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Porphyromonas gingivalis-stimulated macrophage subsets exhibit differential induction and responsiveness to interleukin-10

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Running Title: Macrophage tolerisation by \textit{P. gingivalis}

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Abbreviations: \textit{Mφ}s, macrophages; IL-10, Interleukin-10; IL-10R, Interleukin-10 Receptor, TNF\alpha, tumour necrosis factor-\alpha; CP, chronic periodontitis; PG-LPS, Porphyromonas gingivalis lipopolysaccharide; TLR, Toll-like receptor; ET, endotoxin tolerance; STAT-3, signal transducer and activation of transcription-3; NF\kappa B, nuclear factor-kappa B; SOCS-3, suppressor of cytokine signalling-3; PAMPs, pathogen associated molecular patterns.

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Abstract:

Objectives: Oral mucosal macrophages (Mφs) determine immune responses; maintaining tolerance whilst retaining the capacity to activate defences against pathogens. Mφ responses are determined by two distinct subsets; pro-inflammatory M1- and anti-inflammatory/regulatory M2-Mφs. Tolerance induction is driven by M2 Mφs, whereas M1-like Mφs predominate in inflammation, such as that exhibited in chronic Porphyromonas gingivalis (PG) periodontal infection. Mφ responses can be suppressed to benefit either the host or the pathogen. Chronic stimulation by pathogen associated molecular patterns (PAMPs), such as LPS, is well established to induce tolerance. The aim of this study was to investigate the P. gingivalis-driven induction of and responsiveness to the suppressive, anti-inflammatory cytokine, IL-10, by Mφ subsets.

Methods: M1- and M2-like Mφs were generated in vitro from the THP-1 monocyte cell line by differentiation with PMA and Vitamin D3, respectively. Mφ subsets were stimulated by PG-LPS in the presence or absence of IL-10.

Results: PG-LPS differentially induced IL-10 secretion and endogenous IL-10 activity in M1- and M2-like subsets. In addition, these subsets exhibited differential sensitivity to IL-10-mediated suppression of TNFα, where M2 Mφs where sensitive to IL-10 and M1 Mφs were refractory to suppression. In addition, this differential responsiveness to IL-10 was independent of IL-10-binding and expression of the IL-10 receptor signal transducing subunit, IL-10Rβ, but was in fact dependent on activation of STAT-3.
Conclusion: *P. gingivalis* selectively tolerises regulatory M2 M\(\phi\)s with little effect on pro-inflammatory M1 M\(\phi\)s; differential suppression facilitating immunopathology at the expense of immunity.

Key words: Periodontitis, Macrophage, Inflammation, Endotoxin Tolerance, Interleukin-10.

Introduction: Chronic periodontitis (CP) is a chronic relapsing-remitting inflammatory disease of periodontal tissues, cycling between bouts of inflammation and immune suppression, resulting in destruction of the periodontium and ultimately tooth loss. CP results from persistent microbial challenge by dysbiotic biofilms in which *Porphyromonas gingivalis* (PG) is an important member, inducing host inflammatory responses (Ximenez-Fyvie et al., 2000; Ezzo & Cutler, 2003). PG is an intracellular oral pathogen, which infects mucosal macrophages (M\(\phi\)s) (Cutler et al., 1993; Yilmaz et al., 2002). Clearance of which would normally require cell mediated immune responses, however PG-lipopolysaccharide (PG-LPS) induces immune-deviation towards non-clearing humoral responses, facilitating pathogen persistence (Pulendran et al., 2001). PG-LPS also exhibits low endotoxin activity and structural variation, hence differential utilisation of TLR2 and TLR4 (Darveau et al., 2004); thus, subverting both adaptive and innate immunity to survive in mucosal tissue. Immunosuppression can drive this immune deviation. PG-LPS suppresses M\(\phi\) responses by inducing endotoxin tolerance (ET); where LPS pre-exposure rendered innate immune cells refractory to subsequent endotoxin challenge (Biswas & Lopez-Collazo, 2009). ET may be
beneficial or harmful to both host and pathogen; suppressing harmful over-exuberant
pro-inflammatory tissue-destructive responses in the host (Foster & Medzhitov, 2009),
whereas, simultaneously favouring pathogen persistence by suppressing protective
inflammatory responses. Oral mucosal Mφs are important to ET; their activation status
determining whether the mucosal environment is beneficial to the host or pathogen.
PG modulates host cell function to benefit its own survival (Yilmaz, 2008).
Interestingly, PG weakly induces pro-inflammatory cytokines, favouring insufficient
clearing responses, bacterial proliferation and persistence. The cytokines produced
to this expanded bacterial number contribute to localised tissue destruction
characteristic of CP (Gemmell et al., 1997; Garlet, 2010).

Mφs densely populate oral mucosa, responding to PG by producing pro-inflammatory
cytokines (TNFα, IL-1α, IL-1β, IL-18, IL-6, IL-12, IL-8, CCL2, CXCL10, MCP-1, IL-32)
and lower anti-inflammatory cytokine levels (IL-10, TGFβ)(Zhou et al., 2005; Barksby
et al., 2009): a profile suggestive of the M1 pro-inflammatory Mφ subset. In contrast,
Mφs from non-infected homeostatic mucosal tissue, exhibit a cytokine phenotype
resembling the anti-inflammatory/ regulatory M2 subset (TGFβ, IL-10 and low levels
of pro-inflammatory cytokines) (Merry et al., 2012). PG-LPS-stimulated M1 Mφ
cytokine profiles suggest M1 association with pro-inflammatory pathology, whereas
M2 Mφs display a profile associated with regulatory/homeostatic conditions (Foey &
Crean, 2013). These differential Mφ responses correspond to expression of TLR2,
TLR4 and their co-receptor molecule, CD14, driving potent inflammatory responses.
CD14^{high} M1 Mφs produce high level pro-inflammatory- and low level regulatory-
cytokines, which if uncontrolled, results in higher levels of inflammation and
periodontal disease (Tervonen et al., 2007). Conversely, CD14\textsuperscript{low} M\textsubscript{ϕ}s predict a non-pathogenic, homeostatic mucosal effector phenotype (Smith et al., 2005). Thus, mucosal M\textsubscript{ϕ} effector phenotype (inflammatory vs regulatory) may be controlled by regulation of TLR-mediated signals.

M\textsubscript{ϕ}s can be tolerised by several mechanisms, including down-regulation of pattern recognition receptors (PRRs), induction of pro-inflammatory cytokine antagonists, endogenous inhibitors of PRR-mediated signalling and suppressive cytokines (TGF\textbeta, IL-10) (Biswas & Lopez-Collazo, 2009). The relevance of ET in the pathology of CP is the subject of intense research efforts. PG-LPS is predominantly recognised by TLR2, instead of TLR4. In CP, TLR2\textsuperscript{+} and TLR4\textsuperscript{+} monocytes are recruited into gingival lamina propria however, diseased tissue was generally tolerised by down-regulation of TLR2, TLR4, TLR5 and MD-2. Functionally, PG-LPS pre-treatment of monocytes suppressed subsequent stimulation of both pro-inflammatory (TNF\textalpha, IL-1\beta, IL-6, IL-8) and anti-inflammatory cytokines (IL-10) (Muthukuru et al., 2005). PG-LPS tolerisation of M\textsubscript{ϕ}s however, was more complex where cytokines were differentially suppressed, dependent on M\textsubscript{ϕ} subset (Foey & Crean, 2013).

The anti-inflammatory cytokine, IL-10, suppresses M\textsubscript{ϕ} inflammatory responses (Moore et al., 2001) and may be an important mediator of ET. The potential therapeutic value of IL-10, coupled with its limited success in clinical trials (Fedorak et al., 2000; Tilg et al., 2002), suggest the need to investigate its functionality and signalling in chronic inflammatory diseases such as CP. IL-10 signals are transduced by an IL-10R\textalpha and
IL-10Rβ receptor complex preceding a signal cascade involving JAK1/TYK2 and STAT3 (Finbloom & Winestock, 1995). Crucially, STAT3 is associated with IL-10 expression (Benkhart et al., 2000; Staples et al., 2007), anti-inflammatory function, inhibition of Mφ activation (Donnelly et al., 1999) and the M2 Mφ subset. STAT3 conditional knockout in murine Mφs, rendered Mφs refractory to IL-10 and development of chronic enterocolitis (Riley at al., 1999; Takeda et al., 1999).

Our previous observations demonstrated differential Mφ subset sensitivity to PG-LPS induced ET (Foey & Crean, 2013). It is probable that this different responsiveness to tolerisation is, in part, reflected by a subset-specific sensitivity to IL-10 anti-inflammatory/regulatory responses, as a consequence of differential IL-10 induction and signal transduction pathways. Thus, the aim of this study was to investigate the production of and responsiveness to the regulatory cytokine IL-10 in functionally disparate Mφ subsets, relevant to mucosal Mφ effector function in the context of P. gingivalis infection and chronic periodontitis.

**Materials and methods:**

**Monocyte and macrophage (Mφ) culture**

The human monocytic cell line, THP-1, was obtained from ECACC (Salisbury, UK) and used between passages 7 and 25. THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% v/v foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza, Wokingham, UK), here on referred to as R10. Cells were plated out at 1x10^5 cells/well in R10, in 96 well flat-bottomed tissue
culture plates. Pro-inflammatory (M1-like) Mφs and anti-inflammatory (M2-like) Mφs were generated by THP-1 monocyte differentiation in the presence of 25 ng/ml PMA or 10 nM 1,25-(OH)_{2}-Vitamin D_{3} (Sigma-Aldrich, Poole, UK) for 3 and 7 days, respectively (Daigneault et al., 2010). PMA and Vitamin D_{3}-differentiated Mφs were phenotyped according to gene expression and compared favourably to the established phenotype of conventional M1 and M2 subsets: PMA M1-like Mφs were TNFα^{hi}, IL-8^{hi}, IL-12^{hi}, iNOS^{hi}, IL-10^{lo}, Arginase^{lo} whereas Vitamin D_{3} M2-like Mφs were TNFα^{lo}, IL-8^{lo}, IL-10^{hi}, Arginase^{hi}, CD206^{hi}.

**Bacteria and pathogen associated molecular patterns (PAMPs)**

Due to the ability of *P.gingivalis* to induce inflammatory factors via membrane receptors, the effects of *P. gingivalis* lipopolysaccharide (PG-LPS) were studied. PG-LPS was obtained from Autogen Bioclear, Calne, UK. This PAMP was extracted from *P. gingivalis* strain ATCC 33277 originally isolated from human gingival sulcus (American Type Culture Collection). The suppliers extracted PG-LPS by successive enzymatic hydrolysis and purification by Phenol-TEA-DOC protocol (Hirschfeld et al., 2000), prior to being made commercially available.

**Activation of monocyte and macrophage cytokine production**

THP-1-derived M1- and M2-like Mφs were stimulated by the bacterial PAMP; 100 ng/ml PG-LPS and cultured for 18 hours (determined as optimal concentration and time period for expression of the inflammatory cytokines TNFα, and IL-10, data not
shown). Supernatants were harvested and stored at -20°C until required for cytokine assay by sandwich ELISA.

Induction of endogenous IL-10 activity and modulation of macrophage TNFα production by exogenous IL-10

Endogenous cell-associated IL-10 was assayed based on anti-inflammatory activity of IL-10 to suppress LPS-induced TNFα. M1 and M2 Mϕs were pre-treated with 10 μg/ml 9D7 neutralising anti-IL-10 antibody (Biolegend, San Diego, USA) or irrelevant, isotype-matched control antibody for 30 minutes prior to stimulation with 100ng/ml PG-LPS and cultured for 18 hours at 37°C, 5% CO₂. In addition, Mϕ sensitivity to exogenous IL-10 was assayed where M1 and M2s were pre-treated with exogenously added IL-10 (0, 0.2, 2 and 20 ng/ml)(NIBSC, Potter’s Bar, UK) for 30 minutes prior to stimulation with 100ng/ml PG-LPS and cultured for 18 hours. Supernatants were collected and assayed for TNFα secretion by sandwich ELISA. Mϕ viability was routinely checked by either MTT assay or trypan blue exclusion. A physiologically-relevant response was indicated, as no significant reductions in viability were observed for any treatments used in this study (viability routinely >85%).

Cytokine measurement

TNFα and IL-10 were analysed by sandwich ELISA using commercially available capture and detection antibodies (BD-Pharmingen, Oxford, UK). Protocols were followed according to manufacturer’s instructions and compared to standard curves (range of 7 to 5,000 pg/ml), using international standards available from NIBSC.
Colorimetric development was measured spectrophotometrically by an OPTImax tuneable microplate reader at 450 nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp., Sunnyvale, CA, USA).

Flow cytometric analysis of IL-10 binding and IL-10Rβ

IL-10 binding to the ligand-binding IL-10Rα chain was determined using Fluorokine Biotinylated human interleukin-10 (R&D Systems, Abingdon, UK) according to manufacturer’s instructions. Briefly, Mφs were washed and resuspended to 1x10⁵ cells per tube. Biotinylated IL-10 was added to the cells and incubated on ice for 60 mins. Avidin-FITC was then added and incubated for 30 mins on ice. Finally, cells were washed, resuspended in FACS buffer for analysis. Specificity of IL-10 binding was determined using anti-IL-10 blocking antibody added to biotinylated IL-10 prior to introduction to Mφs. Measurement of the IL-10 signalling chain (IL-10Rβ) used a directly conjugated, PE-anti-IL-10Rβ antibody according to manufacturer’s instructions. Briefly, Mφs were washed and resuspended to 1x10⁵ cells per tube. PE-anti-IL-10Rβ was added to the cells and incubated on ice for 60 mins. Stained cells were washed, resuspended in FACS buffer and analysed using a BD FACS Aria II flow cytometer. Data collected represented net mean fluorescent intensities using a live gating strategy set for FSC/SSC.

Western blot analysis of phospho-STAT3

M1 and M2-like Mφs were seeded in 12 well plates at 1x10⁶ cells/ml. Mφs were pre-treated with IL-10 or anti-IL-10 for 1 hour prior to stimulation with 100 ng/ml PG-LPS.
for 30 mins (optimal stimulation for STAT3 activation). Following stimulation, cells were lysed on ice for 15 min in lysis buffer (1% NP-40, 200 mM NaCl, 0.1mM ethylenediaminetetra-acetic acid, 1mM dithiothreitol, 1mM Na$_3$VO$_4$, 1mM NaF, 1mM phenylmethylsulphonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 10 µg/ml aprotinin). Lysed samples (10µg) were separated on a 12% sodium dodecyl sulphate-polyacrylamide gel and Western blotted onto PVDF membrane (Thermo Fisher Scientific, Cramlington, UK). Phosphorylated STAT3 was detected using anti-phosphoSTAT3 (Tyr 705) antibody (New England BioLabs Ltd., UK) followed by anti-rabbit HRP conjugate (New England BioLabs Ltd., UK) and enhanced chemiluminescence (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, UK). STAT3 total protein was detected for the purpose of loading controls to the corresponding phospho-Western. Protein bands were scanned and visualised for chemiluminescence by a Typhoon Trio variable mode imager (GE Healthcare, UK).

**Statistical analysis**

Measure of statistical significance was analysed using a balanced analysis of variance (General Linear Model, Minitab version 16) followed by a multiple comparison test (LSD, least significant difference). Significance was set at p<0.05 (*p<0.05, **p<0.01 and ***p<0.001).
Results:

PG-LPS differentially induces MΦ subset IL-10 production

M1 and M2 MΦs produce different cytokine profiles; M1s exhibit a predominantly pro-inflammatory profile whereas M2s express a more anti-inflammatory/regulatory profile. This experiment was undertaken to establish whether M1 and M2 MΦs respond differently with respect to induction of IL-10 in response to PG. Indeed, PG-LPS induced distinct subset-specific cytokine profiles. PG-LPS induced minimal secretion of IL-10 by M1-like MΦs (secretion significant, p=0.0051, but close to lower level of sensitivity of ELISA), compared to M2 MΦs (figure 1a). M2-secreted IL-10 however, was measured at relatively low levels (120±10 pg/ml, p<0.0001). In addition to secretion, considering membrane bound IL-10 has been demonstrated (Fleming et al., 1999), this endogenous activity was also investigated using neutralising anti-IL-10 antibody in the context of TNFα suppression. No endogenous activity was exhibited by unstimulated MΦs (figure 1b & 1c). Upon PG-LPS stimulation however, MΦ subsets exhibited differential expression of endogenous IL-10 activity. PG-LPS failed to induce an endogenous suppressive IL-10 activity in M1s; induction of TNFα did not exhibit a statistically significant change (p=0.247) between isotype-matched control and neutralising anti-IL-10 antibody (Figure 1b). PG-LPS did, however, induce an endogenous IL-10 activity expressed by M2-like MΦs. Neutralisation of IL-10 activity augmented TNFα secretion by 20% (p=0.011, figure 1c).

[ADD FIGURE 1 HERE]
M1- and M2-like Mφs differentially respond to IL-10

Upon PG-LPS challenge, Mφ subsets differentially produce secreted IL-10 and endogenous IL-10 activity. The responsiveness of these Mφ subsets to the anti-inflammatory cytokine, IL-10, was investigated by IL-10 ability to suppress TNFα. In the case of pro-inflammatory M1-like Mφs, IL-10 failed to significantly suppress PG-LPS-induced TNFα secretion to a concentration of 20 ng/ml (figure 2). In contrast, M2-like Mφs were sensitive to the anti-inflammatory effects of IL-10; where IL-10 dose-dependently suppressed PG-LPS-induced TNFα by 75% at 20 ng/ml (p<0.01) (figure 2).

M1- and M2-like Mφs display similar IL-10 binding capability and signalling receptor, IL-10Rβ expression

This differential IL-10-sensitivity suggested different binding capabilities between M1- and M2-like Mφs. IL-10 binds the IL-10R α-subunit; it was hypothesised that IL-10Rα, hence IL-10 binding capability varied between these subsets. This was investigated by IL-10 ligand-binding, which demonstrated IL-10 binding was both unaltered by PG-LPS stimulation or affected by Mφ subset (figure 3a). There was no significant difference in binding activity between unstimulated M1 and M2 Mφs and that this binding activity was not significantly altered by PG-LPS for M1 (p=0.165) or M2 (p=0.749) Mφs.
In light of the differential IL-10-sensitivity exhibited by these Mϕ subsets, which failed to be reflected by IL-10-binding activity (IL-10Rα), a further investigation was undertaken to observe whether this responsiveness was as a consequence of IL-10 signal transducing subunit, IL-10Rβ. Both M1 and M2s expressed IL-10Rβ in the absence of PG-LPS, with M2 Mϕs expressing greater levels of this receptor subunit (MFI = 2000 AU compared to 1000 AU for M1s) (figure 3b). Upon PG-LPS stimulation however, both M1 and M2s exhibited significant down-regulation in IL-10Rβ (M1s, p=0.0414 and M2s, p=0.0035).

[ADD FIGURE 3 HERE]

STAT-3 is differentially activated by PG-LPS and IL-10 in M1- and M2-like Mϕs

Down-regulation of the receptor signalling subunit in both M1 and M2 Mϕs does not adequately explain differential responsiveness to IL-10. Downstream of IL-10 receptor signalling, STAT3 mediates anti-inflammatory responses. Thus, STAT-3 activation may determine this differential sensitivity to IL-10. Phospho-Western blot analysis showed M1 Mϕs failed to activate STAT3 in response to PG-LPS in the presence or absence of IL-10. IL-10 alone only superficially activated STAT3 (figure 4a). In contrast, PG-LPS clearly activated STAT3 in M2 Mϕs, augmented by exogenous IL-10. The PG-LPS-induced phospho-STAT3 was dependent on endogenous IL-10; neutralisation of which abrogated STAT3 activation (figure 4b).

[ADD FIGURE 4 HERE]
DISCUSSION:

Previous studies from this laboratory have shown M1- and M2-like Mφs to exhibit differential sensitivity to ET (Foey & Crean, 2013). In an attempt to elucidate mechanisms underlying this differential sensitivity, this investigation has drawn several conclusions with respect to Mφ responses to the oral pathogen, *P. gingivalis*. Firstly, PG-LPS activation induces Mφ subset-specific responses, where M2-like Mφs preferentially secrete IL-10 as well as an endogenous cell-associated activity, compared to M1-like Mφs. Secondly, Mφs also displayed a selective, subset-specific responsiveness to IL-10. M1 Mφs were refractory to the anti-inflammatory activity of IL-10 whereas M2s were sensitive. Finally, differential IL-10 responsiveness was not as a consequence of selective expression of IL-10-binding activity (IL-10Rα) or signal transduction receptor (IL-10Rβ), but reflected by activation of the downstream transcription factor, STAT3.

The pathological role for Mφs in chronic periodontitis is well established. Indeed, Mφ numbers have been shown to be increased above healthy control levels in CP gingival tissue biopsy samples (Gemmell et al., 2001; Lappin et al., 2000). *P. gingivalis*, the keystone pathogen to periodontitis, drives the gingipain-mediated activation of the Mφ urokinase plasminogen activator (uPA) system, resulting in a proteolytic cascade involved in inflammatory destruction and loss of bone architecture (Fleetwood et al., 2015). In addition, Mφ depletion has been demonstrated to be associated with a reduction in inflammation and alveolar bone resorption, where the M1 subset predominates in response to *P. gingivalis* infection, resulting in an inflammatory phenotype defined by the increased secretion of TNFα, IL-1β, IL-6, IL-12p70, MCP-1.
and MIP-1α (Lam et al., 2014). The potential predominance of an M1-like subset in CP conforms to phenotypic observations of *P. gingivalis* stimulation of murine Mφ studies, where both PG-LPS and *P. gingivalis* bacteria induced expression of TNFα, IL-12 and iNOS by IFNγ-primed M1 Mφs (Holden et al., 2014; Lam et al., 2016). In this investigation, PG-LPS stimulation of human M1 and M2 Mφ subsets demonstrated differing cytokine effector profiles; where M1s (representative of recruited, pro-inflammatory pathological Mφs) exhibited a pro-inflammatory phenotype (TNFα, IL-1β, IL-6, NFκB, IL-10) and M2s (representative of regulatory, anti-inflammatory mucosal Mφs) tended to be more anti-inflammatory/regulatory (TNFα, IL-1β, IL-6, NFκB, IL-10, TGFβ) (Foey & Crean, 2013; Holden et al., 2014). Mucosal Mφs are considered to exist in these discrete functional subsets, governed by environmental stimuli (Smythies et al., 2005; Foey, 2012). This investigation demonstrated IL-10 production conforms to this functional subset dichotomy with respect to M2 Mφs exhibiting higher levels of both secreted and endogenous cell-associated IL-10 when stimulated by PG-LPS. Conversely, M1 Mφs failed to display an endogenous IL-10 activity and resulted in low level secretion.

IL-10 regulates/suppresses pro-inflammatory responses (Fiorentino et al., 1991). This anti-inflammatory effect can be elicited at many levels; including, down-regulating TNFα secretion, suppressing TNFα activity via inducing TNF-R shedding or by modulating NFκB-signalling events driving TNFα production (Schottelius et al., 1999). Although these Mφ subsets have been characterised with respect to TNFα and IL-10, the level of cross-regulation and plasticity exhibited by these subsets is highlighting the importance to characterise subset responsiveness to regulatory cytokines such as...
Our data clearly establishes a differential responsiveness of $M_\Phi$ subsets to IL-10, where M1s were refractory and M2s sensitive to the anti-inflammatory effects of IL-10. This sensitivity of M2s to IL-10 suppression of TNF$\alpha$ would appear to be independent of subset-specific binding activity and signal transduction receptor and is reflected by differential utilisation of downstream intracellular signalling pathways.

The desired outcome would be to harness anti-inflammatory properties of IL-10; controlling inflammatory mechanisms in chronic diseases such as CP. This data introduces two problems: 1) lack of $M_\Phi$ subset selectivity of IL-10 binding and 2) the IL-10-responsive subset is the M2 regulatory subset whereas the M1 pro-inflammatory pathological subset is unresponsive. This study may suggest why IL-10 treatment has exhibited limited success in suppressing $M_\Phi$-driven chronic inflammatory diseases such as RA, CD and psoriasis (Fedorak et al., 2000; Tilg et al., 2002; McInnes et al., 2001). The specificity of IL-10-mediated anti-inflammatory response would appear to result from differential activation and utilisation of STAT3 (Benkhart et al., 2000). PG-LPS activates STAT3 in M2 $M_\Phi$s, this response is indirect through IL-10; neutralisation of which abrogated the activation response. Due to the short activation time used in this experiment (30 mins), the IL-10-dependency does not result from de novo IL-10 synthesis but is likely to result from a pre-existing endogenous IL-10 activity observed for M2s. Interestingly, PG-LPS stimulation in the presence of exogenous IL-10 dramatically augmented STAT3 activation. Coupled with the fact that results showed a PG-LPS-induced decrease in IL-10R$\beta$ and no change for IL-10R$\alpha$, the PG-LPS augmentation of STAT3 activation would appear to be both IL-10-dependent in early responses and IL-10-independent at later times.
IL-10 and STAT3 are not only directly involved in anti-inflammatory and regulatory responses but indirectly via effects on Mφ polarisation. Both IL-10 and STAT3 influence Mφ plasticity and differentiation towards the M2 effector subset. Conditional knock-out of Mφ STAT3, renders mice refractory to IL-10 and develop chronic enterocolitis (Riley et al., 1999; Takeda et al., 1999); although STAT3 is necessary for IL-10 signalling, additional pathways are required for Mφ inhibition. One such mechanism may involve IL-10-inducible STAT3-regulated gene expression of SOCS3, which negatively feeds-back to regulate IL-10 signalling (Donnelly et al., 1999; Cassatella et al., 1999; Auernhammer et al., 1999). SOCS3 may not just regulate anti-inflammatory responses but also M2 to M1 switch, driving Mφ plasticity towards this pro-inflammatory phenotype (Liu et al., 2008). Manipulation of Mφ effector phenotype via controlling plasticity between M1 and M2 subsets, or specific Mφ subset tolerance induction, hence determining immune response as either pro-inflammatory/immune activatory or anti-inflammatory/tolerogenic, would greatly benefit therapeutic management of inflammatory pathologies such as CP.

Intuitively CP, a relapsing/remitting chronic inflammatory disease, is constantly switching between inflammation and tolerance: inflammation targets the pathogen and results in collateral damage of host tissue whereas ET results in tissue repair and a chance for pathogen numbers to recover. This differential Mφ sensitivity to the anti-inflammatory effects of IL-10 however, is suggestive of a disease stage whereby homeostatic M2-like Mφs are suppressed by IL-10 whereas the pro-inflammatory
nature of M1 MΦs is untouched. Such a differential suppression may be beneficial for controlling pathogen numbers but will be detrimental to tissue repair.

In conclusion, this investigation further characterises M1- and M2-like MΦ subsets with respect to PG-LPS-induced activation/suppression decisions. Selective MΦ subset tolerisation and responsiveness to anti-inflammatory cytokines affords us a better understanding of the role of distinct MΦ subsets in driving both activatory and suppressive stages in CP. Investigation of subset sensitivity to IL-10-mediated suppression observed that the subset least sensitive to IL-10 suppression was the pro-inflammatory pathological subset, M1. This would suggest that such mechanisms of ET may be detrimental to the host tissue and its repair. Selective manipulation of MΦ subset suppression, hence the balance of activation and suppression between such subsets, may represent a translatable future therapeutic approach for the control of such destructive oral inflammatory pathologies but can only realistically be employed upon full understanding of the mechanisms driving such relapsing/remitting episodes characteristic of CP.

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Conflict of Interest Disclosure: There are no conflicts of interest with this study.
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**Figure legends:**

**Figure 1.** M1 & M2 MΦs display differential IL-10 profiles in response to PG-LPS. THP-1-derived M1 and M2 MΦs were generated by differentiating THP-1 monocytes with either 25ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days or 10nM 1,25-
(OH)₂ vitamin D₃ for 7 days, respectively. Secretion of IL-10 (a) is depicted where M1 (bold) and M2 (shaded) Mϕ subsets were stimulated with or without (control) 100ng/ml PG-LPS. Endogenous IL-10 activity, upon treatment with 10µg/ml neutralising anti-IL-10 compared to an isotype-matched control antibody (IC), is represented for PG-LPS-stimulated and unstimulated M1 (b) and M2 (c) macrophage TNFα secretion. Cytokine production is expressed as the mean±SD in pg/ml for IL-10 (a) and TNFα (b & c). Data displayed represents triplicate samples for n=3 replicate experiments. Significant differences in cytokine production between activated M1 and M2 Mϕs and unstimulated controls and between isotype control and neutralising IL-10 antibody treatment are indicated as *p<0.05, **p<0.01, ***P<0.001 and ns, not significant.

**Figure 2.** Differential sensitivity of PG-LPS-induced Mϕ TNFα to IL-10. M1 (bold) and M2 (shaded) Mϕ subsets were stimulated with 100ng/ml PG-LPS for 24hours in the presence or absence of IL-10 at concentration range from 0 to 20ng/ml. Anti-inflammatory effect of IL-10 on TNFα cytokine production is expressed as percentage of LPS-induced TNFα production originally represented as mean±SD in pg/ml. Data displayed represents triplicate samples for n=3 replicate experiments. Significant effects compared to PG-LPS control in the absence of IL-10 (0ng/ml) is indicated for Mϕ subsets as **p<0.01 and ns, not significant.

**Figure 3.** M1 and M2 Mϕs exhibit similar IL10R subunit expression in response to PG-LPS. THP-1-derived M1 and M2 Mϕs were generated by differentiating THP-1 monocytes with either 25ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days or
10nM 1,25-(OH)$_2$ vitamin D$_3$ for 7 days, respectively. FITC-labelled IL-10 binding to the IL-10R$\alpha$ ligand-binding subunit (a) and anti-IL-10R$\beta$ antibody binding of the signalling subunit (b) is depicted in net MFI±SD for M1 and M2 M$\Phi$s in the presence or absence of PG-LPS (100 ng/ml) stimulation. Data displayed represents triplicate samples for n=3 replicate experiments. Significant differences in binding activity between PG-LPS stimulated and non-stimulated M$\Phi$ controls are indicated as *p<0.05, **p<0.01 and ns, not significant.

**Figure 4. STAT3 is differentially activated by PG-LPS and IL-10 in M1 and M2 M$\Phi$s.** THP-1-derived M1 and M2 M$\Phi$s were plated out at 5x10$^6$ cells per well in a flat-bottomed 12 well plate and treated with PG-LPS (100 ng/ml) in the presence or absence of exogenously added IL-10 (10ng/ml) or neutralising anti-IL-10 antibody (10$\mu$g/ml 9D7). After 30 mins stimulation time, cell lysates were harvested. Western blot analysis of activated phospho-STAT3 shows IL-10-mediated activation of STAT3 in M1 (a) and M2 (b) M$\Phi$s. Loading controls are presented as total STAT3 blots below the corresponding phospho-Westerns. Data displayed are representative of three replicate experiments.
Foey et al. 2016 Figure 1.

a) IL-10 (pg/ml)

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b) M1-like macrophages

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c) M2-like macrophages

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Foey et al., 2016 Figure 2.
Figure 3.

(a) IL-10-FITC binding Net MFI

(b) IL10Rβ chain (Net MFI)
Foey et al., 2016 Figure 4.

a) M1-like macrophages

b) M2-like macrophages

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