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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 *P. gingivalis*

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Abbreviations: *Mφs*, macrophages; *IL-10*, Interleukin-10; *IL-10R*, Interleukin-10 Receptor, *TNFα*, tumour necrosis factor-α; *CP*, chronic periodontitis; *PG-LPS*, *Porphyromonas gingivalis* lipopolysaccharide; *TLR*, Toll-like receptor; *ET*, endotoxin tolerance; *STAT-3*, signal transducer and activation of transcription-3; *NFκB*, 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 (M ϕ s) determine immune responses; maintaining tolerance whilst retaining the capacity to activate defences against pathogens. M ϕ responses are determined by two distinct subsets; pro-inflammatory M1- and anti-inflammatory/regulatory M2-M ϕ s. Tolerance induction is driven by M2 M ϕ s, whereas M1-like M ϕ s predominate in inflammation, such as that exhibited in chronic *Porphyromonas gingivalis* (PG) periodontal infection. M ϕ 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 M ϕ subsets.

Methods: M1- and M2-like M ϕ s were generated *in vitro* from the THP-1 monocyte cell line by differentiation with PMA and Vitamin D₃, respectively. M ϕ 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 TNF α , where M2 M ϕ s were sensitive to IL-10 and M1 M ϕ s 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-10R β , but was in fact dependent on activation of STAT-3.

Conclusion: *P.gingivalis* selectively tolerises regulatory M2 Mφs with little effect on pro-inflammatory M1 Mφs; 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 (Mφs) (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 Mφ 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^{low} M2 Mφs predict a non-pathogenic, homeostatic mucosal effector phenotype (Smith et al., 2005). Thus, mucosal Mφ effector phenotype (inflammatory vs regulatory) may be controlled by regulation of TLR-mediated signals.

Mφs 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 (TGFβ, 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⁺ and TLR4⁺ 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 (TNFα, IL-1β, IL-6, IL-8) and anti-inflammatory cytokines (IL-10) (Muthukuru et al., 2005). PG-LPS tolerisation of Mφs however, was more complex where cytokines were differentially suppressed, dependent on Mφ subset (Foey & Crean, 2013).

The anti-inflammatory cytokine, IL-10, suppresses Mφ 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-10Rα and

IL-10R β 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 M ϕ activation (Donnelly et al., 1999) and the M2 M ϕ subset. STAT3 conditional knockout in murine M ϕ s, rendered M ϕ s refractory to IL-10 and development of chronic enterocolitis (Riley et al., 1999; Takeda et al., 1999).

Our previous observations demonstrated differential M ϕ 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 M ϕ subsets, relevant to mucosal M ϕ effector function in the context of *P. gingivalis* infection and chronic periodontitis.

Materials and methods:

Monocyte and macrophage (M ϕ) 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 μ g/ml streptomycin (Lonza, Wokingham, UK), here on referred to as R10. Cells were plated out at 1×10^5 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 D₃ (Sigma-Aldrich, Poole, UK) for 3 and 7 days, respectively (Daigneault et al., 2010). PMA and Vitamin D₃-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 D₃ 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 TNF α production by exogenous IL-10

Endogenous cell-associated IL-10 was assayed based on anti-inflammatory activity of IL-10 to suppress LPS-induced TNF α . M1 and M2 M ϕ s were pre-treated with 10 μ g/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, M ϕ 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 TNF α secretion by sandwich ELISA. M ϕ 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

TNF α 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-10R β

IL-10 binding to the ligand-binding IL-10R α chain was determined using Fluorokine Biotinylated human interleukin-10 (R&D Systems, Abingdon, UK) according to manufacturer's instructions. Briefly, M ϕ s were washed and resuspended to 1×10^5 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 M ϕ s. Measurement of the IL-10 signalling chain (IL-10R β) used a directly conjugated, PE-anti-IL-10R β antibody according to manufacturer's instructions. Briefly, M ϕ s were washed and resuspended to 1×10^5 cells per tube. PE-anti-IL-10R β 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 M ϕ s were seeded in 12 well plates at 1×10^6 cells/ml. M ϕ s 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 Na₃VO₄, 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 M ϕ subset IL-10 production

M1 and M2 M ϕ s 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 M ϕ s 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 M ϕ s (secretion significant, $p=0.0051$, but close to lower level of sensitivity of ELISA), compared to M2 M ϕ s (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 TNF α suppression. No endogenous activity was exhibited by unstimulated M ϕ s (figure 1b & 1c). Upon PG-LPS stimulation however, M ϕ subsets exhibited differential expression of endogenous IL-10 activity. PG-LPS failed to induce an endogenous suppressive IL-10 activity in M1s; induction of TNF α 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 M ϕ s. Neutralisation of IL-10 activity augmented TNF α 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 TNF α . In the case of pro-inflammatory M1-like Mφs, IL-10 failed to significantly suppress PG-LPS-induced TNF α 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 TNF α by 75% at 20 ng/ml ($p < 0.01$) (figure 2).

[ADD FIGURE 2 HERE]

M1- and M2-like Mφs display similar IL-10 binding capability and signalling receptor, IL-10R β 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-10R α , 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 M ϕ subsets, which failed to be reflected by IL-10-binding activity (IL-10R α), a further investigation was undertaken to observe whether this responsiveness was as a consequence of IL-10R signal transducing subunit, IL-10R β . Both M1 and M2s expressed IL-10R β in the absence of PG-LPS, with M2 M ϕ s 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-10R β (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 M ϕ s

Down-regulation of the receptor signalling subunit in both M1 and M2 M ϕ s 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 M ϕ s 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 M ϕ s, 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 M ϕ s 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 M ϕ responses to the oral pathogen, *P. gingivalis*. Firstly, PG-LPS activation induces M ϕ subset-specific responses, where M2-like M ϕ s preferentially secrete IL-10 as well as an endogenous cell-associated activity, compared to M1-like M ϕ s. Secondly, M ϕ s also displayed a selective, subset-specific responsiveness to IL-10. M1 M ϕ s 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-10R α) or signal transduction receptor (IL-10R β), but reflected by activation of the downstream transcription factor, STAT3.

The pathological role for M ϕ s in chronic periodontitis is well established. Indeed, M ϕ 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 M ϕ 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, M ϕ 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 TNF α , IL-1 β , IL-6, IL-12p70, MCP-1

and MIP-1 α (Lam et al., 2014). The potential predominance of an M1-like subset in CP conforms to phenotypic observations of *P.gingivalis* stimulation of murine M ϕ studies, where both PG-LPS and *P.gingivalis* bacteria induced expression of TNF α , IL-12 and iNOS by IFN γ -primed M1 M ϕ s (Holden et al., 2014; Lam et al., 2016). In this investigation, PG-LPS stimulation of human M1 and M2 M ϕ subsets demonstrated differing cytokine effector profiles; where M1s (representative of recruited, pro-inflammatory pathological M ϕ s) exhibited a pro-inflammatory phenotype (TNF α^{hi} , IL-1 β^{lo} , IL-6 ^{lo} , NF κ B ^{hi} , IL-10 ^{lo}) and M2s (representative of regulatory, anti-inflammatory mucosal M ϕ s) tended to be more anti-inflammatory/regulatory (TNF α^{lo} , IL-1 β^{hi} , IL-6 ^{hi} , NF κ B ^{lo} , IL-10 $^{+}$, TGF β^{+}) (Foey & Crean, 2013; Holden et al., 2014). Mucosal M ϕ s 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 M ϕ s exhibiting higher levels of both secreted and endogenous cell-associated IL-10 when stimulated by PG-LPS. Conversely, M1 M ϕ s 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 TNF α secretion, suppressing TNF α activity via inducing TNF-R shedding or by modulating NF κ B-signalling events driving TNF α production (Schottelius et al., 1999). Although these M ϕ subsets have been characterised with respect to TNF α 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

IL-10. Our data clearly establishes a differential responsiveness of M ϕ 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 α 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 ϕ 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 ϕ -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 ϕ 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 β and no change for IL-10R α , 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.

381

382 IL-10 and STAT3 are not only directly involved in anti-inflammatory and regulatory
383 responses but indirectly via effects on M ϕ polarisation. Both IL-10 and STAT3
384 influence M ϕ plasticity and differentiation towards the M2 effector subset. Conditional
385 knock-out of M ϕ STAT3, renders mice refractory to IL-10 and develop chronic
386 enterocolitis (Riley et al., 1999; Takeda et al., 1999); although STAT3 is necessary
387 for IL-10 signalling, additional pathways are required for M ϕ inhibition. One such
388 mechanism may involve IL-10-inducible STAT3-regulated gene expression of SOCS3,
389 which negatively feeds-back to regulate IL-10 signalling (Donnelly et al., 1999;
390 Cassatella et al., 1999; Auernhammer et al., 1999). SOCS3 may not just regulate anti-
391 inflammatory responses but also M2 to M1 switch, driving M ϕ plasticity towards this
392 pro-inflammatory phenotype (Liu et al., 2008). Manipulation of M ϕ effector phenotype
393 via controlling plasticity between M1 and M2 subsets, or specific M ϕ subset tolerance
394 induction, hence determining immune response as either pro-inflammatory/immune
395 activatory or anti-inflammatory/tolerogenic, would greatly benefit therapeutic
396 management of inflammatory pathologies such as CP.

397

398 Intuitively CP, a relapsing/remitting chronic inflammatory disease, is constantly
399 switching between inflammation and tolerance: inflammation targets the pathogen and
400 results in collateral damage of host tissue whereas ET results in tissue repair and a
401 chance for pathogen numbers to recover. This differential M ϕ sensitivity to the anti-
402 inflammatory effects of IL-10 however, is suggestive of a disease stage whereby
403 homeostatic M2-like M ϕ s 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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556

557 **Figure legends:**

558 **Figure 1. M1 & M2 M ϕ s display differential IL-10 profiles in response to PG-LPS.**

559 THP-1-derived M1 and M2 M ϕ s were generated by differentiating THP-1 monocytes
 560 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) M ϕ subsets were stimulated with or without (control) 100ng/ml PG-LPS. Endogenous IL-10 activity, upon treatment with 10 μ g/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 TNF α secretion. Cytokine production is expressed as the mean $\pm$ SD in pg/ml for IL-10 (a) and TNF α (b & c). Data displayed represents triplicate samples for n=3 replicate experiments. Significant differences in cytokine production between activated M1 and M2 M ϕ s 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 M ϕ TNF α to IL-10. M1 (bold) and M2 (shaded) M ϕ 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 TNF α cytokine production is expressed as percentage of LPS-induced TNF α production originally represented as mean $\pm$ 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 M ϕ subsets as **p<0.01 and ns, not significant.

Figure 3. M1 and M2 M ϕ s exhibit similar IL10R subunit expression in response to PG-LPS. THP-1-derived M1 and M2 M ϕ s were generated by differentiating THP-1 monocytes with either 25ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days or

584 10nM 1,25-(OH)₂ vitamin D₃ for 7 days, respectively. FITC-labelled IL-10 binding to
585 the IL-10R α ligand-binding subunit (a) and anti-IL-10R β antibody binding of the
586 signalling subunit (b) is depicted in net MFI $\pm$ SD for M1 and M2 M ϕ s in the presence
587 or absence of PG-LPS (100 ng/ml) stimulation. Data displayed represents triplicate
588 samples for n=3 replicate experiments. Significant differences in binding activity
589 between PG-LPS stimulated and non-stimulated M ϕ controls are indicated as *p<0.05,
590 **p<0.01 and ns, not significant.

591

592 **Figure 4. STAT3 is differentially activated by PG-LPS and IL-10 in M1 and M2**
593 **M ϕ s.** THP-1-derived M1 and M2 M ϕ s were plated out at 5x10⁶ cells per well in a flat-
594 bottomed 12 well plate and treated with PG-LPS (100 ng/ml) in the presence or
595 absence of exogenously added IL-10 (10ng/ml) or neutralising anti-IL-10 antibody
596 (10 μ g/ml 9D7). After 30 mins stimulation time, cell lysates were harvested. Western
597 blot analysis of activated phospho-STAT3 shows IL-10-mediated activation of STAT3
598 in M1 (a) and M2 (b) M ϕ s. Loading controls are presented as total STAT3 blots below
599 the corresponding phospho-Westerns. Data displayed are representative of three
600 replicate experiments.

Foey et al. 2016 Figure 1.

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Foey et al., 2016 Figure 2.

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Foey *et al.*, 2016 Figure 3.

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Foey et al., 2016 Figure 4.