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## Repeated exposure to *Porphyromonas gingivalis* LPS during macrophage polarisation leads to diminished inflammatory cytokine production

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### Abstract

**Objective:** The objective of the present study was to determine the effects of concurrent LPS and cytokine priming, reflective of the *in vivo* milieu, on macrophage production of key periodontitis associated cytokines TNF, IL-1 $\beta$  and IL-6.

**Design:** THP-1 cells were pre-treated with combinations of *Porphyromonas gingivalis* and *Escherichia coli* lipopolysaccharide (LPS), concurrently with polarising cytokines IFN $\gamma$  and IL-4, or PMA as a non-polarised control. Production of key periodontitis associated cytokines in response to subsequent LPS challenge were measured by enzyme – linked immunosorbent assay.

**Results:** Compared with cells incubated with IFN $\gamma$  or IL-4 alone in the “polarisation” phase, macrophages that were incubated with LPS during the first 24 h displayed a down-regulation of TNF and IL-1 $\beta$  production upon secondary LPS treatment in the “activation” phase. In all three macrophage populations (M0, M1 and M2), pre-treatment with *P. gingivalis* LPS during the polarisation process led to a significant decrease in TNF production in response to subsequent activation by LPS ( $p = 0.007$ ,  $p = 0.002$  and  $p = 0.004$ , respectively). Pre-treatment with *E. coli* LPS also led to a significant down-regulation in TNF production in all three macrophage populations ( $p < 0.001$ ). Furthermore, the presence of *E. coli* LPS during polarisation also led to the down-regulation of IL-1 $\beta$  in the M1 population ( $p < 0.001$ ), whereas there was no measurable effect on IL-1 $\beta$  production in M0 or M2 macrophages. There was no significant effect on IL-6 production.

**Conclusions:** Macrophages become refractory to further LPS challenge, whereby production of key periodontitis associated cytokines TNF and IL-1 $\beta$  is reduced after exposure to LPS during the polarisation phase, even in the presence of inflammatory polarising cytokines. This diminished cytokine response may lead to the reduced ability to clear infection and transition to chronic inflammation seen in periodontitis.

### Key words

Macrophage, *Porphyromonas gingivalis*, periodontitis, cytokine, LPS

## Introduction

In healthy oral tissues, there is a fine balance in the interplay between host immune responses and resident microbes. When a shift occurs in the microbial community, or there is a dysfunctional immune response, homeostasis is disrupted and disease ensues. Periodontitis is a chronic inflammatory disease characterised by loss of periodontal ligament attachment to the tooth surface and irreversible, osteoclast driven alveolar bone resorption. The aetiology of periodontal disease is complex and incompletely understood. It has been established, however, that the presence of a subgingival dental plaque biofilm, concurrent with a susceptible host immune response, are required for progression of gingivitis into periodontitis [1]. Several “keystone” pathogens have been implicated in the initiation of periodontitis, including *Porphyromonas gingivalis*, among others [1].

Bacteria in the gingival sulcus are protected from mechanical removal by tooth brushing, meaning that tissues of the periodontium are constantly exposed to bacteria and their associated antigens. Bacterial stimulation of host tissues is met with a retaliatory host inflammatory response. When inflammation is initiated, monocytes are recruited from the circulation into the tissues, where they differentiate into macrophages [2-4], directed by membrane bound and soluble factors that are encountered in their microenvironment. Dependant on these factors, macrophages will polarise within a spectrum of functional phenotypes, from pro-inflammatory (classically activated or M1) to anti-inflammatory, or regulatory (alternatively activated or M2) effector cells [5-8, 9[Murray, 2014 #1313, 10[Murray, 2014 #1313]. Classically activated macrophages are polarized by granulocyte macrophage-colony stimulating factor (GM-CSF) and IFN $\gamma$ , whilst alternatively activated macrophages (M2), can be polarized by M-CSF, IL-4, IL-13, IL-10 and immune complexes [5, 6, 11, 12].

Via the production of cytokines and chemokines, macrophages can orchestrate innate and adaptive immune responses in an attempt to control infection and repair damaged tissue. M1 type macrophages express high levels of pro-inflammatory cytokines, including TNF, IL-1 $\beta$ , and IL-6, which are attributed to inflammation and tissue destruction seen in periodontal disease [9, 13-15]. M2 type macrophages exhibit anti-inflammatory properties and express the regulatory cytokines, IL-10 and TGF- $\beta$ , which negatively regulate M1 activity and contribute to the wound healing process. Both M1 and M2 macrophages are likely to be present in periodontal lesions [16]. Aberrant macrophage function has been implicated in the breakdown of immune tolerance [2, 17] and progression into chronic inflammation. Thus, periodontal disease is the manifestation of a dysregulated immune response, which fails to clear infection by periodontal pathogenic bacteria: A robust inflammatory response is need to clear infection, but is lacking in patients who succumb to periodontal disease. Various mechanisms have been proposed for this lack of robust response, including endotoxin tolerance. Endotoxin tolerance occurs when monocytes or macrophages are repeatedly exposed to bacterial products, such as

LPS. It has been shown previously that monocytes and macrophages can become tolerant to LPS from periodontal pathogen, *P. gingivalis*.

*P. gingivalis* has emerged as a popular periodontal research target because of its interesting immunomodulatory properties and implications as a keystone pathogen. The bacterium has a unique LPS structure and induces cytokine responses in a different way to that of the prototypical LPS from *Escherichia coli* [18-20], likely due to the different lipid A structures between these bacteria [18, 21]. Previous studies have examined the ability of periodontal pathogens to induce endotoxin tolerance in monocytes (if an infiltrating monocyte encounters LPS *before* any maturation / polarisation factors in an inflammatory lesion), or the effects of endotoxin tolerance on already polarised macrophages (encountering LPS *after* becoming polarised) [22-26]. However, *in vivo*, infiltrating monocytes are likely to encounter both LPS *and* polarising cytokines at the same time, rather than encountering first one factor then the other in a linear fashion. No studies to date have described what effect this would have on the resulting polarised macrophages ability to produce the robust inflammatory response required to clear the infection. Thus, the present study aimed to examine the effects of concomitant exposure of monocytes to LPS and cytokines during the macrophage polarisation process on resulting macrophage production of key periodontitis associated cytokines (TNF, IL-1 $\beta$  and IL-6).

## Methods

### General cell culture

THP-1 cells, a human pro-monocytic cell line derived from peripheral blood monocytic leukaemia (ECACC 88081201), were maintained in RPMI 1640 (Lonza, Slough, UK) supplemented with 10% foetal bovine serum (LabTech, UK) and 1% L-glutamine (Lonza, UK) in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Cells were initially seeded at 4 x 10<sup>5</sup> cells / mL, routinely sub-cultured using 1:4 split ratios and maintained for no more than 10 passages.

### Generation of M1- and M2- like THP-1 macrophages

The methodology was adapted from Tjiu *et al* [27]. 5 x 10<sup>5</sup> THP-1 were seeded in tissue culture plates and incubated with 5 ng/ml PMA for 6 h. Next, all the culture media was removed, including any non-adhered cells and was replaced with fresh media containing either (a) 5 ng/ml PMA, (b) 20 ng/ml IFN $\gamma$  + 5 ng/ml PMA (c) 20 ng/ml IL-4 + 5 ng/ml PMA for a further 18 h to generate unpolarised, M1-like and M2-like macrophages, respectively. To measure cytokine responses of polarised THP-1 cells to stimulation with Ultra-pure *P. gingivalis* LPS: a penta- acyltated, di-phosphorylated lipid A isoform [17] (InvivoGen, CA), or *E. coli* K12 (InvivoGen, CA) LPS, cells were polarised according to the aforementioned protocol and stimulated with 1 $\mu$ g/mL LPS. Cells incubated in media alone served as negative controls. Upon

completion of the incubation period, cell free supernatants were harvested and stored at -20°C until assay for cytokines by Enzyme Linked Immunosorbent Assay (ELISA).

To measure the effects of LPS present in the polarising conditions, cells were cultured in the presence or absence of 1 µg/ml *P. gingivalis* or *E. coli* LPS, either alone or in concert with polarising cytokines. Following polarisation, culture medium was removed and cells were washed 3 times in PBS. Fresh media was added containing either 1 µg/ml *P. gingivalis* or *E. coli* LPS to activate the macrophages. Media alone (no LPS) served as a negative control. After 24 h incubation, cell free supernatants were harvested and stored at -20°C until assay for TNF, IL-1β and IL-6 by ELISA.

### Enzyme Linked Immunosorbent Assay (ELISA)

Paired antibody ELISAs (R&D, UK) were used to determine protein expression levels of TNF, IL-1β and IL-6 in cell free supernatants, according to manufacturer guidelines. Absorbance was measured at 450nm in a Versa Max microplate reader (Molecular Devices, UK). Standard curve analysis was performed using SoftMax pro software and unknown concentrations of cytokines in the samples were calculated from the standard curve.

### Quantitative real time PCR

Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (as previously described [28]). RNA quantity was determined using NanoVue plus (GE Healthcare) spectrophotometer and purity was estimated using the A260/A280 ratio. cDNA was generated by reverse transcription reaction (Veriti, Applied Biosystems). Real-time PCR was performed using Power SYBR green PCR master-mix (Applied Biosystems). Test genes IL-10 (*IL10*) and TGF-β (*TGFB1*) were normalised against housekeeping gene, β-actin (*ACTB*). Primers were designed using Applied Biosystems software and purchased from Eurofins MWG Operon (UK) as illustrated in Table 1.

Table 1: Primer sequences.

Gene	Forward	Reverse
IL10	5'- CAAACCAAACCAAGACAGACT - 3'	R: 3'- CAGGAGGACCAGGCAACAGA - 5'
<i>TGFB1</i>	5'- AGTTCAAGCAGAGTACACACAGCAT -3'	3'- AGAGCAACACGGGTTTCAGGTA -5'
<i>ACTB</i>	5'- ATTGCCGACAGGATGCAGAA -3'	3'- CTGATCCACATCTGCTGGAA - 5'

Data was acquired and analysed on the Applied Biosystems StepOne software (Applied Biosciences, UK). Human  $\beta$  actin 1 was used as the internal reference gene (housekeeping gene), and non-polarised (PMA treated) macrophages were used as calibrator samples. Differences in gene expression levels were calculated relative to the calibrator sample using the  $\Delta\Delta$  Ct method.

### **Statistical analysis**

For comparison between two independent treatment groups, Student's t-test (if the data were parametric) or Mann Whitney U test (if data were non-parametric) were used. In experiments where more than two treatment groups tested, a one-way analysis of variance (if the data were parametric) with the Holm-Sidak method for all pairwise comparison procedure, or the Kruskal-Wallis analysis of variance (if the data was non-parametric) were used to test for differences between groups. Results were considered significant if the p value was  $\leq 0.005$ .

## **Results**

### **M1 and M2 macrophages exhibit differential inflammatory cytokine responses to *P. gingivalis* LPS**

In the present study, THP-1 cells were treated with PMA for 6 h to differentiate them from pro-monocytes into adherent macrophage-like cells. M1- and M2- like macrophage populations were generated by treating the cells for a further 18 h with PMA and IFN $\gamma$  (M1) or IL-4 (M2), respectively (method adapted from [29]).

To measure the effects of *P. gingivalis* LPS on inflammatory cytokine production, cells were treated with 1 $\mu$ g/mL *P. gingivalis* LPS for 24 h. M1- like macrophages exhibited significantly higher levels of TNF, IL-1 $\beta$  and IL-6 production than the M2- like population (figure 1a), whereas M2 cells expressed higher levels of IL-10 and TGF- $\beta$  mRNA than M1 (figure 1b). Basal levels of both IL-10 and TGF-  $\beta$  mRNA however were similar between M1 and M2 macrophages (figure 1b). Cells treated with IFN or IL-4 exhibited cytokine profiles typical of M1 and M2 like macrophages, respectively. As previously described <sup>197</sup>, TNF, IL-1 $\beta$  and IL-6 production was up-regulated to a greater extent by *E. coli* LPS than *P. gingivalis* LPS. This was evident in all macrophage polarisation states (figure 1a).

### **Inflammatory cytokine production by M0, M1 and M2 macrophages is modulated by the presence of *P. gingivalis* during polarisation**

The present study revealed differential inflammatory cytokine production between polarised M1- and M2- like macrophages in response to *P. gingivalis* LPS. To reflect *in vivo* conditions, where naïve monocytes are recruited from bone marrow to the

site of inflammation, the next experiments set out to see if this pattern of polarisation and inflammatory cytokine production could be modulated by exposure to LPS before the macrophages had polarised to either subset. To test this, macrophages were polarised as above, in the presence or absence of 1µg/mL *P. gingivalis* LPS. A third population of “unpolarised (M0)” macrophages were generated by treatment with PMA only, to test whether LPS alone had polarising properties. Following polarisation, culture media were removed and cells were washed 3 x in PBS. To stimulate inflammatory cytokine production, the cells were treated again with 1ug/mL *P. gingivalis* LPS for 24 h. Cell free supernatants were collected and assayed for key periodontitis and M1 macrophage associated cytokines TNF, IL-1β and IL-6 by ELISA.

In all three macrophage populations (M0, M1 and M2), pre-treatment with *P. gingivalis* LPS during the polarisation process led to a significant decrease in TNF production in response to subsequent activation by *P. gingivalis* LPS (Figure 2.  $p = 0.007$ ,  $p = 0.002$  and  $p = 0.004$ , respectively). When treated with PMA alone, the “M0” macrophages produced 268 pg/mL TNF in response to *P. gingivalis* LPS challenge. However, when treated with PMA + *P. gingivalis* LPS, TNF production was diminished 10-fold to 27 pg/mL. When cells were polarised with IFNγ and *P. gingivalis* LPS, TNF production was also down-regulated (4-fold) in response to secondary challenge with *P. gingivalis* LPS. The same effect was seen in M2 macrophages (5.5- fold decrease). M1 cells also down regulated their expression of IL-1β. There was no significant effect on levels of IL-1β in the M0 and M2 macrophages. All cell types produced very little IL-6, so any diminishing effect could not be detected.

### **Inflammatory cytokine production by M0, M1 and M2 macrophages is modulated by the presence of *E. coli* LPS during polarisation**

To assess whether the TNF down-regulation described above was specific to *P. gingivalis*, the experiments were repeated, but this time with the addition of *E. coli* LPS instead of *P. gingivalis* LPS during polarisation. At the end of the polarisation phase, culture media were removed and inflammatory cytokine production was stimulated by treatment with *E. coli* LPS. When compared to cells polarised with cytokines alone, the addition of *E. coli* LPS in the polarising media led to a significant down-regulation in TNF production in all three macrophage populations (figure 3.  $p < 0.001$ ). Furthermore, the presence of *E. coli* LPS during polarisation also led to the down-regulation of IL-1β in the M1 population ( $p < 0.001$ ), whereas there was no measurable effect on IL-1β production in M0 or M2 macrophages. Similarly, to polarisation with *P. gingivalis* LPS, the addition of *E. coli* LPS had no significant effect on IL-6 production compared with cytokines alone.

## Cross-modulation of inflammatory cytokine production by different LPS species

Data from the experiments above reported that polarisation in the presence of *P. gingivalis* or *E. coli* LPS led to a down-modulation of TNF (and in the case of M1-macrophages polarised with *E. coli*, IL-1 $\beta$ ). *E. coli* LPS is the archetypal TLR4 ligand, whilst *P. gingivalis* LPS is purported to activate macrophages via TLR2. There is conflicting evidence in the literature regarding *P. gingivalis* LPS – TLR2 /4 signalling, with the likely explanation relating to the multiple lipid A moieties produced by *P. gingivalis* having different receptor binding properties. The next aim of the present study was to see if the two bacterial LPS species with different activating capacities were able to cross-modulate macrophage activation by one another.

To assess this, macrophage populations were polarised in the presence (or absence) of either *P. gingivalis* or *E. coli* LPS, as above. Following polarisation, culture media were removed and macrophages were activated by the other LPS species. When M0, M1 and M2 – like macrophages were differentiated with PMA, cytokines and *P. gingivalis* LPS, then subsequently activated by *E. coli* LPS, TNF production was significantly down – regulated in all three macrophage populations (Figure 4a. M0:  $p < 0.001$ , M1:  $p = 0.04$ , M2:  $p < 0.001$ ). In the M2 population, IL-1 $\beta$  production was also down- regulated ( $p=0.049$ ). Similarly, when macrophages were polarised in the presence of *E. coli* LPS, then cross- activated by *P. gingivalis* LPS, TNF production was also down –regulated (Figure 4b. M0:  $p=0.03$ , M1:  $p=0.006$ , M2:  $p=0.024$ ). In the M1 population, IL-1 $\beta$  production was also down regulated ( $p=0.002$ ).

Finally, we set out to test the effects of chronic exposure to low-dose LPS on macrophage inflammatory cytokine production. This time, the experiments were repeated but using 1 ng / mL of *P. gingivalis* or *E. coli* LPS in the polarising media. *P. gingivalis* at a concentration of 1 ng/ mL had no effect on consequent stimulation with either *P. gingivalis* or *E. coli* LPS; levels of cytokine production remained the same regardless of the presence of 1 ng/ mL *P. gingivalis* LPS in the polarising media. In contrast, the presence of *E. coli* in the polarising media led to the down-regulation of TNF production in PMA and M2 – like macrophages, but not in M1 – like macrophages (Figure 5). There was no significant effect on IL-1 $\beta$  or IL-6 production in any cell type (data not shown).

## Discussion

In periodontal disease, infiltrating monocytes mature and polarise into macrophages with a range of effector phenotypes in response to locally derived factors such as IFN $\gamma$  (generating inflammatory M1 macrophages) or IL-4 / 13 (generating reparatory, regulatory M2 macrophages). Given the cocktail of bacterial and host derived factors

present in the periodontal lesion, it is unlikely that infiltrating monocytes will encounter first one factor then the other in a linear fashion, rather, they are likely to encounter both simultaneously. To date, no studies have sought to determine what effects this would have on the ability of the resulting macrophages to produce a robust response, needed to clear the infection. Therefore, we aimed to test what effects exposure to bacterial LPS during the polarisation of monocytes into effector macrophages would have on the resulting macrophages' ability to produce the inflammatory cytokines needed to clear infection. Furthermore, we sought to examine whether the same effect was seen in non-polarised, M1 and M2 macrophages, which have differing roles in disease progression.

Cells from the human monocyte line, THP-1, were treated with PMA, PMA + IFN $\gamma$  or PMA + IL-4 to generate un-polarised, M1 and M2 like macrophages, respectively. Polarised cells were challenged with *P. gingivalis* or *E. coli* LPS and assessed for protein production of key periodontal disease associated cytokines, TNF, IL1 $\beta$  and IL-6. As previously reported, *P. gingivalis* LPS elicited cytokine responses to a lesser extent than *E. coli* LPS [30]. Pre-treatment with IFN $\gamma$  resulted in higher levels of cytokine production than un-polarised and IL-4 pre-treated cells. Holden *et al* also reported a similar finding, that M2- like macrophages produced TNF and IL-1 $\beta$  in response to *P. gingivalis* LPS, but to a lower level than that of M1- like macrophages [22]

To assess the effects of concomitant exposure to LPS and polarising cytokines on macrophage cytokine production, THP-1 cells were given two, 24 hour treatments; the first a "polarising" treatment to differentiate the monocytes into macrophages, followed by an "activator" treatment to elicit cytokine production. "Un-polarised" macrophages were incubated with PMA for 24 h before washing and challenging with LPS for a further 24 h. The addition of priming cytokines (IFN and IL-4) into the differentiation media during the "polarising" treatment phase, lead to cytokine production characteristic of M1 and M2 macrophages in response to LPS added in the "activation" treatment phase. Compared to cells incubated with differentiation media alone in the "polarisation" phase, macrophages that were incubated with LPS during the first 24 h displayed a down-regulation of TNF and IL-1 $\beta$  production upon secondary LPS treatment in the "activation" phase. This effect was measured in response to both LPS species. Un-primed macrophages displayed a cytokine profile similar to that of M2 macrophages, suggesting that in the absence of the M1-priming cytokine IFN $\gamma$ , macrophages default to a less inflammatory phenotype. This also suggests that LPS as a stimulant alone cannot induce full inflammatory M1 macrophage polarisation.

A study by Zaric *et al* (2011) reports that TNF production is down- regulated in response to repeated exposure to *P. gingivalis* and *E. coli* LPS in THP-1 cells, but that IL-8 is down regulated only by repeated exposure to *E. coli* LPS, thus

suggesting only partial tolerance is achieved by *P. gingivalis* LPS [31]. An earlier study by Martin *et al* (2001) reported that endotoxin tolerance induced by *E. coli* LPS in THP-1 led to down-regulation of TNF, IL-1 $\beta$  and IL-6, whereas endotoxin tolerance induced by *P. gingivalis* LPS only resulted in down-regulation of IL-1 $\beta$  production [32]. These findings differ from the present study in that TNF production was down regulated by prior exposure to *P. gingivalis* LPS. However, both studies agree that IL-1 $\beta$  was down regulated by prior exposure to either LPS species. In contrast to the Martin *et al* (2001) study, and in common with results from the present study, Muthukuru *et al* (2005) reported that TNF and IL-1 $\beta$  were down-regulated in response to repeated exposure of PBMCs to *P. gingivalis* LPS [33].

Dobrovolskaia *et al* (2003) reported that both *E. coli* LPS and synthetic TLR2 ligand PAM3CSK4 induced homotolerance but not heterotolerance in murine macrophages [34]. PAM3CSK4 pre-treatment up-regulated TNF production in response to *E. coli* LPS. *P. gingivalis* LPS also induced homotolerance, but like PAM3CSK, *E. coli* pre-treatment up-regulated TNF production in response to secondary stimulus with *P. gingivalis* LPS. In contrast, results from the present study showed that *E. coli* LPS pre-treatment led to a down-regulation of TNF production when subject to a secondary stimulation by *P. gingivalis* LPS. However, the studies are in agreement in that as *P. gingivalis* pre-treatment led to a down-regulation of TNF production when subject to a secondary stimulation by *E. coli* LPS. Differences in responses reported in various studies may be attributable to the diverse macrophage differentiation protocols and different sources (thus antigenic structures) of LPS.

In response to polarisation with either LPS species, the present study showed little difference in their ability to down-regulate cytokines. Previous studies have shown that *P. gingivalis* LPS is a weaker inducer of endotoxin tolerance than *E. coli* LPS [31, 32], and that this is down to differential use of TLRs and intracellular signalling pathways, with *E. coli* LPS reducing degradation of NF $\kappa$ B inhibitors I $\kappa$ K $\alpha$  and I $\kappa$ K $\beta$  and TLR4 surface expression upon secondary stimulation, whereas *P. gingivalis* LPS up-regulates TLR2 and CD14 expression and degrades inhibitory I $\kappa$ K $\beta$ , thereby rendering cells less responsive to tolerance [32].

Finally, the addition of *E. coli* LPS at a concentration of 1ng/mL in the polarising media resulted in the down-regulation of TNF in PMA and M2 like macrophages in response to a secondary LPS challenge (Figure 5). This yields two potentially interesting hypotheses; 1) *E. coli* LPS has the ability to modulate TNF production at low concentrations, whereas *P. gingivalis* LPS can only modulate TNF production at higher concentrations (1  $\mu$ g/mL). 2) Neither LPS significantly modulated TNF production in M1 macrophages. It may be that given their anti-inflammatory nature, M2 like macrophages are more readily tolerised at lower concentrations than M1 to ensure that there is a robust inflammatory response, even to low concentrations of LPS. Translated into the clinical situation, this could mean that if *P. gingivalis* LPS is present in periodontal tissues at very low concentrations, low-level inflammatory

cytokine production may continue and contribute to the tissue damage and bone resorption seen in periodontal disease.

## Conclusion

Data from the present study demonstrated that macrophages become refractory to further LPS challenge after being exposed to LPS during the polarisation phase, even in the presence of polarising cytokines. This suggests that if infiltrating monocytes entering the infected tissues encounter LPS at the same time as host-derived cytokines, they become refractory to further LPS challenge. This may be a protective mechanism engaged by the macrophages to limit collateral tissue damage in periodontal disease, as seen in endotoxin tolerance. It is particularly interesting that M1 like macrophages do not reach their full inflammatory potential if they meet LPS during their polarising stage, even in the presence of potent polarising agent, IFN $\gamma$ . This may account for the change to chronicity witnessed clinically in periodontal diseases; if macrophages cannot reach full inflammatory potential, then infection may not be eradicated and inflammation becomes chronic. Further research to elucidate the mechanisms of macrophage polarisation and function in disease states will lay the groundwork to significantly improve management options for periodontitis, and other chronic inflammatory conditions such as peri-implantitis, and provide novel therapeutic targets for management options of inflammatory oral disorders.

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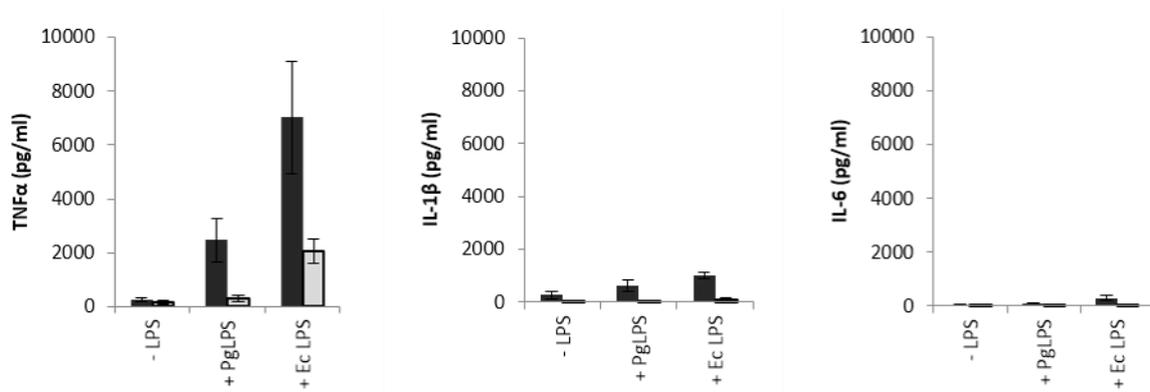
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## Figures

1a



1b

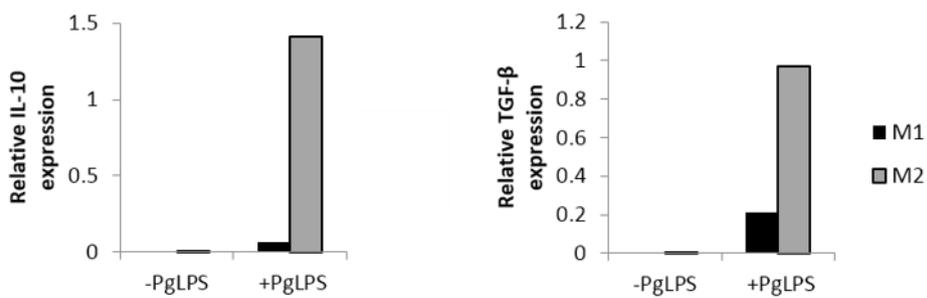


Figure 1: (a) TNF, IL-1 $\beta$  and IL-6 production in response to LPS. THP-1 cells were incubated with IFN $\gamma$  (M1) or IL-4 (M2). After 24 h, culture media were removed and cells were washed x3 in PBS. 1 $\mu$ g/mL Pg or Ec LPS was added to each cell type and incubated for 24 h. Cytokine protein expression was measured by ELISA. Data are means of 3 independent experiments  $\pm$  standard deviation. (b) Gene expression levels of IL-10 and TGF- $\beta$  were measured in M1 and M2 macrophages stimulated with *P. gingivalis* LPS for 6 h. Unstimulated cells served as negative controls.

2.

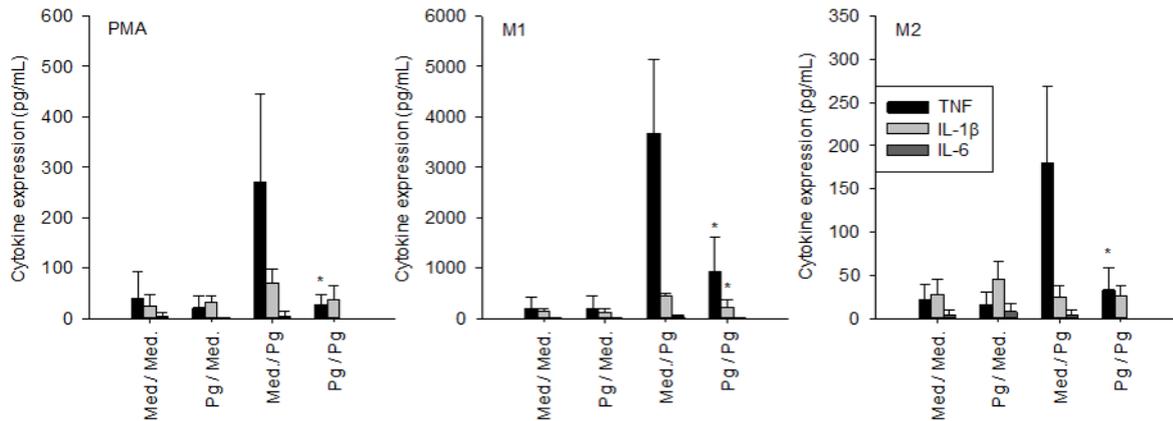


Figure 2: Expression of inflammatory cytokines following repeated exposure of macrophages to *P. gingivalis* LPS. PMA, M1 and M2 macrophages were polarised in the presence or absence of *P. gingivalis* LPS. After 24-hour incubation, media was removed and cells were washed x3 in PBS. Macrophages were then challenged with 1µg/ml *P. gingivalis* LPS for 24 h. Cells incubated for 24 h in normal media (no LPS) served as negative controls. Supernatants were assayed for TNF, IL-1β and IL-6 by ELISA. Data are expressed as the mean of three independent experiments performed in triplicate wells +/- standard deviation. \* indicates a statistically significant change in cytokine expression levels at p < 0.05.

3.

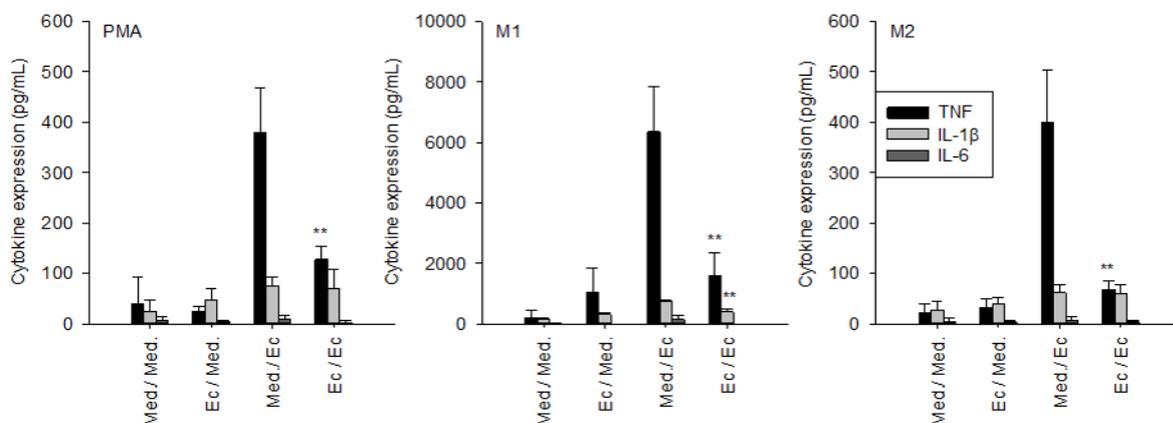
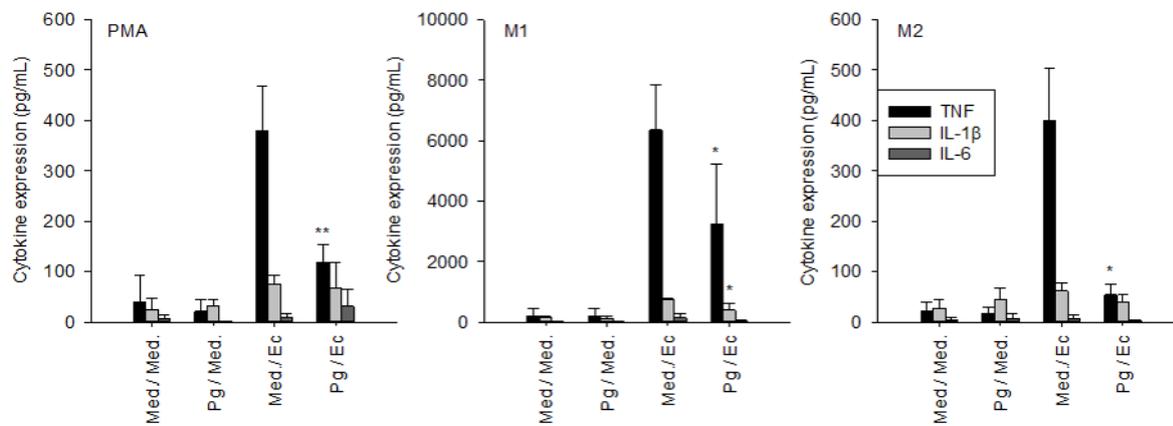


Figure 3: Expression of inflammatory cytokines following repeated exposure of macrophages to *E. coli* LPS. PMA, M1 and M2 macrophages were polarised in the presence or absence of *E. coli* LPS. After 24-hour incubation, media was removed and cells were washed x3 in PBS. Macrophages were then challenged with 1µg/ml *E. coli* LPS for 24 h. Cells incubated for 24 h in normal media (no LPS) served as negative controls. Supernatants were assayed for TNF, IL-1β and IL-6 by ELISA. Data are expressed as the mean of three independent experiments performed in triplicate wells +/- standard deviation. \* indicates a statistically significant change in cytokine expression levels at p < 0.05.

#### 4. a



#### 4. b

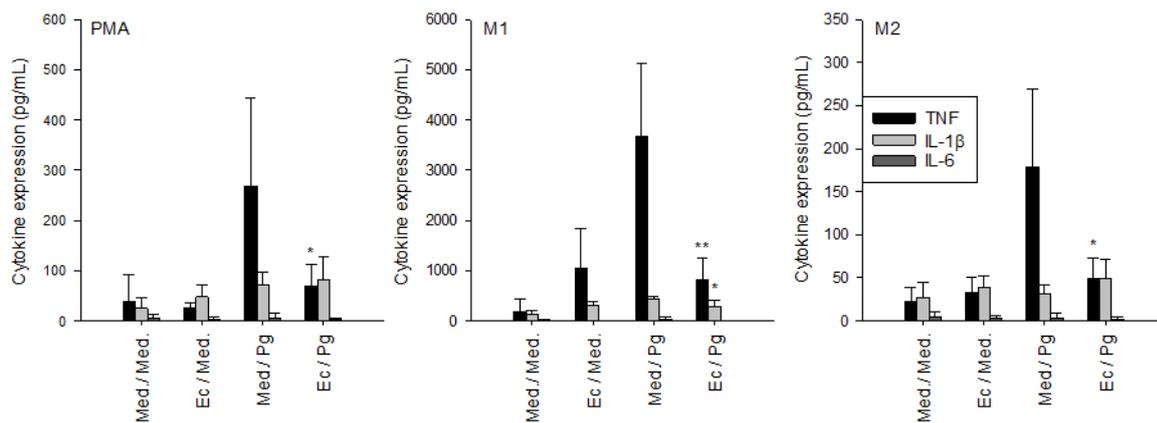
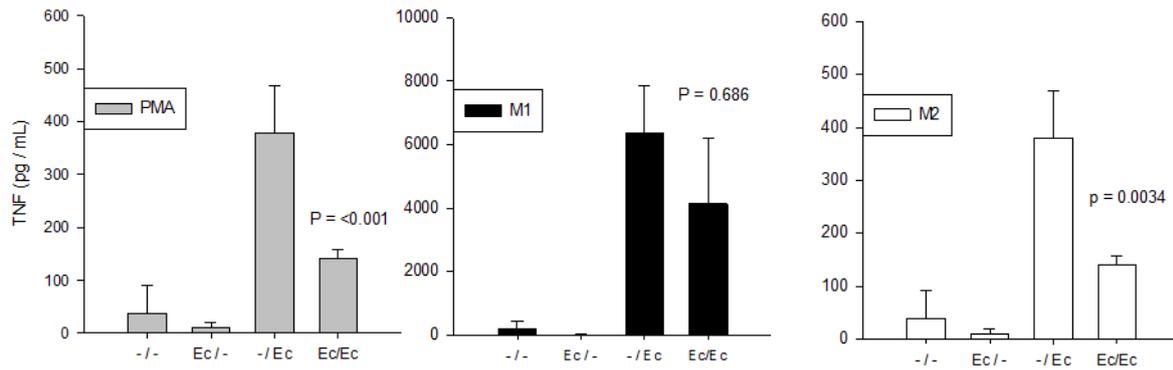


Figure 4: Expression of inflammatory cytokines following cross-exposure of macrophages to *P. gingivalis* and *E. coli* LPS. PMA, M1 and M2 macrophages were polarised in the presence or absence of *P. gingivalis* or *E. coli* LPS. After 24-hour incubation, media was removed and cells were washed x3 in PBS. Macrophages were then cross - challenged with 1µg/ml *E. coli* (a) or *P. gingivalis* LPS (b) for 24 h. Cells incubated for 24 h in normal media (no LPS) served as negative controls. Supernatants were assayed for TNF, IL-1β and IL-6 by ELISA. Data are expressed as the mean of three independent experiments performed in triplicate wells +/- standard deviation. \* indicates a statistically significant change in cytokine expression levels at p < 0.05.

#### 5.



**Figure 5:** Expression of TNF following exposure of macrophages to *E. coli* LPS. PMA, M1 and M2 macrophages were polarised in the presence or absence of *P. gingivalis* or *E. coli* LPS. After 24-hour incubation, media was removed and cells were washed x3 in PBS. Macrophages were then challenged with 1µg/ml *E. coli* LPS for 24 h. Cells incubated for 24 h in normal media (no LPS) served as negative controls. Supernatants were assayed for TNF by ELISA. Data are expressed as the mean of three independent experiments performed in triplicate wells +/- standard deviation. \* indicates a statistically significant change in cytokine expression levels at  $p < 0.05$ .