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Gingipain-dependent degradation of mTOR pathway proteins by the periodontal pathogen *Porphyromonas gingivalis* during invasion

Prachi Stafford[1], **Jon Higham**[1], **Abigail Pinnock**[1], **Craig Murdoch**[1], **C. W. Ian Douglas**[1], **Graham P Stafford**[1,*], and **Daniel W Lambert**[1,*]

[1]Integrated Bioscience, School of Clinical Dentistry, University of Sheffield, Sheffield, S10 2TA, UK

SUMMARY

Porphyromonas gingivalis and *Tannerella forsythia* are Gram-negative pathogens strongly associated with periodontitis. Their abilities to interact, invade and persist within host cells are considered crucial to their pathogenicity, but the mechanisms by which they subvert host defences are not well understood. In this study, we set out to investigate whether *P. gingivalis* and *T. forsythia* directly target key signalling molecules which may modulate the host cell phenotype to favour invasion and persistence. Our data identify, for the first time, that *P. gingivalis*, but not *T. forsythia*, reduces levels of intracellular mammalian target of rapamycin (mTOR) in oral epithelial cells following invasion over a 4 hour time course, via the action of gingipains. The ability of cytochalasin D to abrogate *P. gingivalis*-mediated mTOR degradation suggests that this effect is dependent upon cellular invasion. We also show that levels of several other proteins in the mTOR signalling pathway are modulated by gingipains, either directly or as a consequence of mTOR degradation including p-4E-BP1. Taken together, our data suggests that *P. gingivalis* manipulates the mTOR pathway, providing evidence for a potentially novel mechanism by which *P. gingivalis* mediates its effects on host cell responses to infection.

Keywords

Porphyromonas gingivalis; gingipains; mTOR; periodontitis; degradation

Introduction

Periodontitis is a chronic condition characterised by the inflammation of the ligaments and other structures supporting the teeth. Pathogenesis results from a combination of the host response to microbial challenge and the direct effects of bacterial virulence factors that result in destruction of both hard and soft tissues in the periodontium (Ready *et al.,* 2008). While periodontitis is a polymicrobial disease, the pathogens predominantly responsible are considered to be the red-complex anaerobes *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* (Socransky *et al.,* 1998).

*Corresponding authors: Integrated Bioscience, School of Clinical Dentistry, University of Sheffield, Sheffield, S10 2TA, UK, Phone: 00 44 114 2717959, d.w.lambert@sheffield.ac.uk; g.p.stafford@sheffield.ac.uk.

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Both *P. gingivalis* and *T. forsythia* are Gram-negative bacteria harbouring numerous virulence factors which contribute to tissue destruction and host invasion (Holt and Ebersole 2005, Tanner and Izard 2006, Sharma 2010, Stafford *et al.,* 2012). Examples of such virulence factors include lipopolysaccharide (LPS), gingipain proteases, lipoproteins and fimbriae in the case of *P. gingivalis* (Holt *et al.,* 1999), and the fibronectin binding protein *BspA*, a unique glycosylated S-layer (Posch *et al.,* 2011, Settem *et al.,* 2012) and sialidase for *T. forsythia* (Sharma *et al.,* 1998, Sharma *et al.,* 2005, Roy *et al.,* 2011). LPS on the outer surface of both bacteria as well as BspA have been shown to interact with TLR2 on oral epithelial cells (Burns *et al.,* 2006, Myneni *et al.,* 2011) and trigger IL-6 release. The fimbriae of *P. gingivalis* are also involved in cell invasion and bacterial internalisation (Weinberg *et al.,* 1997). Other well-known virulence factors are the proteases secreted by both bacteria; *P. gingivalis* secretes a suite of cysteine-proteases with trypsin-like activity (Kuramitsu 1998), while *T. forsythia* produces a xenologue of MMP-9 (karilysin) that is known to inhibit components of the complement system, and the protease PrtH that has been identified as a cytocidal toxin (Cct) (Nakajima *et al.,* 2006, Jusko *et al.,* 2012). Thus, it is evident that periodontal pathogens employ several mechanisms to colonise surfaces, invade host cells and evade immune surveillance.

The mammalian target of rapamycin (mTOR), a serine/threonine kinase, integrates several key processes such as cell growth, proliferation, cell motility, cell survival, protein synthesis and transcription (Hay and Sonenberg 2004). mTOR has been implicated in the regulation of pro-inflammatory cytokines expression following bacterial challenge (Säemann *et al.,* 2009) as well as in the autophagic pathway (Jung *et al.,* 2010). Recent studies have also shown that amino acid starvation induced by bacterial pathogens modulates the mTOR pathway (Tattoli *et al.,* 2012). Previous studies have shown that *P. gingivalis* induces autophagy in endothelial cells (Dorn *et al.,* 2001) and affects both cell proliferation and cell growth in osteoblastic/stromal cells, in human trophoblasts (Kato *et al.,* 2008, Inaba *et al.,* 2009) and gingival epithelial cells (Andrian *et al.,* 2006). Mechanisms underlying these changes however, remain to be elucidated. Since mTOR is key to several of the cellular responses elicited by periodontal pathogens such as *P. gingivalis*, this study aimed to determine whether periodontal pathogens are able to influence the mTOR signalling pathway.

Materials and Methods

Reagents and Antibodies

Unless otherwise stated all chemical reagents were from Sigma (Poole, UK) and all mTOR pathway antibodies used were from Cell Signalling (New England Biolabs, Hitchin, UK). Mouse-anti-GAPDH was from Sigma. Antibodies against *P. gingivalis* were prepared within the Sheffield Antibody Unit (BioServ UK Ltd, Sheffield, UK) by inoculating New Zealand rabbits with formalin-fixed whole *P. gingivalis* (strain NCTC11834).

Cell culture

The oral squamous cell carcinoma (OSCC)-derived cell line H357 (a generous gift of Professor S. Prime, University of Bristol, UK) was grown and maintained in Dulbecco’ s

Modified Eagle's medium supplemented with 10% (v/v) foetal bovine sera and 2 mM L-glutamine.

Immortalised human oral keratinocytes (OKF6/Tert2) (Dickson *et al.,* 2000) were kindly provided by Dr. J. Rheinwald (Harvard Medical School, Cambridge, MA, UK) and were grown in defined keratinocyte-SFM supplemented with defined growth supplements (Fisher Scientific). Cells were grown to 70-80% confluence and the media changed every 3-4 days.

Bacterial strains and growth conditions

Bacterial strains used were *T. forsythia* (ATCC 43037) and the *P. gingivalis* strains NCTC11834 and W50 (ATCC 53978) and the derivative W50 isogenic mutants E8 (*rgpA::Em rgpB::Tet*) and K1A (*kgp*::Em mutant) (Aduse-Opoku *et al.,* 2000). Both the parental strain W50 and the E8 and K1A mutants were kindly supplied by Professor. M. Curtis (Barts and The London School of Medicine and Dentistry, UK).

All bacterial strains were grown under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2) at 37°C. *P. gingivalis* strains were grown and maintained on Fastidious Anaerobe agar (FA; Lab M, Bury, UK) supplemented with 5% (v/v) oxylated horse blood (Oxoid, Fisher Scientific, Loughborough, UK). For growth in liquid cultures, *P. gingivalis* was grown in brain heart infusion broth (BHI; Difco laboratories, East Molesey, Surrey, UK) supplemented with 0.5% (w/v) yeast extract, hemin (5μg/ml), vitamin K (0.5 μg/ml) and cysteine (0.1% (w/v)). *T. forsythia* was maintained on FA agar supplemented with 5% (v/v) oxylated horse blood and 0.17 mM N-acetylmuramic acid (NAM). For growth in liquid cultures, *T. forsythia* was grown in tryptic soy broth (TSB) supplemented with 0.5% (w/v) yeast extract, hemin (5μg/ml), vitamin K (0.5 μg/ml), 0.17 mM N-acetylmuramic acid (NAM) and cysteine (0.1% (w/v)).

Construction of an rpgABkgp triple mutant

To create a strain devoid of all gingipains in *P. gingivalis* W50 background, *kgp*, the gene encoding the lysine-specific gingipain was deleted in the *rgpA::EmrgpB::Tet* (E8,Δ*rgpA*Δ*rgpB)* strain. Briefly, DNA regions flanking the *kgp* gene were amplified by PCR using the primers kgp5′ (CTGCAGAAGTTCACTCTTTC) and kgp5′CatRev (CCAGTGATTTTTTTCTCCACTTTAAAACAATTTATGGTCG) for the 5′ flanking region and primers kgp3′Cat (ACGACCATAAATTGTTTTAAAGTGGAGAAAAAAATCACTGG) and kgp3′Rev (GGCTTTACACTACCGCGCTT) for the 3′ flanking region and the Phusion Polymerase (NEB) according to manufacturer's instructions. The chloramphenicol resistance cassette was amplified from plasmid pCM18 (a gift from Dr. N Jakubovics, Newcastle, UK) using primers Catfor (ACAGAATTACTTTACAGCGAGTTTCTCTATTACGCCCCGCCCTGCCACTC) and Catrev (ACAGAATTACTTTACAGCGAGTTTCTCTATTACGCCCCGCCCTGCCACTC). The three PCR products were mixed at equimolar ratios, heated to 95°C, annealed for 1 h at 30°C, followed by an extension step at 42°C for 5 min. Overlapping PCR was then performed to produce a PCR product of 2.8kb using Phusion Polymerase according to the manufacturer's instructions. Following purification (QIAquick PCR Purification Kit,

Qiagen, Crawley, UK), 200ng of the PCR product was electroporated into the E8 strain (BioRad micropulser, 2.5kV) as previously described (Aduse-Opoku *et al.,* 2000). Tc^R Em^R Cm^R colonies were screened by PCR for deletion of the *kgp* gene. The Arg-specific and Lys-specific gingipain activities were determined in chromogenic assays using *N*-benzoyl-DL-Arg-*p*-nitroanilide (BApNA) and N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (TGPLNA; Fisher-Scientific,) as substrates as previously described (Brien-simpson *et al.,* 2001). *P. gingivalis* fixed with 4% (w/v) paraformaldehyde and resuspended at OD_{600} of 0.5 were used as a control in both proteinase assays.

Invasion assay

Invasion of *P. gingivalis* strains NCTC118324, W50 and *T. forsythia* (ATCC 43037) in both H357 and OK-F6 were quantified by an antibiotic protection assay as previously described (Suwannakul *et al.,* 2010, Honma *et al.,* 2011).

Bacterial challenge and treatment

OK-F6 and H357 were seeded at a density of ~500 cells/cm^2 and cultures overnight. After replacing the media with either DMEM or K-SFM, both supplemented with 0.5% (v/v) FCS, the cells were challenged with either *P. gingivalis* NCTC11834 or *T. forsythia* at an MOI: 100 for 4h. After treatment, the cells were washed twice with phosphate buffered saline (PBS) and resuspended in RIPA buffer containing β-mercaptoethanol (1:100) and supplemented with Complete EDTA-free protease cocktail inhibitors (Roche, West Sussex, UK) and PhosStop Phosphatase inhibitor cocktail (Roche) for extraction and solubilisation of total proteins. Both protease inhibitors were used according to manufacturer' s instructions. Lysed cells were stored at −80°C overnight and after centrifugation (14 000 g, 15 min, 4°C), the supernatants containing proteins were removed and stored at −80°C until used. Unchallenged cells were used as a negative control. For experiments in the presence of cytochalasin D, OK-F6 cells were pre-treated for 30 min with 1 μg/ml cytochalasin D (Kinane *et al.,* 2012) after which they were washed three times with PBS before treatment with *P. gingivalis* as described above. Statistical analysis was performed by Student' s*t* test and p value of less than 0.05 (*, $p<0.05$) was considered significant.

Assay for intracellular protein degradation with crude gingipain preparations (supernatants and cell-associated gingipains) of P. gingivalis and extracellular proteases of *T. forsythia*

To prepare crude gingipain preparations, *P. gingivalis* strains W50, E8, K1A and the triple mutant E18 (*rpgABkgp)* were grown as liquid cultures in BHI media overnight to stationary phase. The cultures were then normalised according to their OD_{600} values (OD_{600}=1.0) using BHI before removal of whole cells by centrifugation (14 000 g, 20 min) and filtration through a 0.45 μm filter. Whole bacterial cells were kept on ice for use in assays with OK-F6 cell lysates as ' cell-associated' gingipain preparations. The cleared protein supernatants were assayed for arginine-specific and lysine-specific gingipain protease activity using chromogenic substrates BAPNA and TGPL-pNa as previously described (Brien-simpson *et al.,* 2001) and used immediately. In all assays, the gingipain-containing supernatants were normalised to cell number by dilution with BHI media and in all cases the cell density of the cultures used differed by no more than 10%.

Oral epithelial cell lysates were prepared from OK-F6 cells using identical growth conditions as described above. The cells were then stored at −80°C for no longer than a 1 week before resuspension in PBS. Following centrifugation (14000 g, 10 min, 4°C), the culture supernatant was transferred into a fresh tube and total protein concentration determined using the R_CD_C protein assay (Pierce, Thermo Scientific, Northumberland, UK). OK-F6 cell lysate (20 μg) was incubated with normalised *P. gingivalis* culture supernatant (20, 10 or 2 μl) in the presence of either leupeptin (0.2 mM) or Na-Tosyl-Lysine Choromethyl Ketone (TLCK, 0.5 mM) for 30 min after which Laemlli SDS sample buffer was added and samples denatured at 95°C. Samples were separated by SDS-PAGE and immunoblotted for mTOR or other signalling proteins.

Similarly, to determine the effect of cell-associated or secreted gingipains for the wild-type compared to the isogenic *rgp, kgp* and *rgpkgp* mutants, OK-F6 cell lysates (20 μg) were incubated with either whole, washed bacterial cells (cell-associated activity) or the supernatants (secreted activity) from the same culture, normalised for cell number. Uninoculated bacterial media was used as a control (BHI) and mTOR levels determined by immunoblotting. The effects of any cell-associated and secreted proteases from *T. forsythia* were also investigated as described above following normalisation for cell number.

Western blotting

Total protein was extracted from harvested cells in supplemented RIPA buffer and total protein concentration was determined using the R_CD_C assay (Pierce) according to manufacturer' s instructions. For western blotting, proteins (20 μg) were subjected to SDS-PAGE using (3-8% (w/v) tris-acetate or bis-tris (4-12% (w/v)) gels and transferred to nitrocellulose membrane. Membranes were blocked with TBST (137 mM NaCl, 20 mM Tris, 0.1% (v/v) Tween, pH 7.6) supplemented with 5% (w/v) powdered skimmed milk for 1 h at room temperature before incubation with primary antibody (1:1000 in 5% (w/v) BSA in TBST) overnight at 4°C. After overnight incubation, membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies (anti-rabbit 1:1000 or anti-mouse 1:3000) in blocking buffer for 1 h. Primary mouse anti-GAPDH antibody (1:10 000) was used as a loading control. After washing, protein expression was visualised by incubation with enhanced chemiluminescence (ECL reagents, Pierce) and exposed to CL-Xposure films (Pierce, Thermo Scientific). Blots are representative of at least three independent observations and densitometry was performed with Adobe Photoshop.

RESULTS

P. gingivalis induces mTOR degradation in oral epithelial cells

To examine the effects of *P. gingivalis* and *T. forsythia* on host cell signalling pathways, we first interrogated their ability to invade oral epithelial cells. Both pathogens invaded both the oral squamous cell carcinoma (OSCC)-derived cell line H357 and the immortalised keratinocyte OK-F6 cell line, although higher invasion levels was consistently observed in the OK-F6 cells (Fig. 1A) using a standard antibiotic protection assay.

Following incubation of both H357 and OK-F6 with either live *T. forsythia* or *P. gingivalis* NCTC11834 at an MOI:100 for 4 h, changes in mTOR protein were investigated by immunoblotting. Cells challenged with media only were used as control. Whilst un-degraded mTOR (289 kDa) was the only band detected in both control and *T. forsythia*-treated samples, a marked decrease in mTOR levels together with a characteristic cleavage pattern was observed following treatment with *P. gingivalis* NCTC11834 (Fig. 1B). This degradation was detected both in H357 (Left panel; *Pg* Lane, Fig. 1B) and OK-F6 (Right Panel, *Pg* Lane, Fig. 1B) cells using specific antibodies for both total mTOR and phosphorylated mTOR (p-mTOR), while levels of the GAPDH loading control were unaffected (Fig. 1B).

mTOR degradation is mediated by gingipains

To determine whether the degradation of mTOR was mediated by *P. gingivalis* gingipains, the effects of protease inhibitors on degradation was examined in a cell-free assay. Briefly, total protein extracted from OK-F6 oral epithelial cells was incubated with decreasing volumes of *P. gingivalis* culture supernatants from a liquid culture of *P. gingivalis* NCTC11834 in the presence and absence of protease inhibitors and mTOR levels were assessed by immunoblotting.

In the absence of these inhibitors, a dose-dependent degradation of mTOR was observed; that is, increasing amounts of full-length mTOR were detectable with decreasing volumes of *P. gingivalis* culture containing secreted gingipains (Fig. 2; lanes 1, 4 and 7). In the presence of both 20μl and 10μl of *P. gingivalis* NCTC11834 culture supernatant, mTOR could not be detected whilst in the presence of 2 μl supernatant un-degraded mTOR could be observed (Fig. 2). In the presence of the Arg-gingipain specific inhibitor leupeptin, only a small effect on mTOR degradation was seen (lanes 2, 5 and 8), while a marked reduction in mTOR degradation was observed in the presence of the inhibitor TLCK (lanes 3, 6 and 9), which is a potent inhibitor of Lys-specific gingipain and a weak inhibitor of Arg specific gingipain (Fig. 2).

To further investigate the role of gingipains, the effects of crude gingipain preparations from a panel of isogenic *P. gingivalis* W50 gingipain mutants on mTOR were studied. The strains used were wild-type strain W50, the *rgp*- mutant E8, *kgp*- mutant KIA and a newly created *rgpABkgp* triple mutant (EK18) generated in this study. This *rgpABkgp*$^{-}$ strain produces neither Arg- or Lys-gingipains (Fig. 3A), does not invade cells but has an unaltered growth rate in BHI-medium (data not shown). OK-F6 cell lysates were incubated with either culture supernatants (containing secreted gingipains) or whole bacterial cells (containing cell-associated gingipains) from bacterial liquid cultures grown overnight and normalised by measurement of OD_{600} and probed for mTOR levels by immunoblotting. As expected, full-length mTOR could not be detected following treatment with culture supernatant from the *P. gingivalis* wild-type strain W50 (Fig. 3B). The degradation was slightly decreased when challenged with the *rgp*$^{-}$ E8 mutant, and greatly reduced with the *kgp*$^{-}$ K1A mutant. No degradation was observed following incubation with supernatant from the triple *rgpABkgp* mutant, which is deficient in both Arg- and Lys-gingipains. Degradation was similarly

observed when the epithelial cell lysate was incubated with whole *P. gingivalis* cells (cell-associated activity; Fig.3C).

Effect of *P. gingivalis* on AKT, an upstream modulator of mTOR signalling

To determine the effect upstream of mTOR, the levels of both total and phosphorylated AKT (p-AKT; S473) following invasion with live *P. gingivalis* 4h post-infection was examined by immunoblotting. A slight reduction in both forms were observed (Fig. 4A) whilst GAPDH levels remain unchanged.

Further investigations showed that AKT degradation was influenced by cell-free gingipains as a marked reduction was observed following incubation of (20 μg) cell lysate with culture supernatants containing gingipains from the wild-type strain W50 when compared to the triple gingipain mutant *rgp*$^{-}$*kgp*$^{-}$ (Fig. 4B) while GAPDH levels were unaffected (shown in Fig. 3C, as these were performed on the same samples). Cell-associated and secreted protease containing fractions from *T. forsythia* in the cell-free system under identical conditions did not degrade mTOR (data not shown).

Effect of *P. gingivalis* on downstream mTOR signalling pathway proteins

To explore whether invasion by *P. gingivalis* affected mTOR-associated proteins and signalling pathways downstream of mTOR, total protein extracts from OK-F6 cells were challenged with *P. gingivalis* NCTC11834 and then probed with antibodies to a panel of proteins involved in the mTOR signalling pathway using immunoblotting.

The levels of the mTOR Complex (mTORC) 1-associated protein, raptor, and the mTORC2-associated protein, rictor, were reduced 4h post *P. gingivalis* infection when compared to the control whilst GβL, a positive regulator associated with both complexes, remained unchanged (Fig. 5). In addition, we probed for the phosphorylated form of the downstream target of mTORC1, 4E-BP1 (p-4E-BP1 T37/46) showed that levels of p-4E-BP1 protein expression were also reduced (Fig. 5) compared to uninfected control. Similarly, the levels of β-actin were markedly decreased when compared to the non-treated control sample (Fig. 5).

P. gingivalis internalisation is required for mTOR degradation

To determine whether mTOR degradation was dependent on internalisation of *P. gingivalis* or simply caused by the action of secreted gingipains entering the cell, OK-F6 cells pre-treated with cytochalasin D for 30 min were challenged with *P. gingivalis* for 4 h. Treatment of OK-F6 cells with cytochalasin D caused a 5 fold reduction in *P. gingivalis* invasion when compared to untreated controls (Fig. 6A). In the absence of cytochalasin D (Fig. 6B), mTOR degradation in response of *P. gingivalis* was observed. However, treatment with cytochalasin D markedly inhinited mTOR degradation, suggesting that invasion is required for degradation of mTOR (Fig. 6A).

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DISCUSSION

The aims of this study were to determine the effects of periodontal pathogens *P. gingivalis* and *T. forsythia* on mTOR and its signalling pathways. Having established that both *P. gingivalis* and *T. forsythia* invaded the oral squamous cell carcinoma (OSCC)-derived cell line H357, in keeping with our previous findings (Suwannakul *et al.,* 2010) and the immortalised oral keratinocyte OK-F6 cell line, levels of mTOR were assessed by immunoblotting. The degradation of both total and p-mTOR in the H357 and OK-F6 cell lines indicates that this phenomenon was cell line independent and importantly *P. gingivalis*-dependent as no degradation was observed with *T. forsythia*. A 4h incubation period was chosen to allow sufficient time for invasion (known to occur within 90 min; (Lamont *et al.,* 1995)). Infection was carried out at an MOI:100 as this has previously been shown to be optimal for invasion (Lamont *et al.,* 1995) and frequently used in studies of modulation of host cell phentotype by *P. gingivalis* (Stathopoulou *et al.,* 2009). This MOI does not affect the viability of the epithelial cells following infection with *P. gingivalis* (Madianos *et al.,* 1996, Fravalo *et al.*, 1996). Data by Stathopoulou and colleagues (2009) have also shown that there is no evidence of apoptosis when cells were infected with *P. gingivalis* (MOI: 100) for 4 h. Thus, in our study, *P. gingivalis* challenge at an MOI 100 for 4 h will allow the early cell responses following invasion to be determined without time for apoptosis to influence cell signalling responses.

The differential effect of *T. forsythia* and *P. gingivalis* on mTOR was unexpected as *T. forsythia* is known to secrete proteases such as karilysin (Karim *et al.,* 2010, Cerdà-Costa *et al.,* 2011, Jusko *et al.,* 2012) and *prtH/ cct* (Nakajima *et al.,* 2006). Our findings suggest that although *T. forsythia* invaded H357 and OK-F6 cells as efficiently as *P. gingivalis*, under our experimental conditions, it is unlikely that mTOR is a substrate for the proteases secreted by *T. forsythia*. Another explanation might be that the proteases are not expressed by *T. forsythia* inside epithelial cells. Whilst we have no direct evidence for this, it is noteworthy that both karilysin and PrtH act extracellularly with karilysin inhibiting several pathways of the complement system (Jusko *et al.,* 2012) and inactivating the anti-bacterial peptide LL-37 (Koziel *et al.,* 2010), while PrtH is able to detach epithelial cells from surfaces (Nakajima *et al.,* 2006, Pei and Grishin 2009). These findings also support previously published data that *P. gingivalis* and *T. forsythia* trigger different signalling pathways following invasion to modulate host cell responses (Bodet *et al.,* 2006). Since changes in mTOR levels were only observed with *P. gingivalis*, the rest of the study focussed on investigating changes in mTOR signalling after *P. gingivalis* challenge.

We next set out to investigate the mechanisms underlying the mTOR degradation elicited by *P. gingivalis* in a cell-free assay. The dose-dependent degradation observed in the presence of increasing volumes of *P. gingivalis* NCTC11834 supernatants suggests that this might be mediated by *P. gingivalis* secreted gingipains. This hypothesis was further corroborated by data obtained from examining degradation in the presence of protease inhibitors. The slight decrease in mTOR degradation in the presence of the Arg-gingipain specific inhibitor leupeptin compared to the strong inhibition in degradation in the presence of Lys-specific gingipain inhibitor TLCK, confirmed that degradation of mTOR was gingipain-dependent and suggested that Lys-gingipain might be predominantly responsible for this phenomenon.

We thus hypothesised that the observed degradation is primarily mediated by Lys-specific gingipain; this was corroborated in a cell free assay using a panel of isogenic *P. gingivalis* W50 gingipain mutants. A cell-free assay was chosen to investigate these changes as levels of invasions between the *rgpAB* and *kgp* strains are not comparable (Suwannakul *et al.,* 2010) and these mutants have pleiotropic phenotypes such as alterations in fimbrial maturation that may also influence host-bacterial interactions, which is probably why, the *rgpABkgp* triple mutant is non-invasive (data not shown). The degradation mediated by the wild-type strain W50 shows that this phenomenon is not strain-specific whilst inhibition of degradation with the triple mutant *rgpABkgp*$^{-}$ substantiates the hypothesis that gingipains are fundamental for this process. Since degradation of mTOR in epithelial cell lysate was observed both with bacterial culture supernatants (secreted gingipains) and whole bacterial cells (cell-associated activity), the data taken together, confirmed that mTOR is degraded by both *P. gingivalis* cell-associated and secreted gingipains.

Upstream of mTOR signalling is the serine/threonine kinase AKT, also known as protein kinase B (Peng *et al.,* 2003). mTOR activation is dependent on the binding of PI3K and subsequent phosphorylation of AKT (Peng *et al.,* 2003). The phosphatidylinositol 3-kinase (PI3K)/AKT signalling pathway is involved in several processes including cell proliferation, survival, growth and motility, some of which are mTOR-mediated. The slight reduction in the levels of both total and phosphorylated AKT demonstrated here was also attributed to cell-free gingipains. Our results are in contrast to previous studies which showed that *P. gingivalis* LPS activates the PI3K-AKT pathway in a TLR2-dependent manner in human monocytes (Martin *et al.,* 2003), while increases in AKT after 24h infection with live *P. gingivalis* has been observed in gingival epithelial cells (Yilmaz *et al.,* 2004). The reasons for this difference are unclear but may be due to both the time point at which the observations were made and the cells used.

Since *P. gingivalis* has an effect on mTOR levels, we explored whether invasion of cells by *P. gingivalis* affected both mTOR-complex-associated proteins and proteins downstream of mTOR. The mTOR pathway comprises two distinct multi-protein complexes known as mTORC1 and mTORC2, with the specific functions of the complexes determined by the regulatory proteins they contain. mTORC1 specifically contains raptor (regulatory-associated protein of mTOR) and is essentially involved in cell growth, proliferation and autophagy (Kim *et al.,* 2002, Laplante and Sabatini 2009). Although the exact function of most of the mTOR-binding proteins remains to be elucidated, it has been proposed that raptor regulates the assembly of the complex, recruits mTORC1 substrates, such as 4E-BP1 and p70SO6, and catalyses their phosphorylation (Hara *et al.,* 2002) so is essential for mTOR signalling *in vivo* (Nojima *et al.,* 2003).

Following *P. gingivalis* treatment, the levels of mTORC1 associated protein raptor was reduced. The combined reduction in levels of both mTOR and raptor suggest that *P. gingivalis* is likely to have a downstream effect in the mTORC1 signalling pathway and this was further corroborated by the fact that the levels of phosphorylated-4E-BP1 decreased. The mTOR substrate 4E-BP1 is important in mTORC1-mediated cell proliferation but not cell growth (Dowling *et al.,* 2010) with its phosphorylation being linked to increased cellular proliferation as well as elevated levels of cyclin D1(CCND1) mRNA; CCND1 encodes a

protein normally required for G1/S transition in the cell cycle (Barnhart *et al.,* 2008). Previous studies have reported an increase in cell proliferation following *P. gingivalis* infection in gingival epithelial cells (Kuboniwa *et al.,* 2008) and a decrease in cell proliferation in the extravillous trophoblast cell line, HTR-8 (Inaba *et al.,* 2009). Our observation that levels of phospho-4E-BP1 are reduced could therefore be a plausible explanation for the decrease in cell proliferation reported (Inaba *et al.,* 2009). This suggestion is also supported by previous findings that a decrease in CCND1 is observed following *P. gingivalis* invasion (Kato *et al.,* 2008, Inaba *et al.,* 2009). This, coupled with the gingipain-mediated degradation of mTOR, suggests that following infection, the mTORC1 signalling pathway is severely impacted.

GβL, a positive regulator of mTOR, is associated with both mTORC1 and mTORC2 (Kim *et al.,* 2003). Within mTORC1, GβL interacts directly with mTOR and increases its kinase activity. Following bacterial challenge, levels of GβL remained unchanged showing that the *P. gingivalis* proteolytic effects are specific to a subset of substrates within the mTOR pathway.

Having examined potential effects on mTORC1, we also investigated whether the gingipains were having an effect on the much less well characterised mTORC2 complex. The composition of this complex differs from mTORC1, with rictor, the rapamycin-insensitive companion of mTOR, being a key component that is essential for its function as highlighted by its role in the modulation of the phosphorylation of protein kinase Cα (PKCα) and the actin cytoskeleton (Sarbassov *et al.,* 2004). The significant reduction in rictor levels suggest that the functions mediated by mTORC2 might also be dysregulated. The levels of β-actin was also found to be altered, corroborating the recent findings of Kinane *et al.* (2012) who, using a similar approach, showed actin degradation by gingipains.

To determine whether mTOR degradation was dependent on internalisation of *P. gingivalis* or was a consequence of secreted gingipains, the effect of cytochalasin D on mTOR degradation was investigated. Cytochalasin D is a fungal metabolite that inhibits actin polymerisation (Casella *et al.,* 1981) and is known to inhibit invasion of both oral epithelial cells (Nakagawa *et al.,* 2006) and endothelial cells (Deshpande *et al.,* 1998, Dorn *et al.,* 2001) by *P. gingivalis.* In addition the recent work of Kinane and co-workers showed that intracellular degradation of actin was abrogated following incubation with cytochalasin D in human gingival epithelial cells (Kinane *et al.,* 2012). In our study, the inhibition of mTOR degradation together with a 5-fold reduction in invasion indicates that invasion is required for degradation of mTOR. In addition, we also confirmed the observation by Kinane *et al* (2012) that actin degradation by *P. gingivalis* is inhibited by cytochalasin D. Our data indicate, that the gingipain-dependent degradation of mTOR requires the action of *P. gingivalis* cell-associated or secreted gingipains deployed after invading host cells.

In conclusion, in this study we have highlighted for the first time a direct effect of the periodontal pathogen *P. gingivalis* on the mTOR signalling pathway. In contrast, this was not the case for its fellow red-complex pathogen *T. forsythia*. We have thus further highlighted the fact that these pathogens stimulate differential host cell responses following infection. Our data demonstrate that mTOR degradation by *P. gingivalis* is mediated by both secreted

and cell-associated gingipains, again illustrating that deployment of gingipains is a central strategy used by *P. gingivalis* to manipulate host cell responses (Sheets *et al.,* 2005, Stathopoulou *et al.,* 2009). We further showed that *P. gingivalis* invasion of oral epithelial cells is required for mTOR degradation. We also observed changes in the levels of mTOR associated proteins such as raptor (mTORC1), rictor (mTORC2) and observed alterations in downstream signalling proteins such as 4E-BP1 (mTORC1 dependent pathway) and actin, suggesting that *P. gingivalis*-mediated mTOR degradation is one facet of how cellular responses are influenced by infection.

Our study adds to the mechanistic picture of how *P. gingivalis,* and specifically its gingipains, exerts its myriad of cellular responses. Specifically, degradation of mTOR may contribute to the modulation of some of the cellular changes such as proliferation, survival and induction of autophagy, which are observed following *P. gingivalis* invasion and internalisation (Dorn *et al.,* 2001, Yamatake *et al.,* 2007, Jung *et al.,* 2010, Mizushima and Komatsu 2011, Yuk *et al.,* 2012). While a link between *P. gingivalis* and autophagy in other cells such as the coronary artery endothelial cells has been established (Dorn *et al.,* 2001, Bélanger *et al.,* 2006), recent studies have also documented autophagy in the gingival fibroblasts of periodontal patients (Bullon *et al.,* 2012). It is therefore tempting to speculate that the data presented here indicate that there may be a link between *P. gingivalis* internalisation and persistence in oral epithelial cells that is related to its ability to engage autophagy pathways. Furthermore, it is becoming clear that autophagy may play a role in a wide range of infections including the alterations of intracellular defence pathway following infection by bacteria such as *Mycobacterium tuberculosis* (Gutierrez *et al.,* 2004) or viruses (Grose 2010). Work is ongoing in our laboratories to further investigate the influence of *P. gingivalis*-induced mTOR degradation on cellular processes, including cell proliferation and autophagy.

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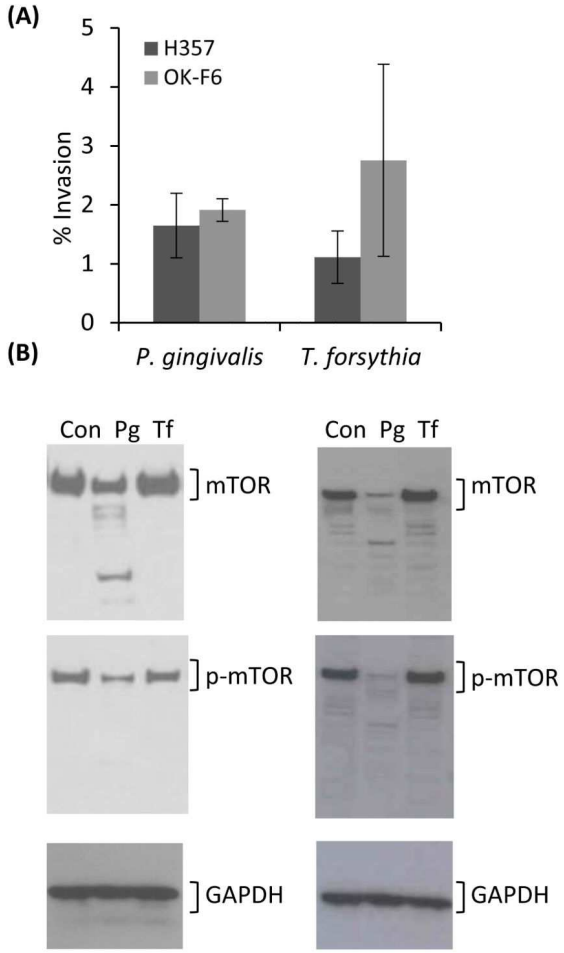

Fig. 1. *P. gingivalis* degrades mTOR in both H357 and OK-F6 cells

(A) Invasion efficiencies of H357 and OK-F6 cells by *P. gingivalis* strains NCTC11834 and *T. forsythia* (ATCC 43037) were compared in an antibiotic protection assay and calculated from colony forming units (CFU) recovered intracellularly as a percentage of the total bacteria inoculated following incubation of the cells with bacteria for 90 min. Error bars represent standard errors and are representative of at least 3 separate observations. (B) Cells were seeded at a density of 500 cells/cm^2 and following challenge with either *P. gingivalis* NCTC11834 or *T. forsythia* (ATCC 43037) at an MOI:100 for 4 h, cells were washed twice in PBS and protein extracted using RIPA buffer supplemented with protease inhibitors. 20 μg of each sample was immunoblotted with both total and phosphorylated-mTOR antibody and visualised by chemiluminescence. GAPDH was used as loading control. Blots are representative of at least 3 independent observations.

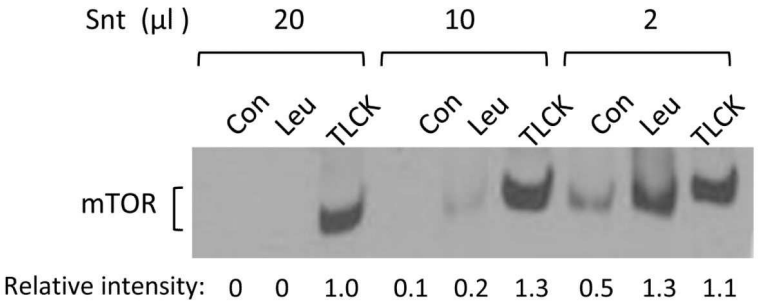

Fig 2. Protease inhibitors impede *P. gingivalis* mediated mTOR degradation in oral epithelial cells

Total protein was extracted from OK-F6 cells and quantified. *P. gingivalis* culture supernatants were then incubated with OK-F6 cell lysate in the presence of leupeptin (Leu) or. TLCK (TLCK), mTOR levels were probed for by blotting and visualised by chemiluminescence. Controls (Con) were cell lysates treated with culture supernatants in the absence of inhibitors. Blots are representative of at least 3 independent observations and relative intensities (compared to the control) are indicated.

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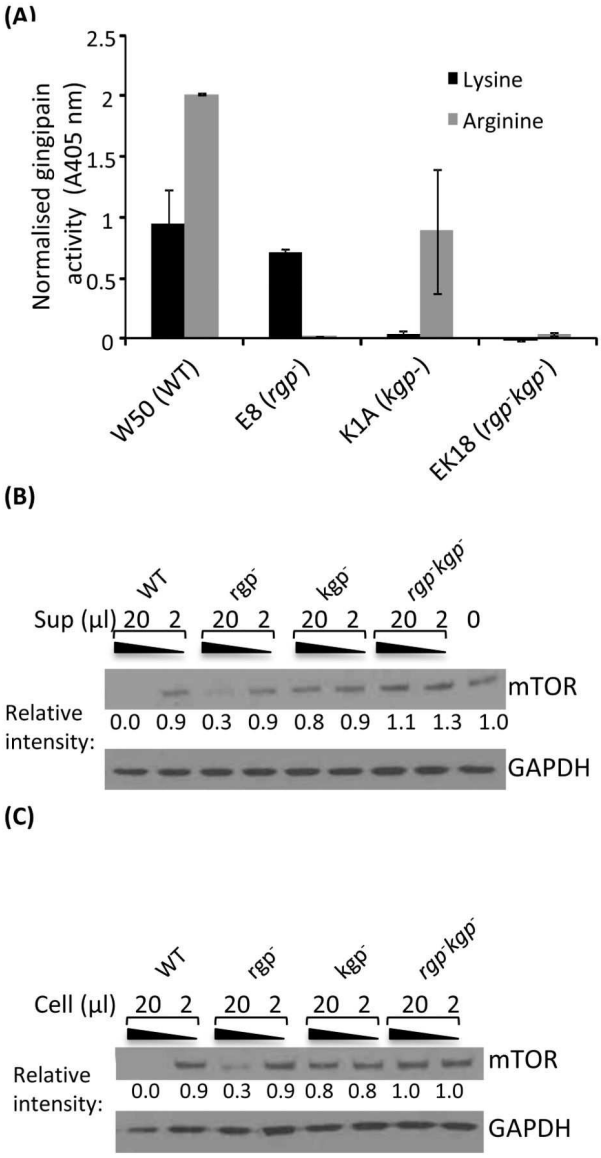

Fig. 3. mTOR degradation is mediated gingipains

(A) A triple *rgp*$^{-}$*kgp*$^{-}$ gingipain mutant was created in *P. gingivalis* W50 *rgpA::EmrgpB::Tet* (E8,Δ*rgpA*Δ*rgpB)* strain by introducing a chloramphenicol cassette within the *kgp* gene. *P. gingivalis* colonies were screened for Tc^{R} Em^{R} Cm^{R} resistance to confirm *kgp* gene deletion and gingipain activities were determined in chromogenic assays using *N*-benzoyl-DL-Arg-*p*-nitroanilide (BApNA) and N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (TGPLNA) as substrates. (B) OK-F6 cells were seeded overnight in K-SFM containing 0.5% FCS (v/v) and after washing the cells were harvested and lysed into PBS. Cell lysates (20 µg) were treated with either culture supernatants containing secreted gingipains (cell-free gingipains) or with (C) whole bacterial cells from the same culture harbouring cell-associated gingipains from the *P. gingivalis* strains W50, E8 (*rpg*$^{-}$), KIA (*kgp*$^{-}$) and E18 (*rgp*$^{-}$*kgp*$^{-}$) for 30 min in the presence of cysteine after which mTOR levels were determined by blotting and visualised by chemiluminescence. GAPDH was used as a loading control. Cells treated with

BHI were used as control. Blots are representative of at least 3 independent observations and relative intensities (compared to the control) are indicated.

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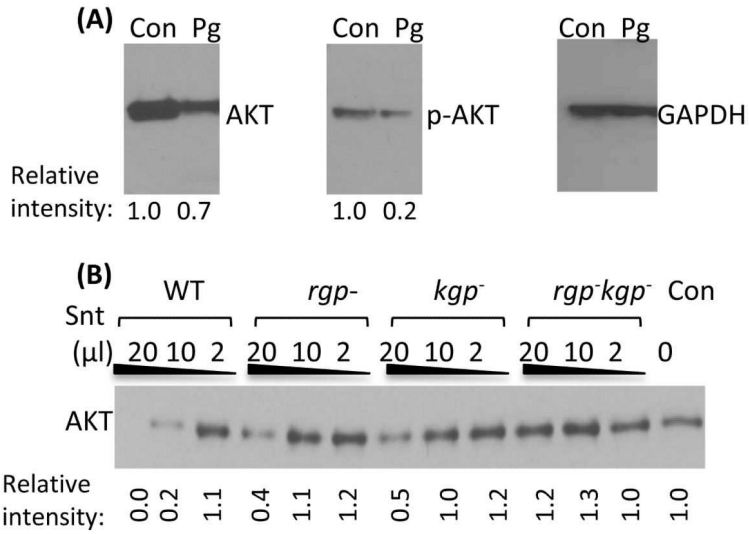

Fig. 4. AKT is degraded following *P. gingivalis* treatment by both lysine- and arginine-specific gingipains

(A) Cells were seeded at a density of 500 cells/cm^2 and following challenge with *P. gingivalis* NCTC11834 (Pg; MOI:100) for 4 h, cells were washed twice in PBS, scrapped in RIPA buffer supplemented with protease inhibitors and total proteins determined. Cells challenged with media only were used as control (Con). 20 µg of each sample was immunoblotted with both total and phosphorylated-AKT (S473) antibodies and visualised by chemiluminescence. GAPDH was used as a loading control. (B) OK-F6 cells were seeded overnight in K-SFM containing 0.5% (v/v) FCS and after washing the cells were scraped into PBS. Cell lysates (20 µg) were treated with culture supernatants from the *P. gingivalis* strains W50, E8 (*rpg*$^-$), KIA (*kgp*$^-$) and E18 (*rgp*$^-$*kgp*$^-$) for 30 min in the presence of cysteine after which mTOR levels were determined by blotting and visualised by chemiluminescence. Cell lysate treated only with culture media was included as control (Con). GAPDH was used as a control but is shown in Fig. 3B, since identical samples were used for these experiments (3B and 4B. Blots are representative of at least 3 independent observations and relative intensities (compared to the control) are indicated.

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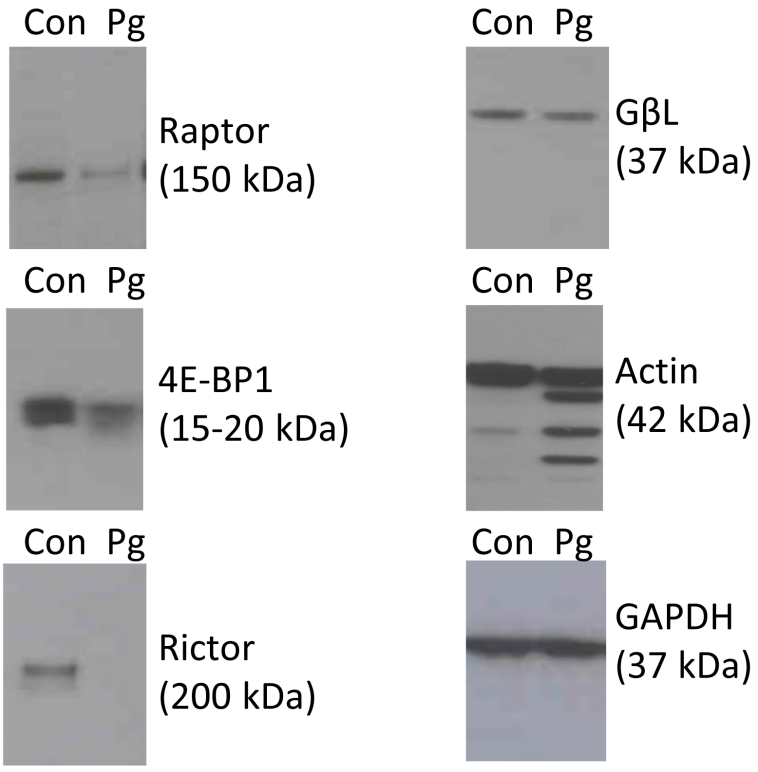

Fig. 5. *P. gingivalis* alters the levels of signalling proteins in the mTOR pathway

(A) OK-F6 cells (~500 cell/cm^2) seeded in K-SFM supplemented with 0.5% FCS (v/v) were challenged with *P. gingivalis* NCTC11834 (Pg; MOI:100) for 4 h (Pg). Unchallenged cells were included as control (Con). The media was discarded and after washing the cells twice in PBS, the cells were scrapped in RIPA buffer supplemented with protease inhibitors. 20 μg of each sample was immunoblotted with antibodies against the mTORC1 associated protein Raptor, the mTORC2 associated protein Rictor, GβL (associated with both mTOR complexes), phosphorylated-4E-BP1 (a transcription factor which acts downstream of mTOR; (T37/46)) and β-actin. GAPDH was included as a loading control. Blots were visualised by chemiluminescence. Blots are representative of at least 3 independent observations.

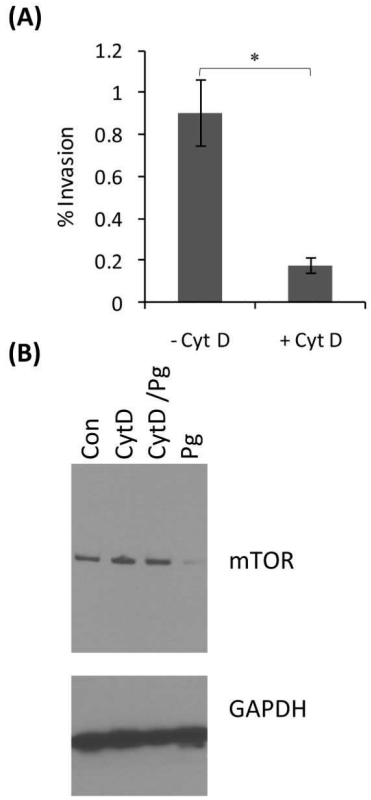

Fig. 6. Cytochalasin D inhibits invasion of oral epithelial cells by *P. gingivalis*

(A) Invasion efficiencies of H357 and OK-F6 cells by *P. gingivalis* strains NCTC11834 in the presence of cytochalasin D were compared in an antibiotic protection assay. Colony forming units (CFU) recovered intracellularly as a percentage of the total bacteria inoculated were calculated following incubation of the cells with bacteria for 90 min. Error bars represent standard errors and are representative of at least 3 separate observations. Percentage invasion is shown and reduction in invasion was found to be statistically significant (*, $p<0.05$). (B) Cytochalasin D inhibits *P. gingivalis*-mediated mTOR degradation. OK-F6 cells were seeded overnight at a density of ~500 cells/cm^2 and the following day the media was replaced with K-SFM containing 0.5% (v/v) FCS. The cells were pre-treated with cytochalasin D (1 μg/ml) for 30 min after which they were challenged with *P. gingivalis* (MOI:100) for 4h (CytD/Pg). Total proteins were extracted in RIPA buffer supplemented with protease inhibitors and β-mercaptoethanol and 20 μg of each sample was probed for mTOR and visualised by chemiluminescence. Cells were treated with *P. gingivalis* only in the absence of cytochalasin D (Pg), cells treated with media (Con) and cells treated with cytochalasin D only (Cyt D). GAPDH was included as a loading control. Blots are representative of at least 3 independent observations.