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

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15 **Running Title:** Macrophage tolerisation by *P. gingivalis*

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27 **Abbreviations:** M $\phi$ s, macrophages; IL-10, Interleukin-10; IL-10R, Interleukin-10 Receptor, TNF $\alpha$ ,  
28 tumour necrosis factor- $\alpha$ ; CP, chronic periodontitis; PG-LPS, *Porphyromonas gingivalis*  
29 lipopolysaccharide; TLR, Toll-like receptor; ET, endotoxin tolerance; STAT-3, signal transducer and  
30 activation of transcription-3; NF $\kappa$ B, nuclear factor-kappa B; SOCS-3, suppressor of cytokine signalling-  
31 3; PAMPs, pathogen associated molecular patterns.

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33 words. Abstract word count = 239 words. Introduction, materials & methods, results and discussion  
34 word count = 3,265. 39 references, 4 figures, 0 tables.

36    **Abstract:**

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

48    **Methods:** M1- and M2-like M $\phi$ s were generated *in vitro* from the THP-1 monocyte cell  
49    line by differentiation with PMA and Vitamin D<sub>3</sub>, respectively. M $\phi$  subsets were  
50    stimulated by PG-LPS in the presence or absence of IL-10.

51    **Results:** PG-LPS differentially induced IL-10 secretion and endogenous IL-10 activity  
52    in M1- and M2-like subsets. In addition, these subsets exhibited differential sensitivity  
53    to IL-10-mediated suppression of TNF $\alpha$ , where M2 M $\phi$ s were sensitive to IL-10 and  
54    M1 M $\phi$ s were refractory to suppression. In addition, this differential responsiveness  
55    to IL-10 was independent of IL-10-binding and expression of the IL-10 receptor signal  
56    transducing subunit, IL-10R $\beta$ , but was in fact dependent on activation of STAT-3.

57 **Conclusion:** *P.gingivalis* selectively tolerises regulatory M2 M $\phi$ s with little effect on  
58 pro-inflammatory M1 M $\phi$ s; differential suppression facilitating immunopathology at the  
59 expense of immunity.

60

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

63

64 **Introduction:**

65 Chronic periodontitis (CP) is a chronic relapsing-remitting inflammatory disease of  
66 periodontal tissues, cycling between bouts of inflammation and immune suppression,  
67 resulting in destruction of the periodontium and ultimately tooth loss. CP results from  
68 persistent microbial challenge by dysbiotic biofilms in which *Porphyromonas gingivalis*  
69 (PG) is an important member, inducing host inflammatory responses (Ximenez-Fyvie  
70 et al., 2000; Ezzo & Cutler, 2003). PG is an intracellular oral pathogen, which infects  
71 mucosal macrophages (M $\phi$ s) (Cutler et al., 1993; Yilmaz et al., 2002). Clearance of  
72 which would normally require cell mediated immune responses, however PG-  
73 lipopolysaccharide (PG-LPS) induces immune-deviation towards non-clearing  
74 humoral responses, facilitating pathogen persistence (Pulendran et al., 2001). PG-  
75 LPS also exhibits low endotoxin activity and structural variation, hence differential  
76 utilisation of TLR2 and TLR4 (Darveau et al., 2004); thus, subverting both adaptive  
77 and innate immunity to survive in mucosal tissue. Immunosuppression can drive this  
78 immune deviation. PG-LPS suppresses M $\phi$  responses by inducing endotoxin  
79 tolerance (ET); where LPS pre-exposure rendered innate immune cells refractory to  
80 subsequent endotoxin challenge (Biswas & Lopez-Collazo, 2009). ET may be

81 beneficial or harmful to both host and pathogen; suppressing harmful over-exuberant  
82 pro-inflammatory tissue-destructive responses in the host (Foster & Medzhitov, 2009),  
83 whereas, simultaneously favouring pathogen persistence by suppressing protective  
84 inflammatory responses. Oral mucosal M $\phi$ s are important to ET; their activation status  
85 determining whether the mucosal environment is beneficial to the host or pathogen.  
86 PG modulates host cell function to benefit its own survival (Yilmaz, 2008).  
87 Interestingly, PG weakly induces pro-inflammatory cytokines, favouring insufficient  
88 clearing responses, bacterial proliferation and persistence. The cytokines produced  
89 to this expanded bacterial number contribute to localised tissue destruction  
90 characteristic of CP (Gemmell et al., 1997; Garlet, 2010).

91

92 M $\phi$ s densely populate oral mucosa, responding to PG by producing pro-inflammatory  
93 cytokines (TNF $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-18, IL-6, IL-12, IL-8, CCL2, CXCL10, MCP-1, IL-32)  
94 and lower anti-inflammatory cytokine levels (IL-10, TGF $\beta$ ) (Zhou et al., 2005; Barksby  
95 et al., 2009): a profile suggestive of the M1 pro-inflammatory M $\phi$  subset. In contrast,  
96 M $\phi$ s from non-infected homeostatic mucosal tissue, exhibit a cytokine phenotype  
97 resembling the anti-inflammatory/ regulatory M2 subset (TGF $\beta$ , IL-10 and low levels  
98 of pro-inflammatory cytokines) (Merry et al., 2012). PG-LPS-stimulated M1 M $\phi$   
99 cytokine profiles suggest M1 association with pro-inflammatory pathology, whereas  
100 M2 M $\phi$ s display a profile associated with regulatory/homeostatic conditions (Foey &  
101 Crean, 2013). These differential M $\phi$  responses correspond to expression of TLR2,  
102 TLR4 and their co-receptor molecule, CD14, driving potent inflammatory responses.  
103 CD14<sup>high</sup> M1 M $\phi$ s produce high level pro-inflammatory- and low level regulatory-  
104 cytokines, which if uncontrolled, results in higher levels of inflammation and

105 periodontal disease (Tervonen et al., 2007). Conversely, CD14<sup>low</sup> M2 M $\phi$ s predict a  
106 non-pathogenic, homeostatic mucosal effector phenotype (Smith et al., 2005). Thus,  
107 mucosal M $\phi$  effector phenotype (inflammatory vs regulatory) may be controlled by  
108 regulation of TLR-mediated signals.

109

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

122

123 The anti-inflammatory cytokine, IL-10, suppresses M $\phi$  inflammatory responses (Moore  
124 et al., 2001) and may be an important mediator of ET. The potential therapeutic value  
125 of IL-10, coupled with its limited success in clinical trials (Fedorak et al., 2000; Tilg et  
126 al., 2002), suggest the need to investigate its functionality and signalling in chronic  
127 inflammatory diseases such as CP. IL-10 signals are transduced by an IL-10R $\alpha$  and

128 IL-10R $\beta$  receptor complex preceding a signal cascade involving JAK1/TYK2 and  
129 STAT3 (Finbloom & Winestock, 1995). Crucially, STAT3 is associated with IL-10  
130 expression (Berkhout et al., 2000; Staples et al., 2007), anti-inflammatory function,  
131 inhibition of M $\phi$  activation (Donnelly et al., 1999) and the M2 M $\phi$  subset. STAT3  
132 conditional knockout in murine M $\phi$ s, rendered M $\phi$ s refractory to IL-10 and  
133 development of chronic enterocolitis (Riley et al., 1999; Takeda et al., 1999).

134

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

143

144 **Materials and methods:**

145 **Monocyte and macrophage (M $\phi$ ) culture**

146 The human monocytic cell line, THP-1, was obtained from ECACC (Salisbury, UK)  
147 and used between passages 7 and 25. THP-1 cells were maintained in RPMI-1640  
148 medium supplemented with 10% v/v foetal calf serum, 2 mM L-glutamine, 100 U/ml  
149 penicillin and 100  $\mu$ g/ml streptomycin (Lonza, Wokingham, UK), here on referred to as  
150 R10. Cells were plated out at  $1 \times 10^5$  cells/well in R10, in 96 well flat-bottomed tissue

151 culture plates. Pro-inflammatory (M1-like) M $\phi$ s and anti-inflammatory (M2-like) M $\phi$ s  
152 were generated by THP-1 monocyte differentiation in the presence of 25 ng/ml PMA  
153 or 10 nM 1,25-(OH) $_2$ -Vitamin D $_3$  (Sigma-Aldrich, Poole, UK) for 3 and 7 days,  
154 respectively (Daigneault et al., 2010). PMA and Vitamin D $_3$ -differentiated M $\phi$ s were  
155 phenotyped according to gene expression and compared favourably to the established  
156 phenotype of conventional M1 and M2 subsets: PMA M1-like M $\phi$ s were TNF $\alpha$  $^{\text{hi}}$ , IL-  
157 8 $^{\text{hi}}$ , IL-12 $^{\text{hi}}$ , iNOS $^{\text{hi}}$ , IL-10 $^{\text{lo}}$ , Arginase $^{\text{lo}}$  whereas Vitamin D $_3$  M2-like M $\phi$ s were TNF $\alpha$  $^{\text{lo}}$ ,  
158 IL-8 $^{\text{lo}}$ , IL-10 $^{\text{hi}}$ , Arginase $^{\text{hi}}$ , CD206 $^{\text{hi}}$ .

159

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

161 Due to the ability of *P.gingivalis* to induce inflammatory factors via membrane  
162 receptors, the effects of *P. gingivalis* lipopolysaccharide (PG-LPS) were studied. PG-  
163 LPS was obtained from Autogen Bioclear, Calne, UK. This PAMP was extracted from  
164 *P. gingivalis* strain ATCC 33277 originally isolated from human gingival sulcus  
165 (American Type Culture Collection). The suppliers extracted PG-LPS by successive  
166 enzymatic hydrolysis and purification by Phenol-TEA-DOC protocol (Hirschfeld et al.,  
167 2000), prior to being made commercially available.

168

169 **Activation of monocyte and macrophage cytokine production**

170 THP-1-derived M1- and M2-like M $\phi$ s were stimulated by the bacterial PAMP; 100  
171 ng/ml PG-LPS and cultured for 18 hours (determined as optimal concentration and  
172 time period for expression of the inflammatory cytokines TNF $\alpha$ , and IL-10, data not

173 shown). Supernatants were harvested and stored at -20°C until required for cytokine  
174 assay by sandwich ELISA.

175

176 **Induction of endogenous IL-10 activity and modulation of macrophage TNF $\alpha$**   
177 **production by exogenous IL-10**

178 Endogenous cell-associated IL-10 was assayed based on anti-inflammatory activity of  
179 IL-10 to suppress LPS-induced TNF $\alpha$ . M1and M2 M $\phi$ s were pre-treated with 10  $\mu$ g/ml  
180 9D7 neutralising anti-IL-10 antibody (Biolegend, San Diego, USA) or irrelevant,  
181 isotype-matched control antibody for 30 minutes prior to stimulation with 100ng/ml PG-  
182 LPS and cultured for 18 hours at 37°C, 5% CO<sub>2</sub>. In addition, M $\phi$  sensitivity to  
183 exogenous IL-10 was assayed where M1 and M2s were pre-treated with exogenously  
184 added IL-10 (0, 0.2, 2 and 20 ng/ml)(NIBSC, Potter's Bar, UK) for 30 minutes prior to  
185 stimulation with 100ng/ml PG-LPS and cultured for 18 hours. Supernatants were  
186 collected and assayed for TNF $\alpha$  secretion by sandwich ELISA. M $\phi$  viability was  
187 routinely checked by either MTT assay or trypan blue exclusion. A physiologically-  
188 relevant response was indicated, as no significant reductions in viability were observed  
189 for any treatments used in this study (viability routinely >85%).

190

191 **Cytokine measurement**

192 TNF $\alpha$  and IL-10 were analysed by sandwich ELISA using commercially available  
193 capture and detection antibodies (BD-Pharmingen, Oxford, UK). Protocols were  
194 followed according to manufacturer's instructions and compared to standard curves  
195 (range of 7 to 5,000 pg/ml), using international standards available from NIBSC.

196 Colorimetric development was measured spectrophotometrically by an OPTIMax  
197 tuneable microplate reader at 450 nm and analysed by Softmax Pro version 2.4.1  
198 software (Molecular Devices Corp., Sunnyvale, CA, USA).

199

200 **Flow cytometric analysis of IL-10 binding and IL-10R $\beta$**

201 IL-10 binding to the ligand-binding IL-10R $\alpha$  chain was determined using Fluorokine  
202 Biotinylated human interleukin-10 (R&D Systems, Abingdon, UK) according to  
203 manufacturer's instructions. Briefly, M $\phi$ s were washed and resuspended to  $1\times 10^5$  cells  
204 per tube. Biotinylated IL-10 was added to the cells and incubated on ice for 60 mins.  
205 Avidin-FITC was then added and incubated for 30 mins on ice. Finally, cells were  
206 washed, resuspended in FACS buffer for analysis. Specificity of IL-10 binding was  
207 determined using anti-IL-10 blocking antibody added to biotinylated IL-10 prior to  
208 introduction to M $\phi$ s. Measurement of the IL-10 signalling chain (IL-10R $\beta$ ) used a directly  
209 conjugated, PE-anti-IL-10R $\beta$  antibody according to manufacturer's instructions.  
210 Briefly, M $\phi$ s were washed and resuspended to  $1\times 10^5$  cells per tube. PE-anti-IL-10R $\beta$   
211 was added to the cells and incubated on ice for 60 mins. Stained cells were washed,  
212 resuspended in FACS buffer and analysed using a BD FACS Aria II flow cytometer.  
213 Data collected represented net mean fluorescent intensities using a live gating  
214 strategy set for FSC/SSC.

215

216 **Western blot analysis of phospho-STAT3**

217 M1 and M2-like M $\phi$ s were seeded in 12 well plates at  $1\times 10^6$  cells/ml. M $\phi$ s were pre-  
218 treated with IL-10 or anti-IL-10 for 1 hour prior to stimulation with 100 ng/ml PG-LPS

219 for 30 mins (optimal stimulation for STAT3 activation). Following stimulation, cells  
220 were lysed on ice for 15 min in lysis buffer (1% NP-40, 200 mM NaCl, 0.1mM  
221 ethylenediaminetetra-acetic acid, 1mM dithiothreitol, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM NaF, 1mM  
222 phenylmethylsulphonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 10 µg/ml  
223 aprotinin). Lysed samples (10µg) were separated on a 12% sodium dodecyl sulphate-  
224 polyacrylamide gel and Western blotted onto PVDF membrane (Thermo Fisher  
225 Scientific, Cramlington, UK). Phosphorylated STAT3 was detected using anti-  
226 phosphoSTAT3 (Tyr 705) antibody (New England BioLabs Ltd., UK) followed by anti-  
227 rabbit HRP conjugate (New England BioLabs Ltd., UK) and enhanced  
228 chemiluminescence (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, UK).  
229 STAT3 total protein was detected for the purpose of loading controls to the  
230 corresponding phospho-Western. Protein bands were scanned and visualised for  
231 chemiluminescence by a Typhoon Trio variable mode imager (GE Healthcare, UK).

232

### 233 **Statistical analysis**

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

238

239

240

241

242 **Results:**

243 **PG-LPS differentially induces M $\phi$  subset IL-10 production**

244 M1 and M2 M $\phi$ s produce different cytokine profiles; M1s exhibit a predominantly pro-  
245 inflammatory profile whereas M2s express a more anti-inflammatory/regulatory profile.  
246 This experiment was undertaken to establish whether M1 and M2 M $\phi$ s respond  
247 differently with respect to induction of IL-10 in response to PG. Indeed, PG-LPS  
248 induced distinct subset-specific cytokine profiles. PG-LPS induced minimal secretion  
249 of IL-10 by M1-like M $\phi$ s (secretion significant, p=0.0051, but close to lower level of  
250 sensitivity of ELISA), compared to M2 M $\phi$ s (figure 1a). M2-secreted IL-10 however,  
251 was measured at relatively low levels (120±10 pg/ml, p<0.0001). In addition to  
252 secretion, considering membrane bound IL-10 has been demonstrated (Fleming et al.,  
253 1999), this endogenous activity was also investigated using neutralising anti-IL-10  
254 antibody in the context of TNF $\alpha$  suppression. No endogenous activity was exhibited  
255 by unstimulated M $\phi$ s (figure 1b & 1c). Upon PG-LPS stimulation however, M $\phi$  subsets  
256 exhibited differential expression of endogenous IL-10 activity. PG-LPS failed to induce  
257 an endogenous suppressive IL-10 activity in M1s; induction of TNF $\alpha$  did not exhibit a  
258 statistically significant change (p=0.247) between isotype-matched control and  
259 neutralising anti-IL-10 antibody (Figure 1b). PG-LPS did, however, induce an  
260 endogenous IL-10 activity expressed by M2-like M $\phi$ s. Neutralisation of IL-10 activity  
261 augmented TNF $\alpha$  secretion by 20% (p=0.011, figure 1c).

262

263 **[ADD FIGURE 1 HERE]**

264

265 **M1- and M2-like M $\phi$ s differentially respond to IL-10**

266 Upon PG-LPS challenge, M $\phi$  subsets differentially produce secreted IL-10 and  
267 endogenous IL-10 activity. The responsiveness of these M $\phi$  subsets to the anti-  
268 inflammatory cytokine, IL-10, was investigated by IL-10 ability to suppress TNF $\alpha$ . In  
269 the case of pro-inflammatory M1-like M $\phi$ s, IL-10 failed to significantly suppress PG-  
270 LPS-induced TNF $\alpha$  secretion to a concentration of 20 ng/ml (figure 2). In contrast,  
271 M2-like M $\phi$ s were sensitive to the anti-inflammatory effects of IL-10; where IL-10 dose-  
272 dependently suppressed PG-LPS-induced TNF $\alpha$  by 75% at 20 ng/ml ( $p<0.01$ ) (figure  
273 2).

274 **[ADD FIGURE 2 HERE]**

275

276 **M1- and M2-like M $\phi$ s display similar IL-10 binding capability and signalling  
277 receptor, IL-10R $\beta$  expression**

278 This differential IL-10-sensitivity suggested different binding capabilities between M1-  
279 and M2-like M $\phi$ s. IL-10 binds the IL-10R  $\alpha$ -subunit; it was hypothesised that IL-10R $\alpha$ ,  
280 hence IL-10 binding capability varied between these subsets. This was investigated  
281 by IL-10 ligand-binding, which demonstrated IL-10 binding was both unaltered by PG-  
282 LPS stimulation or affected by M $\phi$  subset (figure 3a). There was no significant  
283 difference in binding activity between unstimulated M1 and M2 M $\phi$ s and that this  
284 binding activity was not significantly altered by PG-LPS for M1 ( $p=0.165$ ) or M2  
285 ( $p=0.749$ ) M $\phi$ s.

286

287 In light of the differential IL-10-sensitivity exhibited by these M $\phi$  subsets, which failed  
288 to be reflected by IL-10-binding activity (IL-10R $\alpha$ ), a further investigation was  
289 undertaken to observe whether this responsiveness was as a consequence of IL-10R  
290 signal transducing subunit, IL-10R $\beta$ . Both M1 and M2s expressed IL-10R $\beta$  in the  
291 absence of PG-LPS, with M2 M $\phi$ s expressing greater levels of this receptor subunit  
292 (MFI = 2000 AU compared to 1000 AU for M1s)(figure 3b). Upon PG-LPS stimulation  
293 however, both M1 and M2s exhibited significant down-regulation in IL-10R $\beta$  (M1s,  
294 p=0.0414 and M2s, p=0.0035).

295 **[ADD FIGURE 3 HERE]**

296

297 **STAT-3 is differentially activated by PG-LPS and IL-10 in M1- and M2-like M $\phi$ s**

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

307 **[ADD FIGURE 4 HERE]**

308

309 **DISCUSSION:**

310 Previous studies from this laboratory have shown M1- and M2-like M $\phi$ s to exhibit  
311 differential sensitivity to ET (Foey & Crean, 2013). In an attempt to elucidate  
312 mechanisms underlying this differential sensitivity, this investigation has drawn several  
313 conclusions with respect to M $\phi$  responses to the oral pathogen, *P. gingivalis*. Firstly,  
314 PG-LPS activation induces M $\phi$  subset-specific responses, where M2-like M $\phi$ s  
315 preferentially secrete IL-10 as well as an endogenous cell-associated activity,  
316 compared to M1-like M $\phi$ s. Secondly, M $\phi$ s also displayed a selective, subset-specific  
317 responsiveness to IL-10. M1 M $\phi$ s were refractory to the anti-inflammatory activity of  
318 IL-10 whereas M2s were sensitive. Finally, differential IL-10 responsiveness was not  
319 as a consequence of selective expression of IL-10-binding activity (IL-10R $\alpha$ ) or signal  
320 transduction receptor (IL-10R $\beta$ ), but reflected by activation of the downstream  
321 transcription factor, STAT3.

322

323 The pathological role for M $\phi$ s in chronic periodontitis is well established. Indeed, M $\phi$   
324 numbers have been shown to be increased above healthy control levels in CP gingival  
325 tissue biopsy samples (Gemmell et al., 2001; Lappin et al., 2000). *P.gingivalis*, the  
326 keystone pathogen to periodontitis, drives the gingipain-mediated activation of the M $\phi$   
327 urokinase plasminogen activator (uPA) system, resulting in a proteolytic cascade  
328 involved in inflammatory destruction and loss of bone architecture (Fleetwood et al.,  
329 2015). In addition, M $\phi$  depletion has been demonstrated to be associated with a  
330 reduction in inflammation and alveolar bone resorption, where the M1 subset  
331 predominates in response to *P.gingivalis* infection, resulting in an inflammatory  
332 phenotype defined by the increased secretion of TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p70, MCP-1

333 and MIP-1 $\alpha$  (Lam et al., 2014). The potential predominance of an M1-like subset in  
334 CP conforms to phenotypic observations of *P.gingivalis* stimulation of murine M $\phi$   
335 studies, where both PG-LPS and *P.gingivalis* bacteria induced expression of TNF $\alpha$ ,  
336 IL-12 and iNOS by IFN $\gamma$ -primed M1 M $\phi$ s (Holden et al., 2014; Lam et al., 2016). In  
337 this investigation, PG-LPS stimulation of human M1 and M2 M $\phi$  subsets demonstrated  
338 differing cytokine effector profiles; where M1s (representative of recruited, pro-  
339 inflammatory pathological M $\phi$ s) exhibited a pro-inflammatory phenotype (TNF $\alpha$ <sup>hi</sup>, IL-  
340 1 $\beta$ <sup>lo</sup>, IL-6<sup>lo</sup>, NF $\kappa$ B<sup>hi</sup>, IL-10<sup>lo</sup>) and M2s (representative of regulatory, anti-inflammatory  
341 mucosal M $\phi$ s) tended to be more anti-inflammatory/regulatory (TNF $\alpha$ <sup>lo</sup>, IL-1 $\beta$ <sup>hi</sup>, IL-6<sup>hi</sup>,  
342 NF $\kappa$ B<sup>lo</sup>, IL-10<sup>+</sup>, TGF $\beta$ <sup>+</sup>) (Foey & Crean, 2013; Holden et al., 2014). Mucosal M $\phi$ s are  
343 considered to exist in these discrete functional subsets, governed by environmental  
344 stimuli (Smythies et al., 2005; Foey, 2012). This investigation demonstrated IL-10  
345 production conforms to this functional subset dichotomy with respect to M2 M $\phi$ s  
346 exhibiting higher levels of both secreted and endogenous cell-associated IL-10 when  
347 stimulated by PG-LPS. Conversely, M1 M $\phi$ s failed to display an endogenous IL-10  
348 activity and resulted in low level secretion.

349

350 IL-10 regulates/suppresses pro-inflammatory responses (Fiorentino et al., 1991). This  
351 anti-inflammatory effect can be elicited at many levels; including, down-regulating  
352 TNF $\alpha$  secretion, suppressing TNF $\alpha$  activity via inducing TNF-R shedding or by  
353 modulating NF $\kappa$ B-signalling events driving TNF $\alpha$  production (Schottelius et al., 1999).  
354 Although these M $\phi$  subsets have been characterised with respect to TNF $\alpha$  and IL-10,  
355 the level of cross-regulation and plasticity exhibited by these subsets is highlighting  
356 the importance to characterise subset responsiveness to regulatory cytokines such as

357 IL-10. Our data clearly establishes a differential responsiveness of M $\phi$  subsets to IL-  
358 10, where M1s were refractory and M2s sensitive to the anti-inflammatory effects of  
359 IL-10. This sensitivity of M2s to IL-10 suppression of TNF $\alpha$  would appear to be  
360 independent of subset-specific binding activity and signal transduction receptor and is  
361 reflected by differential utilisation of downstream intracellular signalling pathways.

362

363 The desired outcome would be to harness anti-inflammatory properties of IL-10;  
364 controlling inflammatory mechanisms in chronic diseases such as CP. This data  
365 introduces two problems: 1) lack of M $\phi$  subset selectivity of IL-10 binding and 2) the  
366 IL-10-responsive subset is the M2 regulatory subset whereas the M1 pro-inflammatory  
367 pathological subset is unresponsive. This study may suggest why IL-10 treatment has  
368 exhibited limited success in suppressing M $\phi$ -driven chronic inflammatory diseases  
369 such as RA, CD and psoriasis (Fedorak et al., 2000; Tilg et al., 2002; McInnes et al.,  
370 2001). The specificity of IL-10-mediated anti-inflammatory response would appear to  
371 result from differential activation and utilisation of STAT3 (Benkhart et al., 2000). PG-  
372 LPS activates STAT3 in M2 M $\phi$ s, this response is indirect through IL-10; neutralisation  
373 of which abrogated the activation response. Due to the short activation time used in  
374 this experiment (30 mins), the IL-10-dependency does not result from *de novo* IL-10  
375 synthesis but is likely to result from a pre-existing endogenous IL-10 activity observed  
376 for M2s. Interestingly, PG-LPS stimulation in the presence of exogenous IL-10  
377 dramatically augmented STAT3 activation. Coupled with the fact that results showed  
378 a PG-LPS-induced decrease in IL-10R $\beta$  and no change for IL-10R $\alpha$ , the PG-LPS  
379 augmentation of STAT3 activation would appear to be both IL-10-dependent in early  
380 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 $\phi$  polarisation. Both IL-10 and STAT3  
384 influence M $\phi$  plasticity and differentiation towards the M2 effector subset. Conditional  
385 knock-out of M $\phi$  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 $\phi$  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 $\phi$  plasticity towards this  
392 pro-inflammatory phenotype (Liu et al., 2008). Manipulation of M $\phi$  effector phenotype  
393 via controlling plasticity between M1 and M2 subsets, or specific M $\phi$  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 $\phi$  sensitivity to the anti-  
402 inflammatory effects of IL-10 however, is suggestive of a disease stage whereby  
403 homeostatic M2-like M $\phi$ s are suppressed by IL-10 whereas the pro-inflammatory

404 nature of M1 M $\phi$ s is untouched. Such a differential suppression may be beneficial for  
405 controlling pathogen numbers but will be detrimental to tissue repair.

406

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

420

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427

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556

557 **Figure legends:**

558 **Figure 1. M1 & M2 M $\phi$ s display differential IL-10 profiles in response to PG-LPS.**  
559 THP-1-derived M1 and M2 M $\phi$ 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-

561 (OH)<sub>2</sub> vitamin D<sub>3</sub> for 7 days, respectively. Secretion of IL-10 (a) is depicted where M1  
562 (bold) and M2 (shaded) M $\phi$  subsets were stimulated with or without (control) 100ng/ml  
563 PG-LPS. Endogenous IL-10 activity, upon treatment with 10 $\mu$ g/ml neutralising anti-IL-  
564 10 compared to an isotype-matched control antibody (IC), is represented for PG-LPS-  
565 stimulated and unstimulated M1 (b) and M2 (c) macrophage TNF $\alpha$  secretion. Cytokine  
566 production is expressed as the mean $\pm$ SD in pg/ml for IL-10 (a) and TNF $\alpha$  (b & c). Data  
567 displayed represents triplicate samples for n=3 replicate experiments. Significant  
568 differences in cytokine production between activated M1 and M2 M $\phi$ s and  
569 unstimulated controls and between isotype control and neutralising IL-10 antibody  
570 treatment are indicated as \*p<0.05, \*\*p<0.01, \*\*\*P<0.001 and ns, not significant.

571

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

580

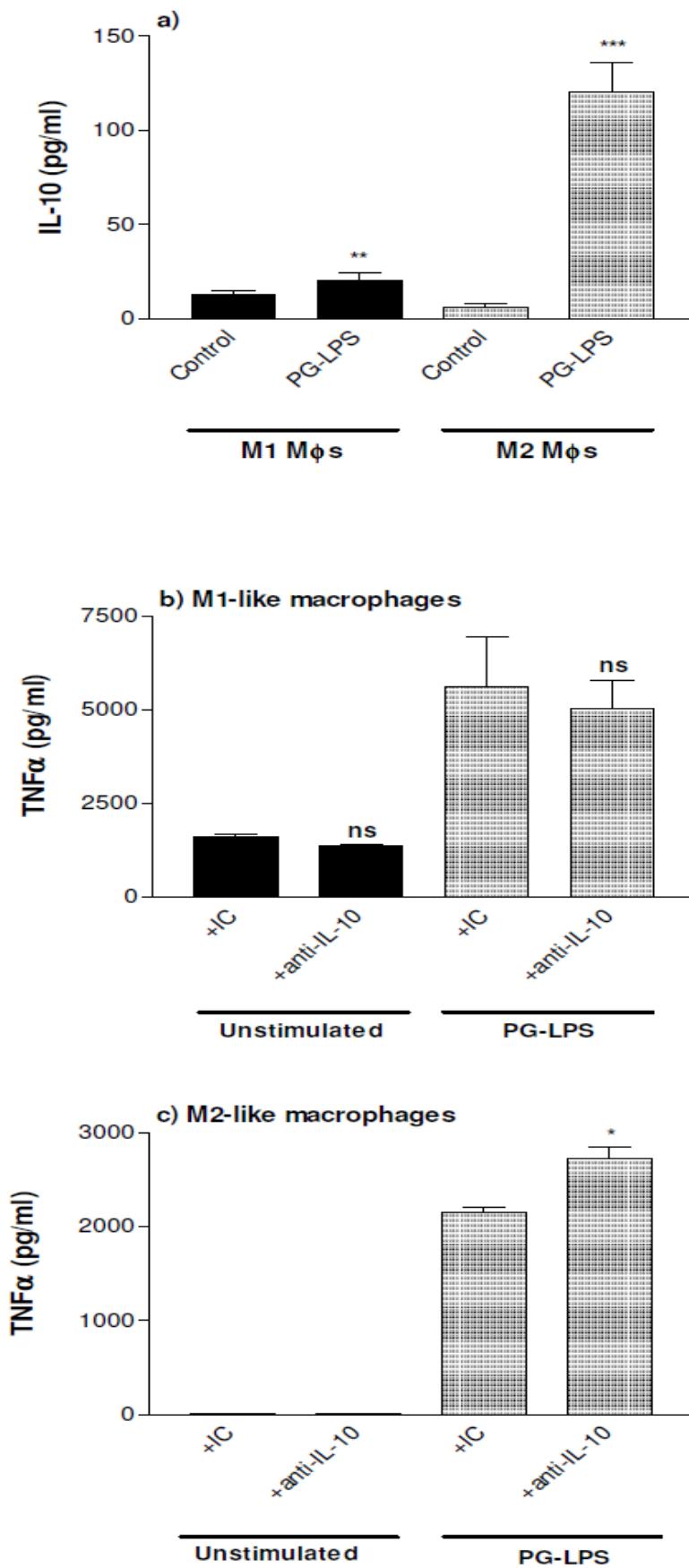
581 **Figure 3. M1 and M2 M $\phi$ s exhibit similar IL10R subunit expression in response**  
582 **to PG-LPS.** THP-1-derived M1 and M2 M $\phi$ s were generated by differentiating THP-1  
583 monocytes with either 25ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days or

584 10nM 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> for 7 days, respectively. FITC-labelled IL-10 binding to  
585 the IL-10R $\alpha$  ligand-binding subunit (a) and anti-IL-10R $\beta$  antibody binding of the  
586 signalling subunit (b) is depicted in net MFI $\pm$ SD for M1 and M2 M $\phi$ 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 $\phi$  controls are indicated as \*p<0.05,  
590 \*\*p<0.01 and ns, not significant.

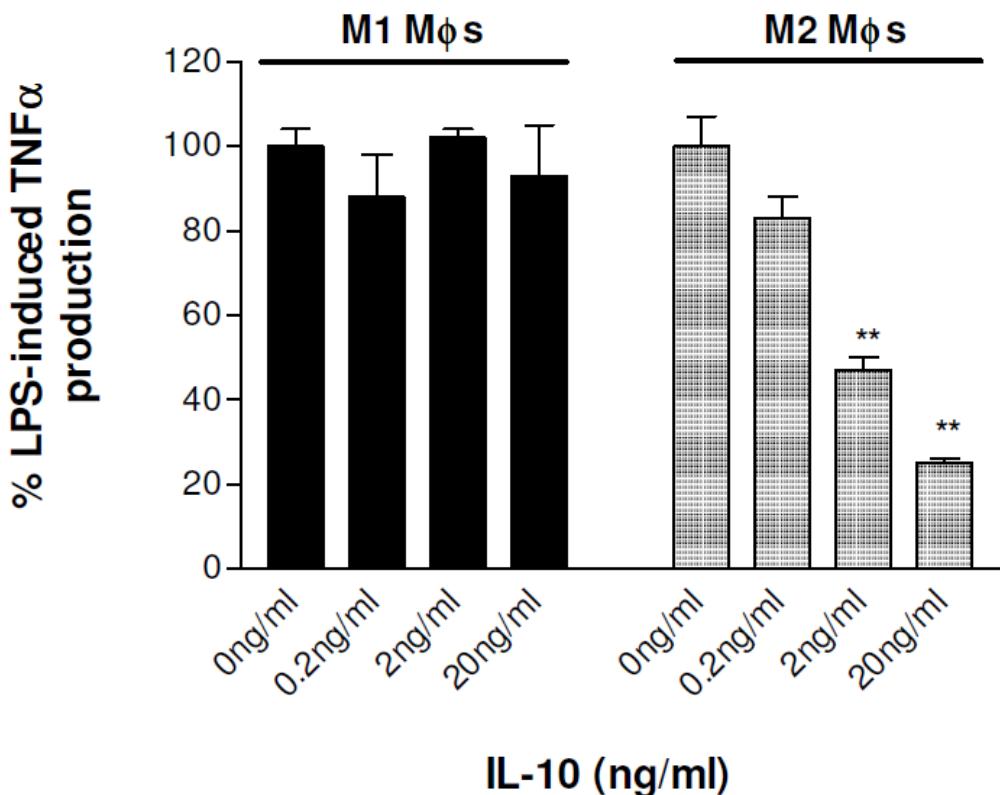
591

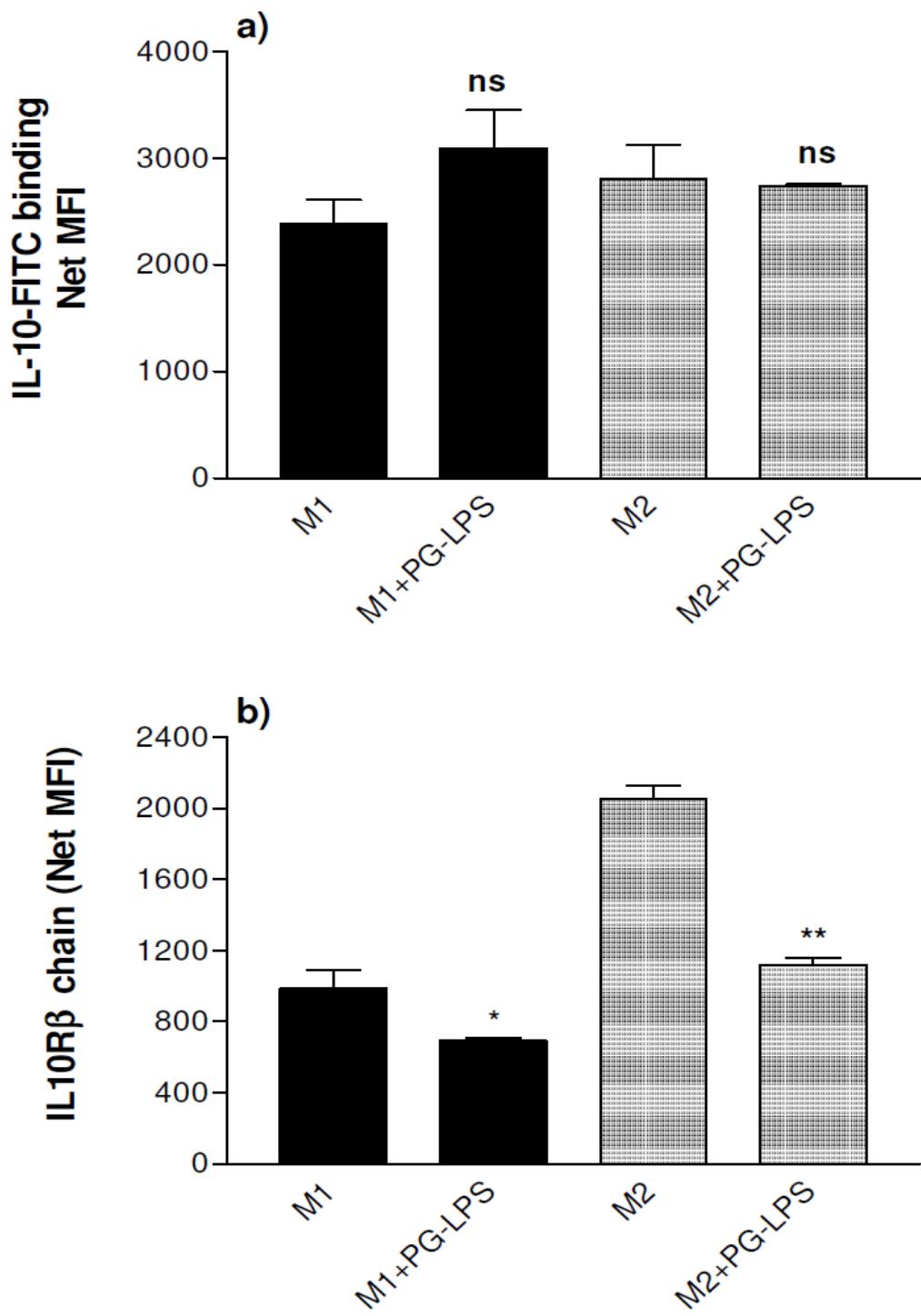
592 **Figure 4. STAT3 is differentially activated by PG-LPS and IL-10 in M1 and M2**  
593 **M $\phi$ s.** THP-1-derived M1 and M2 M $\phi$ s were plated out at 5x10<sup>6</sup> cells per well in a flat-  
594 bottomed 12 well plate and treated with PG-LPS (100 ng/ml) in the presence or  
595 absence of exogenously added IL-10 (10ng/ml) or neutralising anti-IL-10 antibody  
596 (10 $\mu$ 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 $\phi$ 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.



Foey et al., 2016 Figure 2.





604

605

Foey et al., 2016 Figure 4.

