNC8 αβ. Studies have shown that integrin β3 is an essential factor for binding and signaling of several growth factors, including neuregulin-1 (containing an EGF-like domain) (Ieguchi et al. 2010), FGF-1 (Mori et al. 2008) and IGF-1 (Saegusa et al. 2009). The increased expression of growth factors, in response to P. gingivalis, may be a consequence of increased cell metabolism; however, the factors may not be present in their active forms. We have previously shown that patient with periodontitis have increased levels of HGF; however, the biological activity of this growth factor was significantly reduced, compared to healthy volunteers, probably due to proteolytic activity (Lonn et al. 2014). Whether degradation of proinflammatory cytokines and induction of anti-inflammatory cytokines and growth factors is an active immunosuppressive evasion strategy utilised by P. gingivalis remains to be further investigated.

In this study, we show that low concentrations of bacteriocin PLNC8 αβ displays potent antimicrobial action on P. gingivalis and stimulates cell proliferation. PLNC8 αβ efficiently prevented P. gingivalis-mediated cytotoxicity, increased the expression of a large number of proteins associated with cytoskeleton rearrangement and vesicle transport, and restored the levels of inflammatory mediators. Our results show that PLNC8 αβ antagonises the pathogenic activity of P. gingivalis, suggesting different forms of bacteriocin-expressing applications to supplement the host immune responses in periodontitis.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSFD online.

ACKNOWLEDGEMENTS
The Proteomics Core Facility at Sahlgrenska Academy, Gothenburg University, performed the analysis for protein quantification. We are grateful of Inga-Britt and Arne Lundbergs Research foundation for the donation of the Orbitrap Fusion Tríbrid MS instrument. We would like to thank Prof. Gunnel Svensäter and Prof. Julia Davies for their input and collaboration within the KK-Synergy project ‘Biomarkers and biotherapeutics for polymicrobial infections & inflammation’.

FUNDING
This work was supported by the Foundation of Magnus Bergvall [2015–00823] and the Knowledge Foundation [20150244, 20150086], Sweden.

Conflict of interest. None declared.

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