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

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14

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

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26

27 ***Abbreviations:*** *Mφs, macrophages; IL-10, Interleukin-10; IL-10R, Interleukin-10 Receptor, TNFα,*
28 *tumour necrosis factor-α; 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; NFκB, nuclear factor-kappa B; SOCS-3, suppressor of cytokine signalling-*
31 *3; PAMPs, pathogen associated molecular patterns.*

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35

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 D_3, 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 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

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 Mϕs 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 Mϕs densely populate oral mucosa, responding to PG by producing pro-inflammatory
93 cytokines (TNFα, IL-1α, IL-1β, IL-18, IL-6, IL-12, IL-8, CCL2, CXCL10, MCP-1, IL-32)
94 and lower anti-inflammatory cytokine levels (IL-10, TGFβ)(Zhou et al., 2005; Barksby
95 et al., 2009): a profile suggestive of the M1 pro-inflammatory Mϕ subset. In contrast,
96 Mϕs from non-infected homeostatic mucosal tissue, exhibit a cytokine phenotype
97 resembling the anti-inflammatory/ regulatory M2 subset (TGFβ, IL-10 and low levels
98 of pro-inflammatory cytokines) (Merry et al., 2012). PG-LPS-stimulated M1 Mϕ
99 cytokine profiles suggest M1 association with pro-inflammatory pathology, whereas
100 M2 Mϕs display a profile associated with regulatory/homeoatatic conditions (Foey &
101 Crean, 2013). These differential Mϕ responses correspond to expression of TLR2,
102 TLR4 and their co-receptor molecule, CD14, driving potent inflammatory responses.
103 $CD14^{high}$ M1 Mϕs 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^{low} M2 Mϕs predict a
106 non-pathogenic, homeostatic mucosal effector phenotype (Smith et al., 2005). Thus,
107 mucosal Mϕ effector phenotype (inflammatory vs regulatory) may be controlled by
108 regulation of TLR-mediated signals.

109

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

122

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

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

134

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

143

144 **Materials and methods:**

145 **Monocyte and macrophage (Mϕ) 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 μg/ml streptomycin (Lonza, Wokingham, UK), here on referred to as
150 R10. Cells were plated out at $1x10^5$ cells/well in R10, in 96 well flat-bottomed tissue

151 culture plates. Pro-inflammatory (M1-like) Mϕs and anti-inflammatory (M2-like) Mϕs
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 Mϕs were
155 phenotyped according to gene expression and compared favourably to the established
156 phenotype of conventional M1 and M2 subsets: PMA M1-like Mϕs were $TNF\alpha^{hi}$, IL-
157 8^{hi}, IL-12^{hi}, $iNOS^{hi}$, IL-10^{lo}, $Arginase^{lo}$ whereas Vitamin D_3 M2-like Mϕs were $TNF\alpha^{lo}$,
158 IL-8^{lo}, IL-10^{hi}, $Arginase^{hi}$, $CD206^{hi}$.

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 Mϕs 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 TNFα**
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 TNFα. M1and M2 Mϕs were pre-treated with 10 μg/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_2. In addition, Mϕ 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 TNFα secretion by sandwich ELISA. Mϕ 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 TNFα 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-10Rβ**

201 IL-10 binding to the ligand-binding IL-10Rα chain was determined using Fluorokine
202 Biotinylated human interleukin-10 (R&D Systems, Abingdon, UK) according to
203 manufacturer's instructions. Briefly, Mϕs were washed and resuspended to $1x10^5$ 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 Mϕs. Measurment of the IL-10 signalling chain (IL-10Rβ) used a directly
209 conjugated, PE-anti-IL-10Rβ antibody according to manufacturer's instructions.
210 Briefly, Mϕs were washed and resuspended to $1x10^5$ cells per tube. PE-anti-IL-10Rβ
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 Mϕs were seeded in 12 well plates at $1x10^6$ cells/ml. Mϕs 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_3VO_4, 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 Mϕ subset IL-10 production**

244 M1 and M2 Mϕs 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 Mϕs 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 Mϕs (secretion significant, $p=0.0051$, but close to lower level of
250 sensitivity of ELISA), compared to M2 Mϕs (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 TNFα suppression. No endogenous activity was exhibited
255 by unstimulated Mϕs (figure 1b & 1c). Upon PG-LPS stimulation however, Mϕ 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 TNFα 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 Mϕs. Neutralisation of IL-10 activity
261 augmented TNFα secretion by 20% ($p=0.011$, figure 1c).

262

263 ***[ADD FIGURE 1 HERE]***

264

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).

[ADD FIGURE 2 HERE]

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.

287 In light of the differential IL-10-sensitivity exhibited by these Mϕ subsets, which failed
288 to be reflected by IL-10-binding activity (IL-10Rα), a further investigation was
289 undertaken to observe whether this responsiveness was as a consequence of IL-10R
290 signal transducing subunit, IL-10Rβ. Both M1 and M2s expressed IL-10Rβ in the
291 absence of PG-LPS, with M2 Mϕs 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-10Rβ (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 Mϕs**

298 Down-regulation of the receptor signalling subunit in both M1 and M2 Mϕs 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 Mϕs 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 Mϕs, 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 Mϕs 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 Mϕ responses to the oral pathogen, *P. gingivalis*. Firstly,
314 PG-LPS activation induces Mϕ subset-specific responses, where M2-like Mϕs
315 preferentially secrete IL-10 as well as an endogenous cell-associated activity,
316 compared to M1-like Mϕs. Secondly, Mϕs also displayed a selective, subset-specific
317 responsiveness to IL-10. M1 Mϕs 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-10Rα) or signal
320 transduction receptor (IL-10Rβ), but reflected by activation of the downstream
321 transcription factor, STAT3.

322

323 The pathological role for Mϕs in chronic periodontitis is well established. Indeed, Mϕ
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 Mϕ
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, Mϕ 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 TNFα, IL-1β, IL-6, IL-12p70, MCP-1

333 and MIP-1α (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 Mϕ
335 studies, where both PG-LPS and *P.gingivalis* bacteria induced expression of TNFα,
336 IL-12 and iNOS by IFNγ-primed M1 Mϕs (Holden et al., 2014; Lam et al., 2016). In
337 this investigation, PG-LPS stimulation of human M1 and M2 Mϕ subsets demonstrated
338 differing cytokine effector profiles; where M1s (representative of recruited, pro-
339 inflammatory pathological Mϕs) exhibited a pro-inflammatory phenotype (TNFα^{hi}, IL-
340 1β^{lo}, IL-6^{lo}, NFkB^{hi},IL-10^{lo}) and M2s (representative of regulatory, anti-inflammatory
341 mucosal Mϕs) tended to be more anti-inflammatory/regulatory (TNFα^{lo}, IL-1β^{hi}, IL-6^{hi},
342 NFkB^{lo}, IL-10^{+}, TGFβ^{+}) (Foey & Crean, 2013; Holden et al., 2014). Mucosal Mϕs 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 Mϕs
346 exhibiting higher levels of both secreted and endogenous cell-associated IL-10 when
347 stimulated by PG-LPS. Conversely, M1 Mϕs 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 TNFα secretion, suppressing TNFα activity via inducing TNF-R shedding or by
353 modulating NFκB-signalling events driving TNFα production (Schottelius et al., 1999).
354 Although these Mϕ subsets have been characterised with respect to TNFα 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 Mϕ 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 TNFα 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 Mϕ 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 Mϕ-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 Mϕs, 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-10Rβ and no change for IL-10Rα, 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 Mϕ polarisation. Both IL-10 and STAT3
384 influence Mϕ plasticity and differentiation towards the M2 effector subset. Conditional
385 knock-out of Mϕ STAT3, renders mice refractory to IL-10 and develop chronic
386 enterocholitis (Riley et al., 1999; Takeda et al., 1999); although STAT3 is necessary
387 for IL-10 signalling, additional pathways are required for Mϕ 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 Mϕ plasticity towards this
392 pro-inflammatory phenotype (Liu et al., 2008). Manipulation of Mϕ effector phenotype
393 via controlling plasticity between M1 and M2 subsets, or specific Mϕ 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 Mϕ sensitivity to the anti-
402 inflammatory effects of IL-10 however, is suggestive of a disease stage whereby
403 homeostatic M2-like Mϕs are suppressed by IL-10 whereas the pro-inflammatory

404 nature of M1 Mϕs 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 Mϕ subsets with
408 respect to PG-LPS-induced activation/suppression decisions. Selective Mϕ subset
409 tolerisation and responsiveness to anti-inflammatory cytokines affords us a better
410 understanding of the role of distinct Mϕ 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 Mϕ
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.
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427

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555 FimA protein. *Infect. Immun.*, 73(2), 935-943.

556

557 **Figure legends:**

558 **Figure 1. M1 & M2 Mϕs display differential IL-10 profiles in response to PG-LPS.**
559 THP-1-derived M1 and M2 Mϕs 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 $(OH)_2$ vitamin D_3 for 7 days, respectively. Secretion of IL-10 (a) is depicted where M1
562 (bold) and M2 (shaded) Mϕ subsets were stimulated with or without (control) 100ng/ml
563 PG-LPS. Endogenous IL-10 activity, upon treatment with 10μg/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 TNFα secretion. Cytokine
566 production is expressed as the mean±SD in pg/ml for IL-10 (a) and TNFα (b & c). Data
567 displayed represents triplicate samples for n=3 replicate experiments. Significant
568 differences in cytokine production between activated M1 and M2 Mϕs 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 Mϕ TNFα to IL-10.** M1 (bold)
573 and M2 (shaded) Mϕ 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 TNFα cytokine production is expressed as percentage
576 of LPS-induced TNFα production originally represented as mean±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 Mϕ subsets as **p<0.01 and ns, not significant.

580

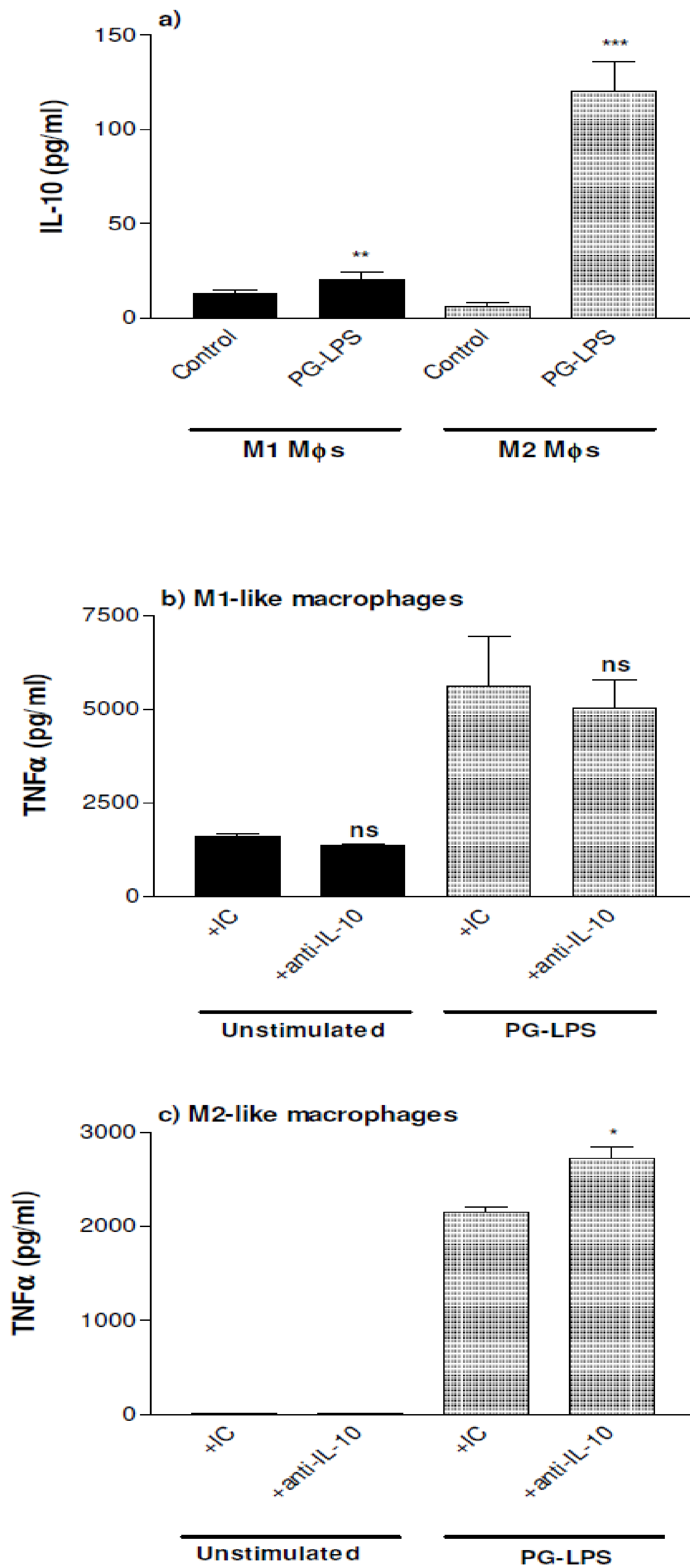
581 **Figure 3. M1 and M2 Mϕs exhibit similar IL10R subunit expression in response**
582 **to PG-LPS.** THP-1-derived M1 and M2 Mϕs were generated by differentiating THP-1
583 monocytes with either 25ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days or

584 10nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. FITC-labelled IL-10 binding to
585 the IL-10Rα ligand-binding subunit (a) and anti-IL-10Rβ antibody binding of the
586 signalling subunit (b) is depicted in net MFI±SD for M1 and M2 Mϕs 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 Mϕ 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 **Mϕs.** THP-1-derived M1 and M2 Mϕs were plated out at $5x10^6$ 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 (10μg/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) Mϕs. Loading controls are presented as total STAT3 blots below
599 the corresponding phospho-Westerns. Data displayed are representative of three
600 replicate experiments.

Foey at el. 2016 Figure 1.



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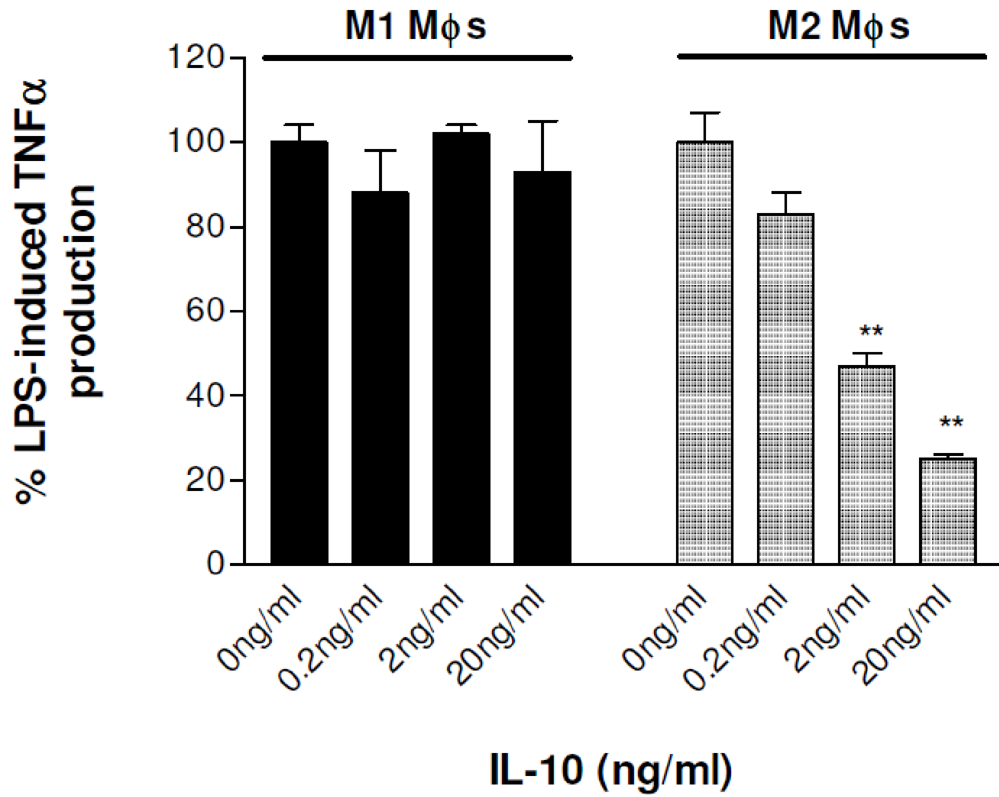

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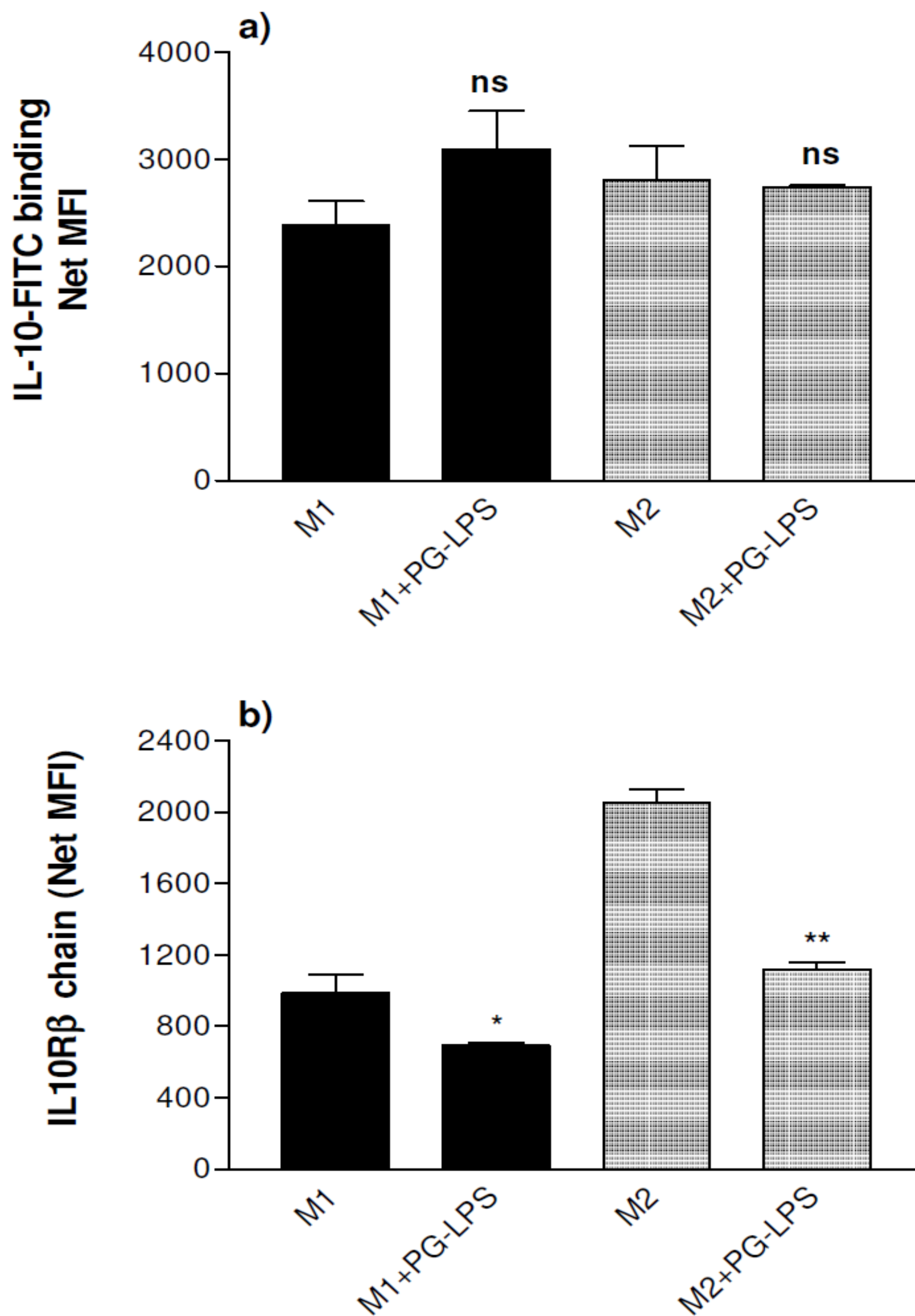

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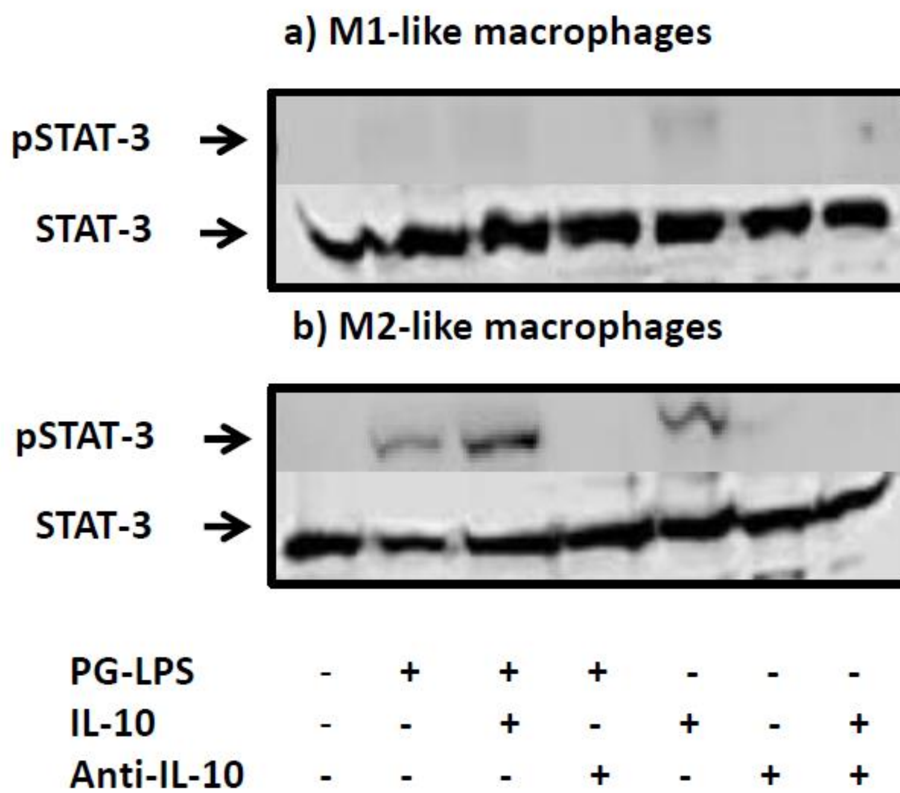
Foey et al., 2016 Figure 4.



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