Pre-conditioning of gingival epithelial cells with sub-apoptotic concentrations of curcumin prevents pro-inflammatory cytokine release.

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Key words

Curcumin, epithelium, gingivitis, biliverdin reductase, haem oxygenase,

Abstract

Background and objective: Plaque-induced gingival inflammation (gingivitis) is ubiquitous in humans. The epithelial barrier reacts to the presence of oral bacteria and induces inflammatory cascades. The objective of this study was to investigate the mechanism by which the small molecule micronutrient curcumin could decrease inflammatory response *in vitro* to oral bacterium heat killed *Fusobacterium nucleatum* as curcumin could be a useful compound for combatting gingivitis already consumed by humans.

Methods: H400 oral epithelial cell line was pre-conditioned with curcumin and the production of cytokines, by enzyme linked immunosorbent assay (ELISA), and translocation of transcription factors used to monitor inflammatory responses. Haem oxygenase (HO-1) expression and molecules that HO-1 releases were evaluated for their potential to reduce the quantity of cytokine production. Immunofluorescence microscopy and Western blotting were used to evaluate changes in transcription factor and enzyme location.

Results: Pre-conditioning of H400 cells with a sub-apoptotic concentration of curcumin (20μM) attenuated secretion of Granulocyte-Macrophage – Colony-Stimulating Factor (GM-CSF) and reduced NFkB nuclear translocation. This pre-conditioning caused an increase in nuclear Nrf2; an initial drop (at 8h) followed by an adaptive increase (at 24h) in glutathione; and an increase in haem oxygenase (HO-1) expression. Inhibition of HO-1 by SnPPIX prevented the curcumin-induced attenuation of GM-CSF production. HO-1 catalyses the breakdown of haem to carbon monoxide, free iron and biliverdin: the HO-1/CO anti-inflammatory pathway. Elevations in carbon monoxide, achieved using carbon monoxide releasing molecule-2 (CORM2) treatment alone abrogated *F. nucleatum* induced cytokine production. Biliverdin is converted to bilirubin by biliverdin reductase (BVR). This pleiotropic protein was found to increase in cell membrane expression upon curcumin treatment

Conclusion: Curcumin decreased inflammatory cytokine production induced by *Fusobacterium nucleatum* in H400 oral epithelial cells. The mechanism of action appears to be driven by the increase of haem oxygenase and the production of carbon monoxide.

Conflict of interest statement

This research has been financially supported by Unilever Oral Care. A.E. Scott is employed by Unilever Oral Care

Introduction

Gingivitis is an inflammatory condition of the gums, induced by the accumulation of a microbial biofilm around the teeth that affects around 90% of adults [1]. Unchecked, this may eventually lead to periodontitis in up to 50% of adults, where the tooth-supporting connective tissues and alveolar bone are destroyed, ultimately leading to tooth loss. Periodontitis is an inflammatory condition of the periodontal tissues, evidenced by an increase in pro-inflammatory cytokines[2] and a large, neutrophil-dominated inflammatory cell infiltrate [3]. The initial local increase in cytokines is likely to emanate from the epithelial cell barrier and transmigrating neutrophils responding to an emerging dysbiosis, following engagement of surface pattern recognition receptors to pathogen associated molecular patterns on the surface of periodontal pathogens such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Specifically, chemokines are released by oral epithelial cells following activation of the Toll-like receptor (TLR; TLR-2, -4 and/or -9) pathways [4] [5] leading to activation of transcription factors such as Nuclear Factor kappa B (NFkB) [6] [7] and Activator Protein 1 (AP-1) [8]. These families of transcription factors are key regulators in the response to infection and are activated by various stimuli including reactive oxygen species (ROS). One of the earliest features of the innate immune response driven by epithelial-derived chemokines is the extravasation of leukocytes, particularly neutrophils, into affected tissues, where they are a source of ROS such as the superoxide anion, hydroxyl radicals and hypochlorous acid [9]. Antioxidant enzymes and peptides such as reduced glutathione (GSH) remove such ROS and abrogate the damage they cause [10]. Recent studies of periodontitis have demonstrated that local levels of glutathione in the epithelial exudate, gingival crevicular fluid, are depleted. However, following successful, non-surgical treatment, glutathione (GSH) levels partially recover and the GSH:GSSG ratio is restored, although total GSH concentrations remain less than in healthy controls [11]. Consistent with this the reducing environment of peripheral blood neutrophils, which is maintained by a high GSH: oxidised glutathione (GSSG) ratio, is compromised in periodontitis patients relative to controls, generating various sequelae of oxidative stress that may damage periodontal tissues[12]. In lung epithelium models, exogenously added GSH can decrease CXCL8 production in response to *Pseudomonas aeruginosa* diffusible material[13], but there is limited data on GSH augmentation strategies in managing periodontitis.

Glutathione is a tripeptide present intra- and extra-cellularly and is highly conserved between species. It is a major contributor to the redox balance within cells, and it is a master regulator of redox-regulated pro-inflammatory gene transcription factors such as NFkB and AP-1, through which it counteracts oxidative stress conditions. It is synthesized in two steps, with the first step, catalysed by gamma-glutamyl cysteine synthetase, being the rate limiting step. The gene regulation of this enzyme

is controlled by the antioxidant response element (ARE) which binds the transcription factor, Nuclear factor (erythroid-derived 2)–related factor 2 (Nrf2). Under normal conditions Nrf2 is sequestered in the cytoplasm by ligation to Kelch-like ECH-associated protein (KEAP1), ubiquitinated and targeted for proteasomal degradation. When a cell is challenged with a primary stressor, e.g. an electrophile or oxidant, KEAP1 is modified at critical thiols thereby releasing Nrf2 and allowing it to relocate to the nucleus[14]. Many genes are regulated by Nrf2, most of which are involved in a protective adaptive response to secondary stressors. Haem oxygenase-1 (*HO-1*) is one such gene upregulated in response to Nrf2 activation. The protein HO-1 catalyses the conversion of haem to carbon monoxide (CO), biliverdin and iron. Induction of HO-1 is considered a rapid protective response against hypoxia, inflammation and inflammatory diseases such as diabetes, obesity, atherosclerosis and other non-communicable diseases (NCD)[,15,16,17]. Of its products, iron is rapidly sequestered by ferritin, which is also upregulated under Nrf2 control [18]. However, CO and biliverdin have been shown to exhibit a number of anti-inflammatory effects. CO inhibits pro-inflammatory cytokine production[19] via modulation of AP-1 [20], Mitogen-activated protein kinases (MAPK) [19] and NFkB [21] pathways. CO can also increase intracellular calcium concentrations [22] which may affect intracellular signalling pathways, such as CaM kinase II and histone deactelyase (HDAC) activity [23, 24]. Biliverdin is rapidly converted into bilirubin by biliverdin reductase (BVR). Bilirubin possesses well documented antioxidant activities and the enzyme biliverdin reductase also acts as a serine threonine kinase possessing its own transcriptional regulation properties [2]5.

Curcumin, a polyphenol found in *Curcuma longa* tubers, has long been used in cooking and traditional medicines. It is a natural pigment with a characteristic yellow-orange colour. It exhibits antibacterial, antifungal, antiviral, antiprotozoal, and antiparasitic properties. Curcumin is lipophilic and is a dimeric derivative of ferulic acid composed of two o-methoxyphenol rings connected by a 1,6-heptadiene-3,5-dione chain. Additionally it has been the subject of research as a cancer therapeutic [26], neuroprotective agent [27] and anti-inflammatory agent [28]. It is a classical Nrf2 agonist acting to release the transcription factor from KEAP1 sequestration [29] and therefore curcumin is of potential importance in downregulating the stress response and consequent inflammation within periodontitis and other “hyperinflammatory” diseases.

In this study we have chosen to use *F. nucleatum* as a key species involved in periodontal diseases. There are many hundred species of bacteria present in the mouth and this bridging organism has been associated with patients with gingivitis and periodontitis: indeed, in periodontitis it helps the insertion of the more prominent periodontopathogen *P. gingivalis* to become established. We have also chosen

to use heat killed *F. nucleatum* so as to minimise other effects that this bacterium may have such as invasion into the epithelium.

The aim of the studies reported here was to determine the potential for curcumin to modulate the initial pro-inflammatory response of oral epithelial cells to challenge by the periodontal pathogen *Fusobacterium nucleatum* in a non-viable form. Herein we demonstrate that in oral epithelial cells, curcumin, at concentrations with no discernible effect on cell viability, is able to decrease the quantity of pro-inflammatory cytokines secreted in response to challenge by heat killed *F. nucleatum*, a process that appears to be mediated through the HO-1 products, CO and biliverdin.

Materials & Methods:

Cell culture

H400 oral epithelial cells were routinely cultured at 37^{o}C in Dulbeco's Modified Eagle's Media (DMEM)/F12 supplemented with 10% foetal calf serum and glutamine in a 5% CO_2 atmosphere. This cell line was derived from an oral squamous cell carcinoma, is adherent and shows a typical polygonal epithelial cell morphology with desmosomal junctions in culture [6, 30] . The cell line was a gift from the originator (Professor S. S. Prime, University of Bristol, UK) but is lodged with the European Collection of Cell Cultures and available commercially. Unless otherwise stated, all experiments were performed on cells plated at a density of 2x10^5/ml into white-walled 96 well plates (Corning; 100μl/well) and allowed to adhere overnight before challenge.

Bacteria

Bacteria (*Fusobacterium nucleatum* American Type Culture Collection (ATCC) #10953) were grown as described previously [31], harvested, washed with sterile distilled water and heat killed for 10min at 100°C. The optical density at 600nm of each stock suspension was measured and used to calculate the approximate numbers of bacteria present [31] for subsequent multiplicity of infection (MOI) calculations.

Preparation of curcumin

Curcumin (Sigma, UK) was dissolved in ethanol, unless otherwise stated. The solution was made at 20mM and diluted with cell culture media 100-fold. Ethanol was used as a vehicle control at an equivalent concentration and never exceeded 1% of the final assay volume.

Viability and caspase activity

Metabolic activity (as a measure of viability) of H400 cells under all treatments was determined by CellTitre-Glo (Promega) and apoptosis was determined by Caspase-Glo 3/7 (Promega). In both cases the manufacturer's protocol was followed. Glo reagents were added at the end of the experiment and luminescence was measured per well for 1s integration, using a Tecan SpectrafluorPlus instrument (Tecan, Germany).

Cytokine and chemokine ELISA enzyme linked immunosorbent assays (ELISA)

To measure the secretion of GM-CSF, Interleukin (IL)-1β, tumour necrosis factor (TNF)-alpha or chemokine (CXCL8, also known as interleukin-8), H400 cells were seeded in 12 well plates (Greiner; $2x10^5$/ml, 1.5ml/well) and left to settle for 24h. Media was exchanged before treatments. Cells were then treated with a variety of compounds (curcumin, tetrahydocurcumin or ferulate 0-50μM) for 8h and then *F. nucleatum* for 16h for GM-CSF, for 48h for IL-1 beta, for 4h for TNFalpha and for 8h for CXCL8 (see supplemental figure 1 for time course analysis). Cytokine levels in cell culture media were determined by enzyme-linked immunosorbent assay (ELISA; Diaclone (IDS Ltd., Boldon, UK) following the manufacturer's instructions. Individual samples were analysed in duplicate and experiments were conducted in triplicate.

NFkB reporter assay

Creation of pGL4.22 3enh transfected H400 cells has been described previously in [7, 32]. pGL4.22 3enh transfected adherent cells were treated with curcumin (20μM) for 8h and then challenged with *F. nucleatum* for 3h (at a range of MOIs). After 3 h, the amount of luciferase produced was determined using ONE-Glo luciferase reagent (Promega, UK). Luminescence was measured per well for 1s integration, using a Tecan SpectrafluorPlus instrument (Tecan, Germany).

TransAM nuclear fractionation and transcription factor detection

Briefly H400 cells (density $2x10^5$ cells/ml, 50ml per 15cm Petri dish) were incubated: with/without curcumin (20μM, 8h) and then challenged with: *F. nucleatum* (1h) for NFkB and AP-1 family members; or with/without curcumin (20μM, 2h) for Nrf2. Nuclear extracts were then prepared by using Active Motif's Nuclear extraction kit (Active Motif, USA). Nuclear extracts were used with TransAM transcription factor ELISAs (Active Motif, USA) to quantify NFkB family members (p65, p50, c-Rel, p52 and Rel-B), AP-1 family members (phosphorylated c-Jun, c-Fos, Fra-1, FosB, JunD and JunB) or Nrf2 in the nucleus. Manufacturer's conditions were followed. Signals were measured using a Tecan SpectrafluorPlus instrument and normalised to protein content determined by BCA assay (Sigma, UK).

Proteasome activity

Proteasome activity of H400 cells treated with curcumin was determined by the Proteasome-Glo 3-Substrate System (Promega, UK) for chymotrypsin, caspase-like and trypsin proteasome activity.

Manufacturer's conditions were followed and luminescence was measured per well for 1s integration, using a Tecan SpectrafluorPlus instrument (Tecan, Germany).

Glutathione

Glutathione in curcumin-treated and control H400 cells pre- and post-bacterial challenge was analysed by GSH-Glo (Promega, UK), following the manufacturer's instructions. Luminescence was measured per well for 1s integration, using a Tecan SpectrafluorPlus instrument (Tecan, Germany).

HO-1 ELISA

To measure the expression of haem oxygenase-1, H400 cells were seeded in 12 well plates (Greiner; $2x10^5$/ml, 1.5ml/well) and left to adhere and grow for 24h. Cells were then treated with a variety of compounds for 8h. Media was removed from the cells and they were washed with ice cold PBS containing protease inhibitors (Complete mini, Roche, UK). Cells were harvested by scraping into extraction buffer (Stressgen). HO-1 content was then determined by HO-1 ELISA (Stressgen, UK) and normalised to protein content, as determined by BCA assay (Sigma, UK). Absorbance was measured using a Tecan SpectrafluorPlus instrument (Tecan, Germany).

Immunofluorescence

H400 oral epithelial cells were seeded (2x 10^5/ml) into 12-well plates (Greiner, Stonehouse, UK) containing acid washed 13mm coverslips and allowed to adhere overnight. Cells were treated as described and then fixed with paraformaldehyde (4%, 10 min, room temperature) and permeabilized with ice-cold methanol (20 min, -20^{o}C). Non-specific binding was blocked by washing with 1% bovine serum albumin (BSA) in PBS and then Nrf2 was located with anti-Nrf2 antibody (ab31163, dilution 1:50, Abcam, UK) or with anti-BVR (B8437, dilution 1:50, Sigma, UK). Secondary antibodies conjugated to Texas Red (AB_2339610, dilution 1:200, Jackson labs, USA) were used to visualise the locations of Nrf2 or Biliverdin reductase (BVR). Nuclei were counterstained with Hoescht stain (10ng/ml). Coverslips were then mounted in MOWIOL® (Calbiochem, Darmstadt, Germany) containing anti-fade compound. Immunostained cells were visualized using a Leica DMRB microscope equipped with a Hamamatsu ORCA camera, and images were captured and processed using OpenLab software

(Improvision, Perkin Elmer, Cambridge, UK) and processed with ImageJ and Abode Photoshop 6.0 (Adobe, San Jose, CA).

Ca^{2+} signalling

Calcium fluctuations in response to curcumin and/or *F. nucleatum* treatment were detected by changes in fluorescence of the calcium sensitive dye FluoForte (Enzo Life Sciences, UK). Adherent H400 cells were preloaded with Fluoforte dye (100µl/well, 1h). Individual wells were then followed fluorescently using a Mithras instrument (Berthold, UK) before stimulants were injected directly into the well. Fluorescent signals were followed for 1min, over which time they stabilised.

HDAC activity

Histone deacetylase (HDAC) class I & II activity of H400 cells treated with curcumin were determined by HDAC-Glo (Promega, UK). Manufacturer's conditions were followed and luminescence was measured per well for 1s integration.

Western blotting for BVR

H400 oral epithelial cells were seeded (2x 10^5/ml) into 6-well plates (Greiner, Stonehouse, UK) and left to settle for 24h. Subsequently cells were treated with curcumin for 8h and then heat-inactivated *F. nucleatum* for 16h, or vehicle. Media was removed and cells washed with PBS prior to membrane isolation (Abcam plasma membrane extraction kit ab65400). Proteins were then solubilised with Laemlli buffer (Sigma, UK) and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 4-20% gels) and electroblotted onto Polyvinylidene fluoride (PVDF). BVR and E-cadherin were detected with anti-BVR (B8437, dilution 1:100 Sigma, UK) and anti-E-cadherin (13-1700, dilution 1:100, Invitrogen, UK). Secondary antibodies conjugated to IR dyes were used to visualise the locations of the proteins of interest. Membranes were visualized using fluorescence detection on the Odyssey Infra-red Imaging System (Licor Biosciences, UK).

Statistics

All data were analysed using one-way analysis of variance (ANOVA), with Graph Pad Prism v 3.03 (Graph Pad, USA).

Results

Sub-apoptotic concentrations of curcumin reduce pro-inflammatory cytokine production in H400 cells

Prior to evaluating the anti-inflammatory properties of curcumin we determined concentrations of curcumin that were not cytotoxic (figure 1 A) nor induced significant levels of apoptosis (figure 1B). Following this 20μM curcumin was used for subsequent experiments. To evaluate the anti-inflammatory effects of curcumin we examined its ability to reduce pro-inflammatory cytokine secretion in response to the periodontally relevant bacterium, *Fusobacterium nucleatum*. GM-CSF secretion was stimulated by *F. nucleatum* at all multiplicities of infection tested (MOI 10-300:1) in a dose-dependent manner (Figure 1 C). Curcumin significantly reduced GM-CSF production across all MOIs tested. Similar responses were seen for TNF-alpha, CXCL8 and IL-1β (figure 1).

Curcumin affects location of NFkB and AP-1 transcription factor families

Concomitant with the decrease in cytokine production was a decrease in NFkB activity determined by reporter assay (figure 2A). Nuclear fractionation and analysis of NFkB family members (figure 2B) and AP-1 family members (figure 2C) revealed there was a significant reduction of p65 and p50 NFkB subunits in the nucleus of cells treated with curcumin prior to *F. nucleatum* stimulation in comparison to *F. nucleatum* stimulation alone. Nuclear phosphorylated c-Jun was the only member of the AP-1 family that was modulated by curcumin and/or *F. nucleatum* treatment. Treatment with either showed a modest increase in phospho-c-Jun, however the treatment of cells with both curcumin and *F. nucleatum* showed a large increase in its nuclear localisation. Following these experiments and those of the range finding of cytokine and chemokine production as MOI of 100:1 was used for the further experiments.

The effect of curcumin on F. nucleatum-induced NFkB activation

In order to establish whether curcumin could alter the activity of proteasomal degradation, which could affect NFkB activation via degradation of IkB, cells were treated with curcumin and subsequently proteasome activity was assessed. Decreases of NFkB activation in the presence of curcumin were not associated with a modulation of proteasome activity (figure 3A) demonstrating that the release of the functional subunits of the transcription factor was not affected by curcumin. The proteasome inhibitor MG132, (200nM) showed at least a 50% inhibition of NFkB activation compared to vehicle controls (supplemental fig 2). Incubation of H400 cells with *F. nucleatum* increased intracellular calcium concentration and this was further enhanced by pre-treatment with curcumin (figures 3B & C). As increases in intracellular calcium may increase CaMkinase II activity and subsequently inhibit histone deactelyase (HDAC) activity, a regulator of NFkB activity, we assessed the effect of curcumin on HDAC

activity. HDAC activity appeared to decrease but not significantly versus control (figure 3D). Trichostatin A was used as a positive control for inhibition of HDAC activity.

Curcumin induces activation of Nrf2, changes glutathione levels and increases HO-1 expression

Curcumin is a well-known inducer of the transcription factor Nrf2, thus we determined if, in the conditions shown to be anti-inflammatory, Nrf2 was induced in H400 cells. Nuclear fractionation and Nrf2-consensus sequence binding assays demonstrated an increase in Nrf2 localisation to the nucleus (figure 4A). Immunocytochemistry also confirmed an increase in the localisation of Nrf2 to the nucleus (figure 4B). Intracellular glutathione, the production of which is determined by the rate limiting enzyme gamma-glutamylcysteine synthetase, a protein controlled by Nrf2, decreased when measured at 8h but was increased in response to curcumin treatment when measured at 24h (figure 4C). This suggests an adaptive stress response from the cells. The decrease in glutathione content at 24h in vehicle control cells (0μM curcumin) is likely to be an effect of the ethanol vehicle [33]. We also demonstrated that HO-1, a protein also controlled by Nrf2, was increased significantly by curcumin incubation (figure 4D). The peak of induction was between 20 and 30μM, the same conditions that elicited anti-inflammatory protection and reduced GM-CSF production. Incubation of H400 cells with tetrahydrocurcumin (THC3) or ferulate (structures illustrated in figure 4E), did not elicit an increase in HO-1 at similar concentrations to those used for curcumin (figure 4D). This confirms that the α, β-unsaturated carbon within the central seven carbon chain (1,6-heptadiene-3,5-dione) are vital for curcumin's mechanism of action as previously described[34].

Effects of HO-1 induction on cytokine production

To determine if HO-1 plays a role in the inhibition of epithelial cytokine production in the presence of curcumin, HO-1 was induced by curcumin in the presence and absence of its inhibitor, Tin protoporphyrin (SnPPIX). Whilst a cytokine response was induced by *F. nucleatum* stimulation, the inhibitor had no effect on GM-CSF production either alone or in the presence of *F. nucleatum*, however it prevented the decrease in GM-CSF production exerted by pretreatment with curcumin (figure 5A) on *F. nucleatum* induced GM-CSF production. HO-1 degrades haem to CO, free iron and biliverdin. Iron can be sequestered by ferritin and biliverdin can be further converted to bilirubin via biliverdin reductase. To test whether CO may have anti-inflammatory effects H400 cells were incubated, without curcumin, with CO releasing molecule CORM2 (100μM)[35] (figure 5B) and challenged with *F. nucleatum*. CORM2 elicited a significant reduction in GM-CSF production in response to *F. nucleatum*.

Curcumin increases BVR expression at the cell membrane

As BVR is required for the conversion of biliverdin to bilirubin, we investigated whether there was an effect on the cellular localisation of BVR by curcumin and/or *F. nucleatum* stimulation. While *F. nucleatum* stimulation had no effect on BVR localisation, curcumin consistently increased the localisation of BVR at the plasma membrane (figure 6A&B). Figure 6 A shows that in the presence of curcumin there is potential for increased antibody staining seen at the membrane as illustrated by the white arrows. In addition, Western blotting was used to identify BVR in different subcellular fractions: quantification of the blots for optical density suggest that there is an increase in the quantity of BVR isolated in membrane fractions.

Discussion

Many chronic inflammatory non-communicable diseases (NCD) are associated with a dysregulated and exaggerated inflammatory response. Periodontitis is one such NCD and a model disease to study inflammatory pathways and their regulation in response to microbial challenge. Here we have employed gingival inflammation to explore the role of curcumin as a modulator of redox-regulated gene transcription factors that are known to control inflammation and its resolution. Periodontitis and gingivitis are characterised by a cytokine cascade and neutrophilic infiltration into the periodontal tissues, in response to bacterial challenge. The exaggerated neutrophil derived oxidative burst [36, 37] and subsequent oxidative stress is likely to be responsible for the tissue destruction prevalent in periodontitis [9,38].

We have demonstrated that sub-apoptotic concentrations of curcumin can decrease intracellular GSH (8h) thereby stimulating an stress response, and cytokine release, particularly GM-CSF, across a range of MOIs by oral epithelial cells, which form the first line of defence against periodontopathogens, such as *Fusobacterium nucleatum*. Curcumin has been used at similar concentrations (1-50µM) in other studies[39], using different cell lines, to demonstrate sub-apoptotic effects that are cytoprotective. This includes the use of 10µM curcumin to prevent GM-CSF production in lymphocytes exposed to dust mite *Dermatophagoides farinea*[40]. In the study presented here, the subsequent increase in GSH (24h) demonstrates the adaptative response by these epithelial cells to the curcumin which may assist in the reduction of GM-CSF production from curcumin pre-treated cells. The GM-CSF gene promoter region contains both NFkB and AP-1 binding motifs[41]. Within 1h of stimulation with *F. nucleatum* we demonstrated an increase in NFkB activity and relocation of the classical NFkB components p65 and p50 to the nucleus. The relocation of these components is dependent upon NFkB release from its inhibitor of kB (IkB), which is complexed in the cytosol. This complex is phosphorylated and subsequently ubiquitinated for targeted proteasomal degradation. Failure to degrade ubiquitinated IkB may prevent activation of the pathway. Proteasome levels are governed by oxidative stress through Nrf2 activation[42], however under our experimental conditions no change in proteasomal activity was seen suggesting that alternate pathways may exist to regulate nuclear NFkB activity; one such pathway is that of histone deacetylases (HDAC). We observed increases in calcium ion concentrations, which can in turn increase CaMKinase II activity and ultimately lead to phosphorylation and deactivation of HDACs. HDAC3 has been shown to deacetylate NFkB and lead to the transcription factor's nuclear export [43]. Here we show that pre-treatment with curcumin decreases HDAC activity in the H400 cell line, which may contribute to the decrease in NFkB induced GM-CSF production.

We did not observe an early increase in AP-1 components with the inflammatory stimulus alone. Pre-incubation of the epithelial cells with curcumin prior to *F. nucleatum* stimulation increased phosphorylated c-Jun alone. Studies have suggested that AP-1 may act as a negative regulator of inflammation [8,44] through c-Fos or Fra-1, but neither of these proteins were increased in the nucleus at 1h post inflammatory stimulation.

Morse et al [20] have shown that CO attenuates lipopolysaccharide (LPS) driven IL-6 production via reduced binding of AP-1 components JunB, JunD, and c-Fos in macrophages. Here we see no evidence for modulation of these AP-1 components in the nucleus at the time point employed (1hour). However, we were able to demonstrate that curcumin pre-treatment does increase nuclear Nrf2 and downstream Nrf2 targets glutathione and haem oxygenase 1 (HO-1). Tetrahydrocurcumin (THC3) or ferulate had no effect demonstrating that the α,β-unsaturated carbon within the central seven carbon chain (1,6-heptadiene-3,5-dione) is vital for curcumin's mechanism of action in this system. Pae et al [45] have also shown that THC3 does not induce HO-1, in rat smooth muscle cells. Interestingly, demethoxycurcumin and bisdemethoxycurcumin, two other turmeric derived curcuminoids retaining the 1,6-heptadiene-3,5-dione structure but with modified methoxy groups, preserve both NFkB inhibitory capacities[46] and HO-1 stimulating properties[47].

HO-1 is a stress response protein that catalyses the breakdown of haem to iron, CO and biliverdin. It is inhibited by metallic protoporphyrins such as SnPPIX [15]. Here we show that the effect of curcumin pre-treatment on *F. nucleatum* induced GM-CSF production is abrogated by use of this inhibitor, indicating that HO-1 production is linked to the anti-inflammatory effects of curcumin within H400 cells. The HO-1 product iron is rapidly sequestered by ferritin [18], however, CO and biliverdin may be involved in the anti-inflammatory effects of HO-1. By using the CO releasing molecule 2 (CORM2), biliverdin and bilirubin alone we attempted to tease out the role of these molecules. Whilst CORM was the only molecule to significantly reduce *F. nucleatum* induced GM-CSF production, both biliverdin and bilirubin had some effect independently. The overarching interaction of these molecules is outlined in figure 7.

CO has previously been shown to decrease proinflammatory cytokine production [19,45] and also to increase intracellular calcium concentrations [22], which in turn may play a role in the regulation of NFkB via HDAC activation and nuclear export of NFkB. In addition, CO is a ubiquitous vascular relaxant and decreases leukocyte rolling, adhesion and neutrophil migration into inflammatory sites [48,49]. Whilst

these properties could not be tested here they may also contribute to the benefits of localized CO production *in situ* within the gingiva.

BVR is a pleiotropic enzyme, exhibiting a catalytic function as well as being a dual specificity kinase [50]. This latter property can turn extracellular stimuli into cellular responses. Here we show that curcumin stimulates the movement of BVR from the cytoplasm to the plasma membrane. This relocation has been reported by Wegiel et al [51] in macrophages in response to LPS but not previously after curcumin stimulation. BVR has also been shown to activate c-Jun, perhaps aiding in the repression of GM-CSF promoters[52]. In addition, Wang et al[53] and Kim et al[54] found that BVR and HO-1 localise to caveolae, caveolin-1 rich membrane domains, where bilirubin may be externalised and mediate extracellular anti-inflammatory and/or anti-oxidant properties. Bilirubin, the product of BVR, can protect cells against almost 10,000-fold higher concentrations of hydrogen peroxide[55] due to the cycling action of biliverdin reductase. Interestingly systemic bilirubin shows an inverse relationship to the prevalence of periodontitis [56].

Our study included the use of a cell line derived from an oral squamous cell carcinoma: H400. This cell line has been used extensively to explore the interactions between oral bacteria and oral epithelial cells. We have demonstrated the same molecular pathways are activated in H400 cells as primary oral epithelial cells[6,57]. The use of the H400 cell line allows high throughput characterisation of cell responses, such as transcriptional and translational events in response to stimulation, prior to future confirmatory studies on primary cells. As noted by the review by Bierbaumer et al [58] there as yet no gold standard cell culture models of oral epithelium.

Curcumin is a pleiotropic molecule, which may affect many different epithelial cell pathways to decrease the cellular effects of inflammatory stimuli, such as *F. nucleatum* and other periodontal pathogens. Although in many systems curcumin will be rapidly metabolised to the less active form THC3, in the oral cavity it may have a role to play in reducing the exaggerated inflammatory burden evident within periodontal diseases, via topical application to gingival tissues. Clinical trials in this area have begun[59] and a meta-analysis of the use of curcumin as a nonsurgical periodontal treatment has reported that curcumin can decrease gingival index, sulcus bleeding index but not bleeding on probing[60]. In conclusion, we have shown that curcumin can decrease inflammatory markers produced by an oral epithelial cell line in response to challenge by *F. nucleatum*. The mechanism by which this occurs appears to be driven by the induction of NrF2 responses. We have demonstrated that HO-1 and the production of this enzymes product carbon monoxide are key players. There is a hint that the role of biliverdin reductase may also be important but more research on this is required.

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Figure legends

Figure 1 A. Cell viability: H400 cells were incubated for 8h with curcumin at the concentrations shown and the ATP content measured using Cell Titre Glo. B. Caspase activity: H400 cells were incubated for 8h with curcumin at the concentrations shown and the caspase3/7 activity measured using Caspase Glo. C. GM-CSF production: H400 cells were incubated for 8h with curcumin (20µM) and then immediately challenged, with heat-killed *F. nucleatum* at the MOIs indicated. Media was recovered and cytokine and chemokine concentration measured by ELISA. For all experiments n=3, mean +/- SEM shown. *p<0.05 **p<0.01

Figure 2 A. NFkB reporter assay: pGL4.22 3enh stably transfected H400 cells were incubated with and without curcumin (20µM) for 8h and then challenged, with heat-killed *F. nucleatum* at the MOI indicated for 3h. Luminescence from generated luciferase was measured using One Glo B. Nuclear NFkB location: H400 cells were incubated for 8h with curcumin (20µM) and then challenged, with heat-killed *F. nucleatum* for 1h MOI 100:1, nuclear extracts were made and NFkB family members were quantified using Trans AM TF ELISA. C. Nuclear AP-1 location: H400 cells were incubated for 8h with curcumin (20µM) and then challenged, with heat-killed *F. nucleatum* for 1h, MOI 100:1, nuclear extracts were made and AP-1 family members were quantified using Trans AM TF ELISA. For all experiments n=3, mean +/- SEM shown. **p<0.01, *p<0.05

Figure 3 A. Proteasome activity: H400 cells were incubated for 8h with curcumin at the concentrations shown and the proteasome activity measured using Proteasome-Glo-3 substrate system. B (time course) & C (maximum at 1 min post heat-killed *F. nucleatum* stimulation). Calcium flux: H400 cells were incubated for 8h with curcumin (20µM) and then challenged, with heat-killed *F. nucleatum*, MOI 100:1, calcium ion flux was measured immediately using Fluoforte. D. HDAC activity: H400 cells were incubated for 8h with curcumin (20µM) and HDAC activity measured using HDAC-Glo. For all experiments n=3, mean +/- SEM shown. ****p<0.0001

Figure 4 A. NrF2 location: H400 cells were incubated for 2h with curcumin (20µM) nuclear extracts were made and Nrf2 was quantified using Trans AM TF ELISA. B H400 cells were incubated for 2h with curcumin (20µM) and then fixed, permeabilised and stained for Nrf2 (red) and the nucleus (Hoescht, blue), see methods for details. Representative images are shown. C GSH content: H400 cells were incubated for 8h or 24h with curcumin at the concentrations shown; GSH was measured using GSH-Glo. D. HO-1 content: H400 cells were incubated for 8h with curcumin, tetrahydrocurcumin 3 (THC3) and ferulic acid, at the concentrations shown, cells were harvested and the protein content and HO-1

content were determined by BCA assay and HO-1 ELISA respectively. E. Structure of curcumin, tetrahydrocurcumin and ferulic acid. For all experiments n=3, mean +/- SEM shown. *p<0.01, **p<0.05

Figure 5 A. GM-CSF production: H400 cells were incubated for 8h with curcumin (20μM) in the presence or absence of SnPPIX (20μM, HO-1 inhibitor) and then challenged, with heat-killed *F. nucleatum* for 16h MOI 100:1. Media was recovered and GM-CSF concentration measured by ELISA. B. GM-CSF production: H400 cells were incubated for 8h with CORM2 (CO releasing molecule 100μM) and then challenged, with heat-killed *F. nucleatum* for 16h MOI 100:1. Media was recovered and GM-CSF concentration measured by ELISA. For all experiments n=3, mean +/- SEM shown. *p<0.01, ***p<0.001

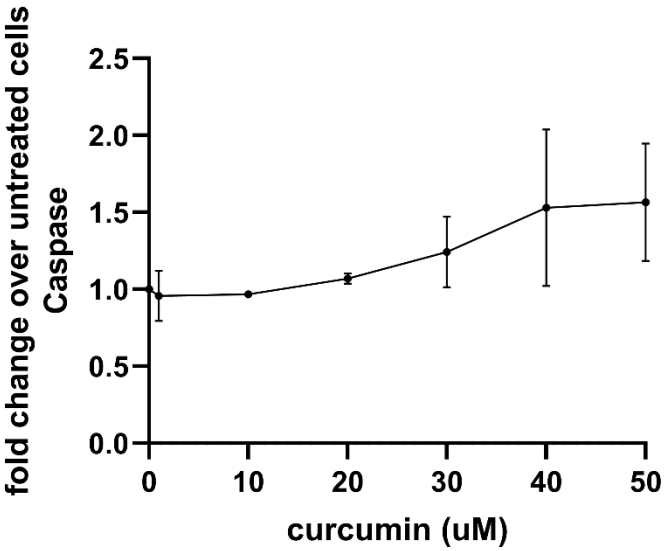
Figure 6 A. H400 cells were incubated for 8h with curcumin (20μM) and then challenged, with heat-killed *F. nucleatum* for 16h MOI 100:1. Cells were fixed, permeabilised and stained for BVR. Representative images are shown. B. H400 cells were incubated for 8h with curcumin (20μM) and then cellular compartments were prepared. Proteins were separated by SDS-PAGE and electroblotted. E-cadherin and BVR were visualised using Licor Odyssey reader. Representative blot (B) shown alongside quantification of the membrane (C) associated BVR band in the two conditions for 3 experiments. WCL: whole cell lysate. For all experiments n=3, mean +/- SEM shown.

Figure 7. Summary of curcumin preconditioning on H400 cells stimulated with *Fusobacterium nucleatum* (heat killed). (1) Under curcumin pre-conditioning Nrf2 is released from Keap1 and translocates to the nucleus nucleus; (2) Nrf2 initiates transcription of genes in the Antioxidant Response Element (ARE), e.g. HO-1 and gamma-glutamyl synthetase preceding GSH production; (3) Production of HO-1; which (4) catalyses haem into biliverdin, iron (Fe^{2+}) and carbon monoxide (CO); (5) CO inhibits the NF-κB pathway, leading to decreased production of inflammatory cytokines; (6) *Fusobacterium nucleatum* (heat killed in these experiments) stimulates NFkB translocation; and (7) production of inflammatory cytokines and chemokines; (8) curcumin induces translocation of some

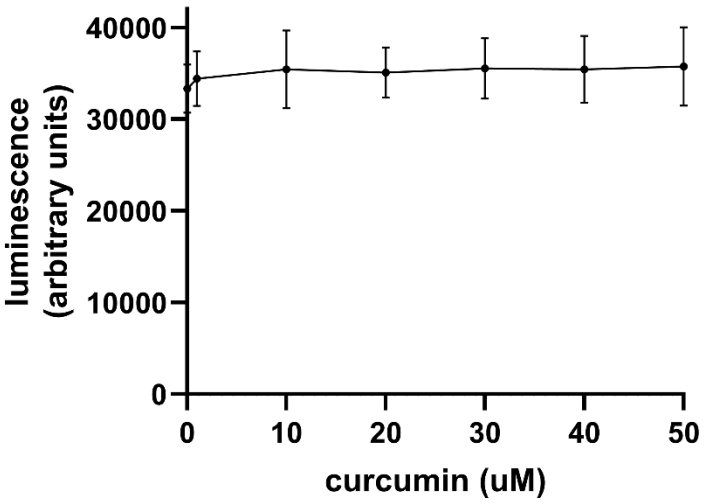
BVR from the cytosol to the membrane with potential to increase extracellular antioxidant power through release of bilirubin.

Figure 1

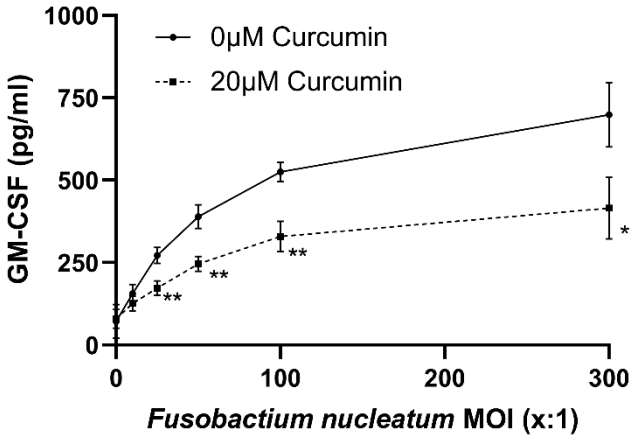
A


fold change over untreated cells
Caspase
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
curcumin (uM)

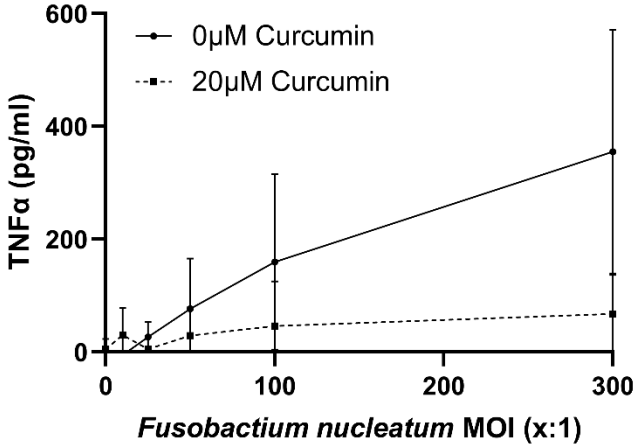
B


luminescence
(arbitrary units)
40000
30000
20000
10000
0
0
10
20
30
40
50
curcumin (uM)

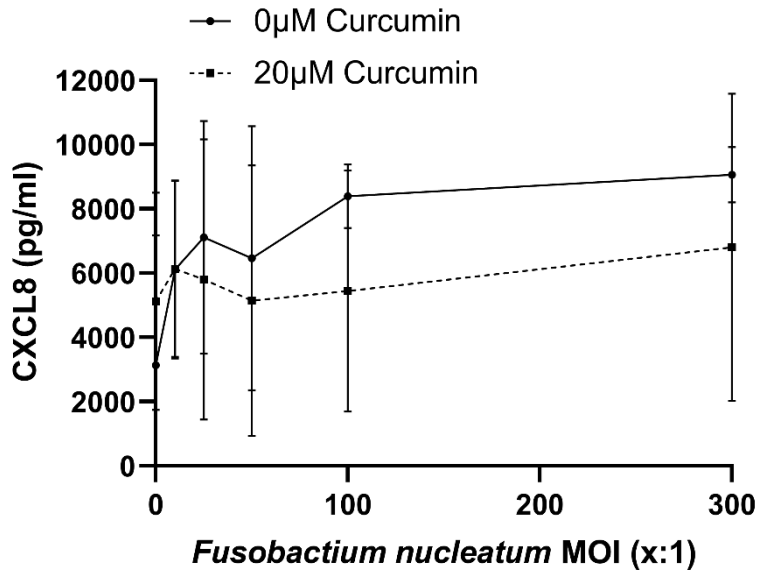
C


1000
750
500
250
0
GM-CSF (pg/ml)
0μM Curcumin
20μM Curcumin
**
**
**
*
0
100
200
300
Fusobactium nucleatum MOI (x:1)

D


600
400
200
0
TNFα (pg/ml)
0μM Curcumin
20μM Curcumin
0
100
200
300
Fusobactium nucleatum MOI (x:1)

E


0μM Curcumin
20μM Curcumin
CXCL8 (pg/ml)
12000
10000
8000
6000
4000
2000
0
0
100
200
300
Fusobactium nucleatum MOI (x:1)

F

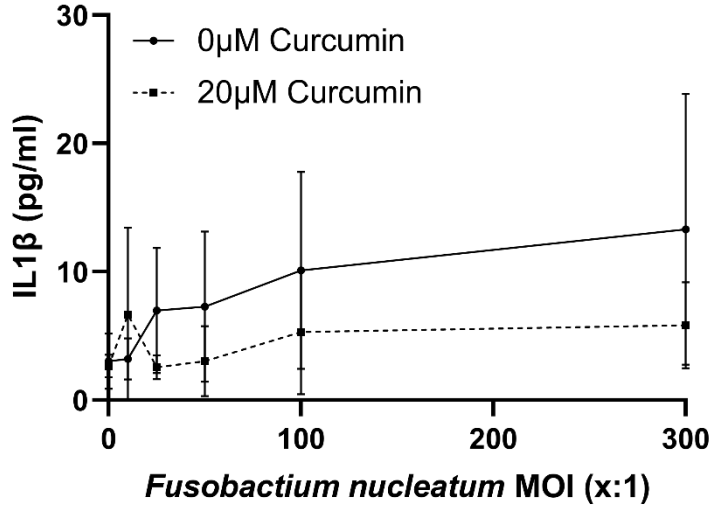
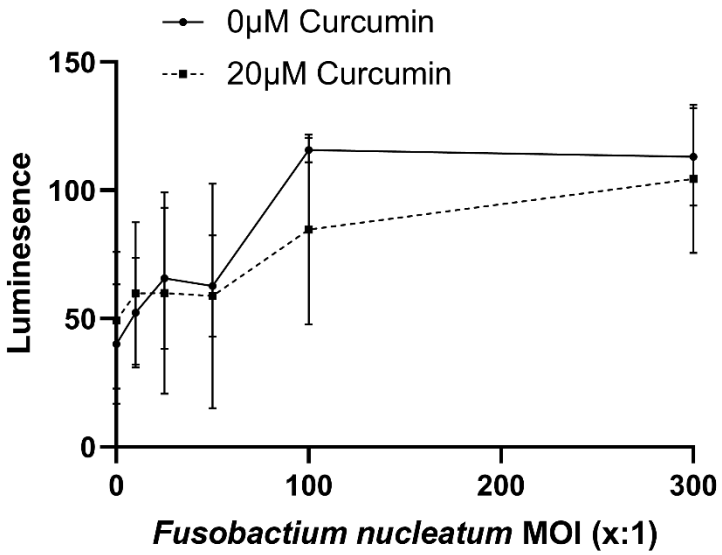
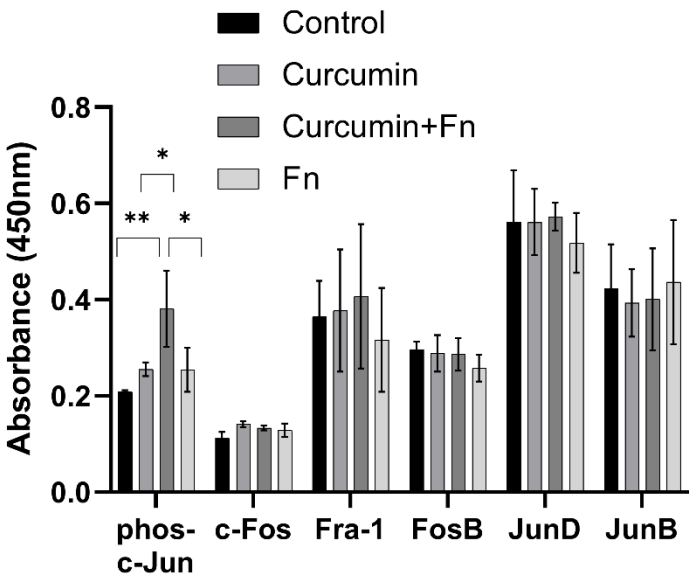

0μM Curcumin
20μM Curcumin
IL1β (pg/ml)
30
20
10
0
0
100
200
300
Fusobactium nucleatum MOI (x:1)

Figure 2

A


0μM Curcumin
20μM Curcumin
Luminesence
150
100
50
0
0
100
200
300
Fusobactium nucleatum MOI (x:1)

B


Control
Curcumin
Curcumin+Fn
Fn
Absorbance (450nm)
0.8
0.6
0.4
0.2
0.0
**
*
*
phos-
c-Jun
c-Fos
Fra-1
FosB
JunD
JunB

C

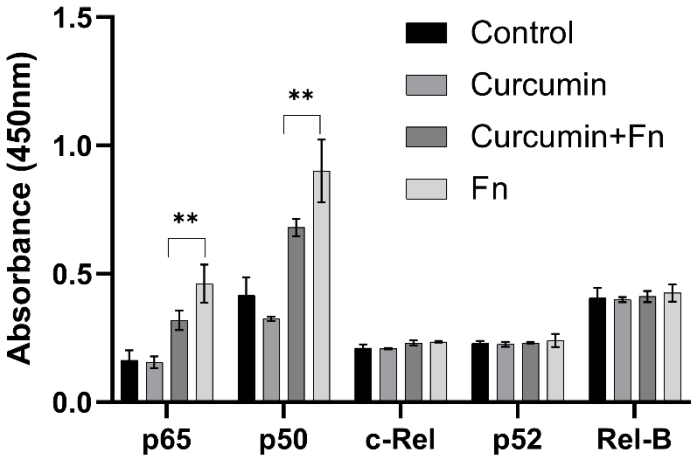
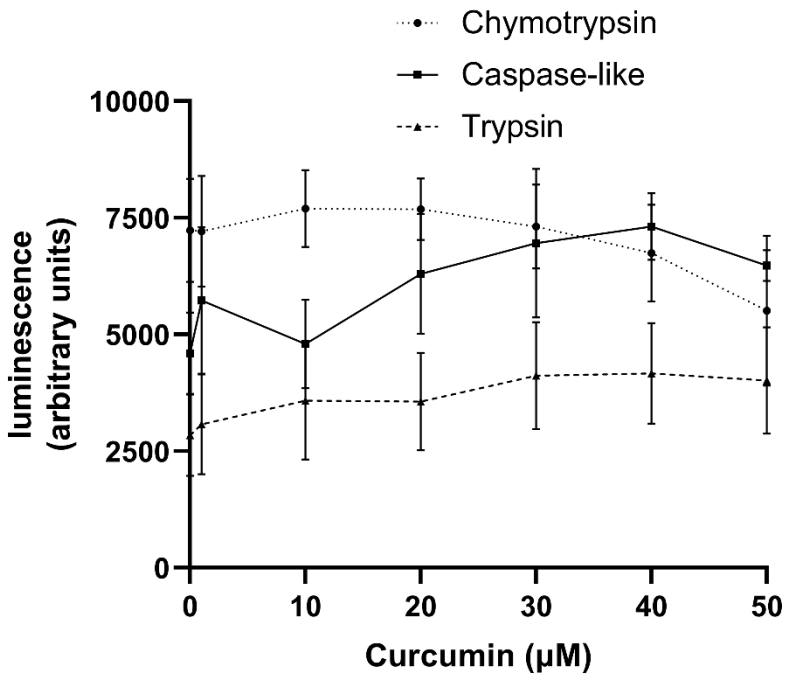
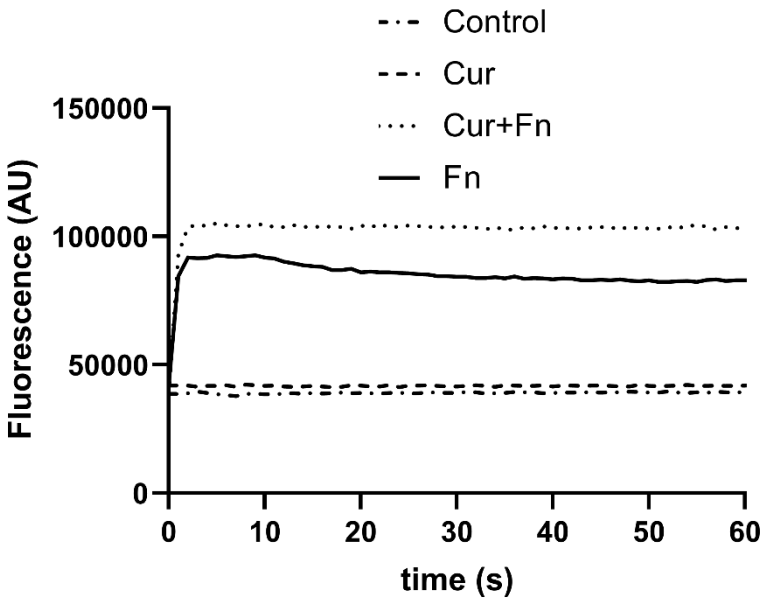

Absorbance (450nm)
1.5
1.0
0.5
0.0
Control
Curcumin
Curcumin+Fn
Fn
**
**
p65
p50
c-Rel
p52
Rel-B

Figure 3

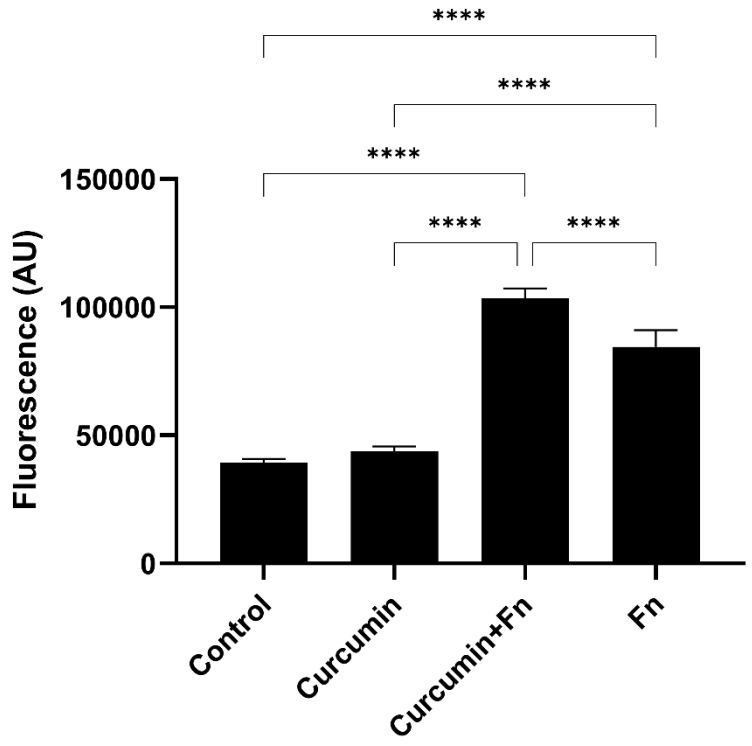
A


Chymotrypsin
Caspase-like
Trypsin
luminescence (arbitrary units)
10000
7500
5000
2500
0
0
10
20
30
40
50
Curcumin (μM)

B


Control
Cur
Cur+Fn
Fn
Fluorescence (AU)
150000
100000
50000
0
0
10
20
30
40
50
60
time (s)

C


****
****
****
****
****
150000
100000
50000
0
Fluorescence (AU)
Control
Curcumin
Curcumin+Fn
Fn

D

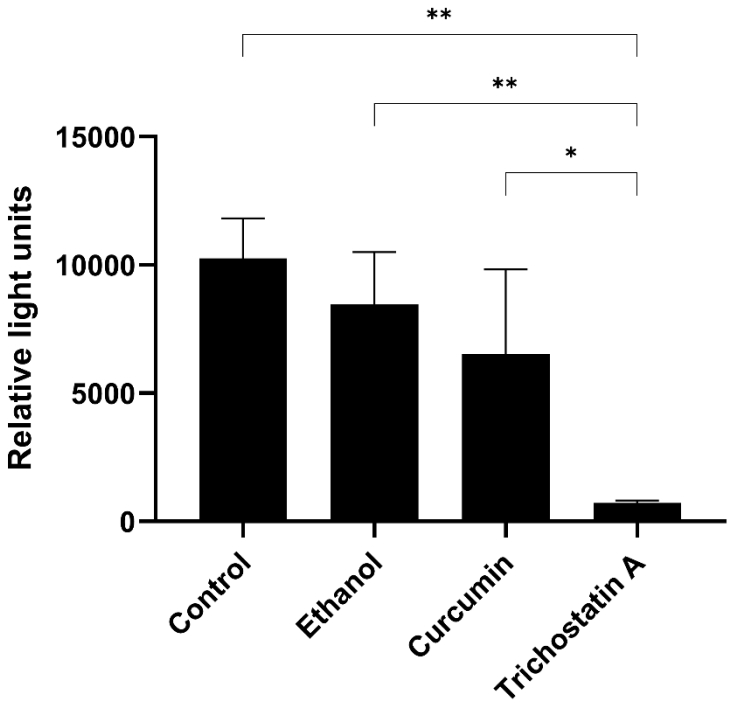
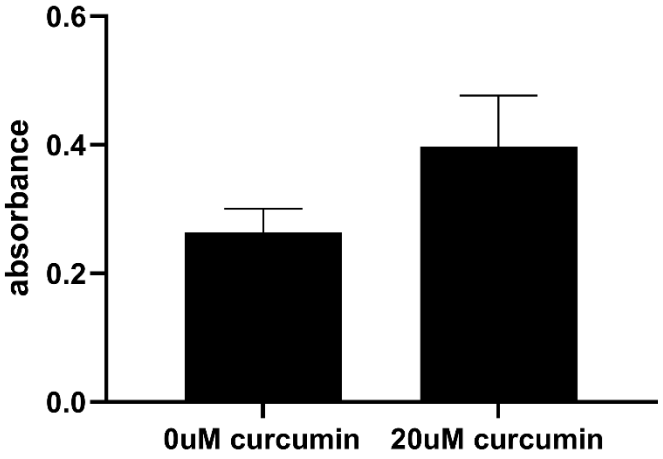
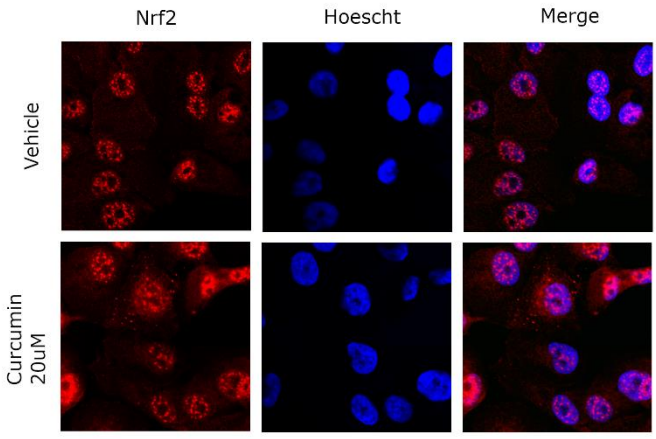

**
**
*
15000
10000
5000
0
Relative light units
Control
Ethanol
Curcumin
Trichostatin A

Figure 4

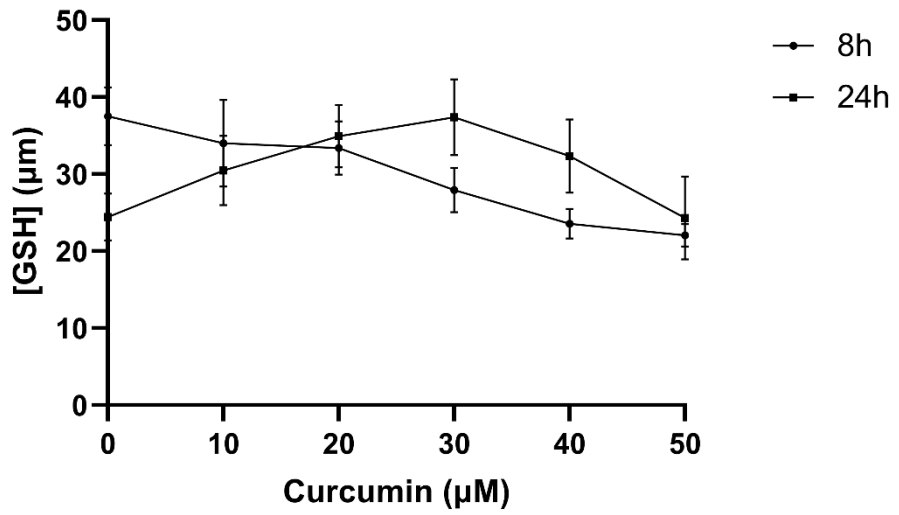
A


0.6
0.4
0.2
0.0
absorbance
0uM curcumin
20uM curcumin

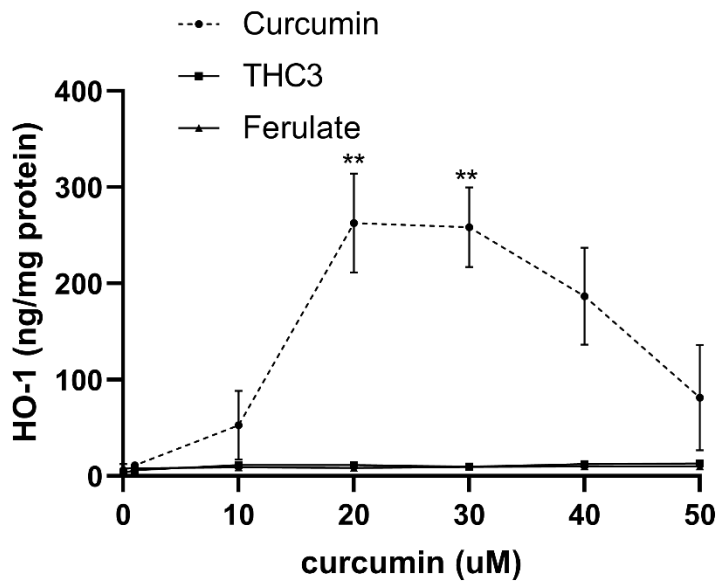
B


Nrf2
Hoescht
Merge
Vehicle
Curcumin 20uM

C

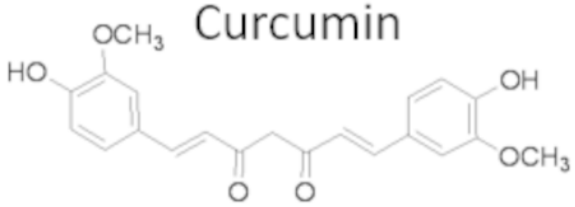

8h
24h
[GSH] (µm)
Curcumin (µM)
0
10
20
30
40
50

D

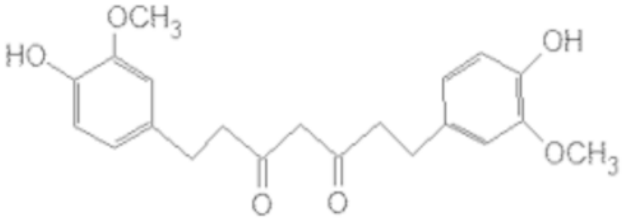

Curcumin
THC3
Ferulate
**
**
HO-1 (ng/mg protein)
curcumin (uM)
0
100
200
300
400
0
10
20
30
40
50

E

Curcumin



Tetrahydrocurcumin



Ferulic acid

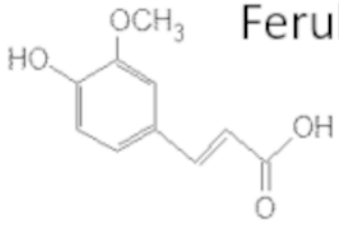
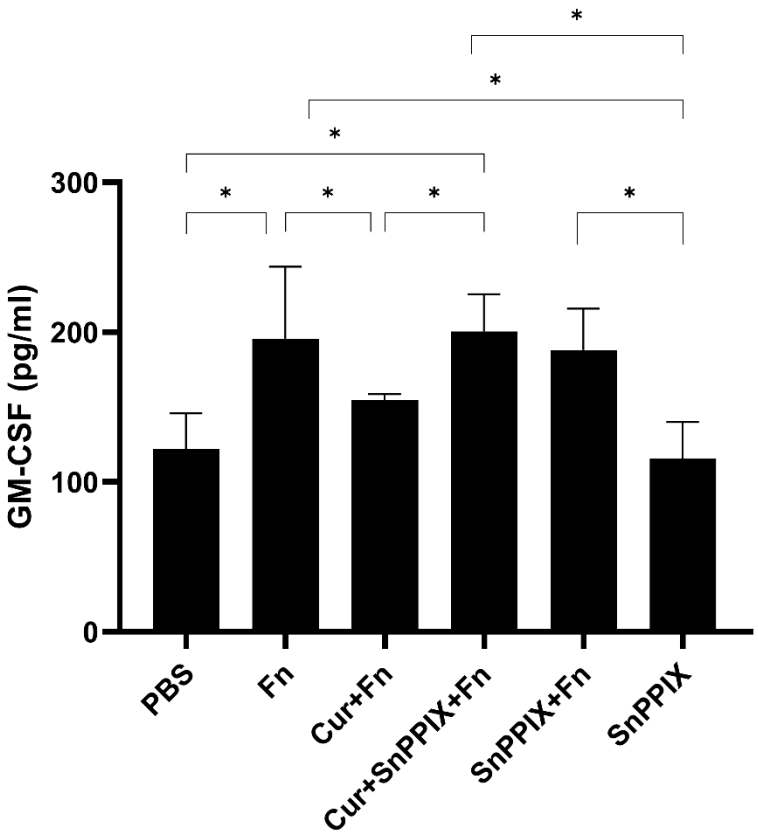


Figure 5

A


GM-CSF (pg/ml)
300
200
100
0
*
*
*
*
*
*
PBS
Fn
Cur+Fn
Cur+SnPPIX+Fn
SnPPIX+Fn
SnPPIX

B

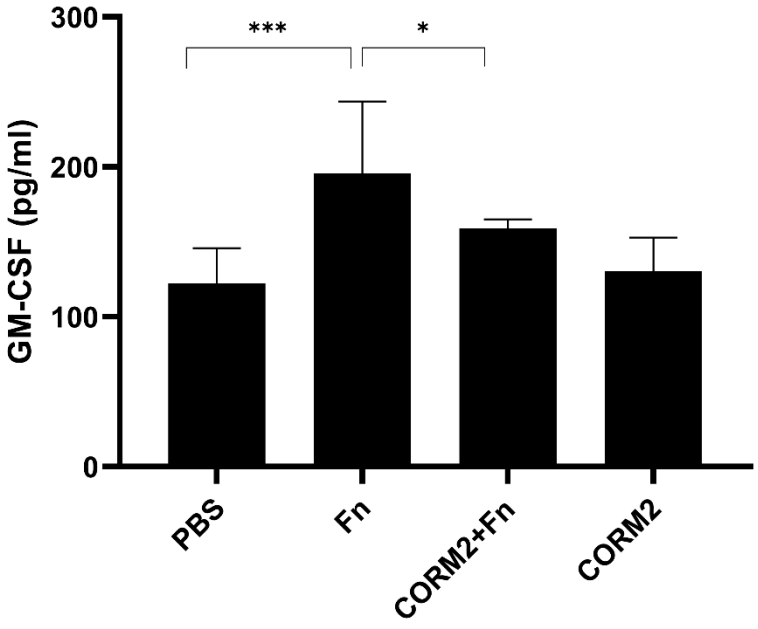
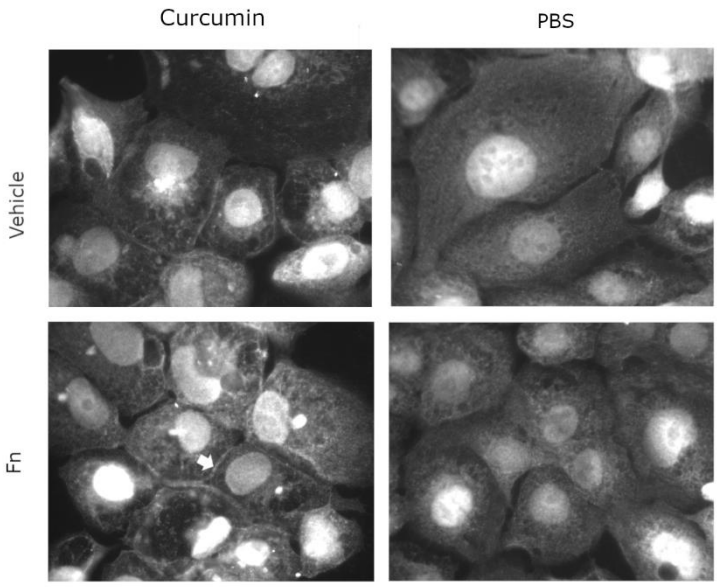
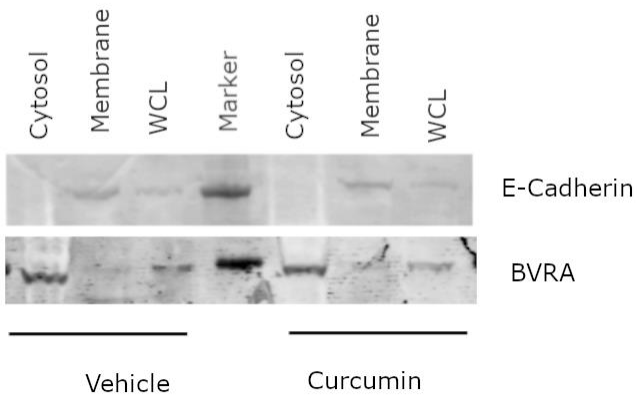

300
200
100
0
GM-CSF (pg/ml)
***
*
PBS
Fn
CORM2+Fn
CORM2

Figure 6

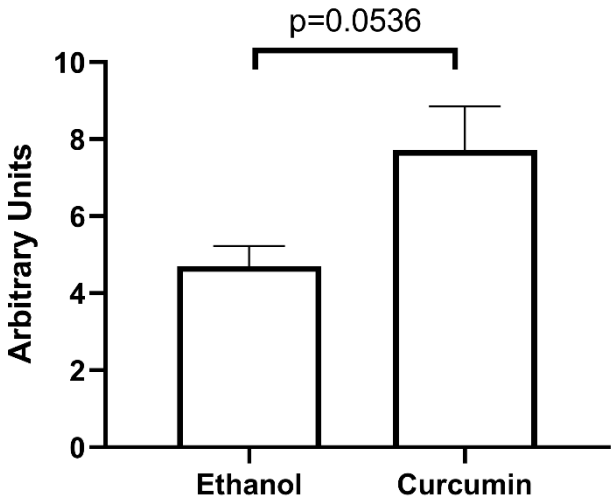
A


Curcumin
PBS
Vehicle
Fn

B


Cytosol
Membrane
WCL
Marker
Cytosol
Membrane
WCL
E-Cadherin
BVRA
Vehicle
Curcumin

C



Supplemental information

Figure 1 *F.nucleatum* stimulated cytokine production by H400 cells. H400 epithelial cells were stimulated with *F. nucleatum* (MOI 300:1) for a range of times. Cytokines were detected by ELISA

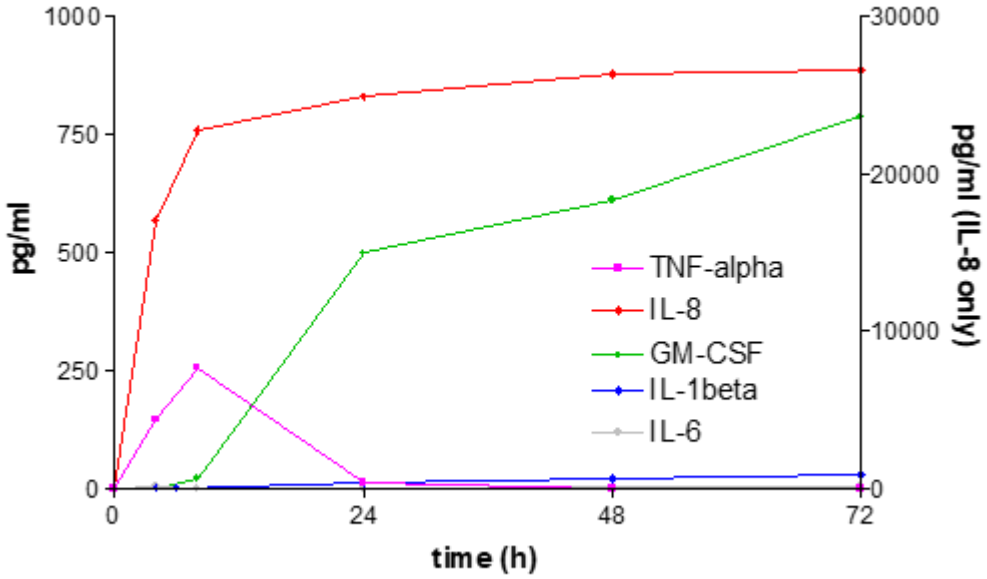


Figure 2 Proteasome activity as inhibited by proteasome inhibitor MG132: H400 cells were incubated with MG132 (200nM) and the proteasome activity measured using Proteasome-Glo-3 substrate system as described in the main methods section.