

1 **Hif-1 $\alpha$  stabilisation polarises macrophages via cyclooxygenase/prostaglandin E2 *in***

2 ***vivo***

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## 29 **Abstract**

30           Macrophage subtypes are poorly characterised in disease systems *in vivo*. The initial  
31 innate immune response to injury and infectious stimuli through M1 polarisation is important  
32 for the outcome of disease. Appropriate macrophage polarisation requires complex  
33 coordination of local microenvironmental cues and cytokine signalling to influence immune  
34 cell phenotypes. If the molecular mechanisms behind macrophage polarisation were better  
35 understood then macrophages could be pharmacologically tuned to better deal with bacterial  
36 infections, for example tuberculosis. Here, using zebrafish *tnfa:GFP* transgenic lines as *in*  
37 *vivo* readouts of M1 macrophages, we show that hypoxia and stabilisation of Hif-1 $\alpha$   
38 polarises macrophages to a *tnfa* expressing phenotype. We demonstrate a novel  
39 mechanism of Hif-1 $\alpha$  mediated macrophage *tnfa* upregulation via a  
40 cyclooxygenase/prostaglandin E2 axis, a mechanism that is conserved in human primary  
41 macrophages. These findings uncover a novel macrophage HIF/COX/TNF axis that links  
42 microenvironmental cues to macrophage phenotype that may have implications in  
43 inflammation, infection and cancer, where hypoxia is a common microenvironmental feature  
44 and where cyclooxygenase and *Tnfa* are major mechanistic players.

45

## 46 **Introduction**

47           The innate immune response to injury and pathogen invasion are complex and are  
48 tightly regulated by coordination of microenvironmental cues and cytokine signalling.  
49 Macrophages are important innate immune cells in disease and their activation states,  
50 commonly termed polarisation states, are especially important during the early response to  
51 injury and infection. Mammalian macrophages have been classified into two broad  
52 polarisation/activation states; M1 (or classically activated) and M2 (alternatively activated)  
53 (1). Pro-inflammatory, or M1, macrophages are highly antimicrobial and can phagocytose  
54 and efficiently kill bacteria. Macrophages are also central players in tissue healing and  
55 restoration of homeostasis post injury/infection, which requires a change in macrophage

56 phenotype to a wound healing, M2, state (2). A diverse variety in macrophage function has  
57 been observed in many *in vitro* systems, yet control of macrophage subsets remains poorly  
58 understood *in vivo*. Current classifications are based on *in vitro* mammalian datasets, using  
59 monocytes that have been polarised to different macrophage phenotypes by addition of  
60 cytokine-cocktails to the media (3). Specific subsets of macrophage polarisation are context  
61 dependent *in vivo* and subtle changes in the microenvironment can lead to different  
62 phenotypes of M1 and M2 macrophages, reflecting a spectrum rather than a binary  
63 classification of behaviours (4). One important microenvironmental factor is hypoxia, which  
64 has profound effects on macrophage phenotypes (5). To fully understand how  
65 microenvironmental cues affect macrophage polarisation, *in vivo* systems are required to  
66 study macrophage behaviour over time and space without disturbance from extrinsic factors.  
67 Determining the molecular triggers and polarisation states of macrophages could open up  
68 possibilities to manipulate macrophage phenotype during disease as novel therapeutic  
69 avenues.

70         The tissue macrophage response to injury/bacterial infections is a rapid, pathogen  
71 recognition receptor (PPR) mediated, switch to an M1 type phenotype, characterised by  
72 production and release of pro-inflammatory mediators, including cytokines such as IL-1 $\beta$ , IL-  
73 6, IL-12 and TNF (6, 7). This phenotypic switch is required for a successful response to the  
74 invading pathogen and for clearance before infection can take hold (8, 9). However, in the  
75 case of intracellular pathogens, such as *Mycobacterium tuberculosis* (Mtb), this macrophage  
76 response is subverted, establishing a protective niche within innate immune cells (10–13).  
77 This bacterial-mediated interference in macrophage polarisation highlights a gap for  
78 potential therapeutic tuning of the macrophage response to allow for bacterial clearance (14,  
79 15).

80         One potential macrophage tuning mechanism is via the Hypoxia Inducible Factor  
81 (HIF) -1 $\alpha$  transcription factor (16). HIF-1 $\alpha$  is a master transcriptional regulator of the cellular  
82 response to low levels of oxygen (hypoxia) (17). Leukocytes, including macrophages and

83 neutrophils, are exquisitely sensitive to tissue hypoxia, a signature of many disease  
84 microenvironments (eg infections and cancer) due to lack of local blood supply and a high  
85 turnover of local oxygen by pathogens (5, 18). Hypoxia and HIF-1 $\alpha$  stabilisation have been  
86 shown to have activating effects on macrophages *in vitro*, increasing their M1 profile and  
87 bactericidal capabilities, yet the pro-inflammatory mechanisms behind this are not well  
88 understood *in vivo* (5).

89 Hypoxia is a microenvironmental signal that increases epithelial  
90 cyclooxygenase/prostaglandin E2 production in cancer models (19–21). Eicosanoids are  
91 lipid signalling molecules and important pro-inflammatory mediators which are produced,  
92 and released, by macrophages during early microbial infection (22, 23). The best  
93 characterised of these are prostaglandins, strong inflammatory mediators that are known to  
94 affect macrophage polarisation states during infection (24). A key family of enzymes for  
95 prostaglandin biosynthesis is the cyclooxygenases (COX). COX-1 and -2 enzymes are  
96 responsible for the production of prostaglandins as breakdown products of arachidonic acid.  
97 Prostaglandins, in turn, are highly immunomodulatory, synergising with cytokines to amplify  
98 pro-inflammatory responses (25). The potential effects of hypoxia-induced prostaglandins on  
99 macrophage polarisation have not been investigated *in vivo*. Recent evidence indicates that  
100 a HIF/COX/TNF axis may exist, with hypoxia-induced TNF expression in osteoblasts (bone  
101 producing cells) being mediated via cyclooxygenase enzymes by an, as yet, unknown  
102 mechanism (26). If observed in macrophages, this HIF/COX/TNF axis could have important  
103 pro-inflammatory outcomes in disease and represent a novel therapeutic avenue.

104 Zebrafish have emerged as a useful model to understand innate immune cells in  
105 their *in vivo* microenvironment (27). Development of multiple innate immune cell transgenic  
106 lines, combined with non-invasive fluorescence imaging, has enabled mechanistic  
107 investigation into macrophage biology (2, 28, 29). Well-characterised models of inflammation  
108 and infection biology have emerged using zebrafish larvae. Mechanistic insights into  
109 neutrophil and macrophage recruitment to an injury site have been gained through use of a  
110 tailfin transection model (30), while host-pathogen interactions have been studied using the

111 natural fish pathogen *Mycobacterium marinum* (Mm) as a model of tuberculosis (27).  
112 Recently, it has been demonstrated that *tnfa* promoter driven fluorescent transgenic lines  
113 display an M1 type phenotype upon sterile injury immune challenge (31, 32). This advance  
114 has enabled us to investigate macrophage polarisation within the zebrafish system during a  
115 host-pathogen immune challenge. We have previously demonstrated that Hif-1 $\alpha$  stabilisation  
116 induces macrophage proinflammatory *il-1 $\beta$*  transcription and that this is host-protective  
117 against Mm infection in the zebrafish model (33). We therefore hypothesised that Hif-1 $\alpha$   
118 stabilisation would promote macrophage pro-inflammatory *tnfa* expression, via a  
119 cyclooxygenase dependent mechanism, that could also be beneficial to the host during  
120 infection.

121 Here, we investigated the regulation of expression of the important M1 pro-  
122 inflammatory cytokine *tnfa* by Hif-1 $\alpha$  stabilisation *in vivo*. We demonstrate that genetic,  
123 pharmacological and hypoxia-mediated Hif-1 $\alpha$  stabilisation upregulated macrophage *tnfa* in  
124 zebrafish. We show that *tnfa* transcription is upregulated in macrophages after injury and  
125 Mm infection (both at early- and granuloma- stage infection). We identify that Hif-1 $\alpha$   
126 activates macrophage *tnfa* via cyclooxygenase/prostaglandin E2, while injury/Mm driven *tnfa*  
127 production does not require active cyclooxygenase. Importantly, this novel macrophage  
128 HIF/COX/TNF axis is conserved in primary human macrophages. These findings have  
129 important implications in inflammation, infection and cancer biology where macrophage  
130 polarisation states are influenced by microenvironmental hypoxia.

131

## 132 **Materials and Methods**

133

### 134 **Zebrafish**

135 Zebrafish were raised and maintained on a 14:10-hour light/dark cycle at 28°C,  
136 according to standard protocols (34), in UK Home Office approved facilities at The Bateson  
137 Centre aquaria at the University of Sheffield. Strains used were Nacre (wildtype),

138 *TgBAC(tnfa:GFP)pd1028*, *Tg(tnfa:eGFP-F)ump5Tg*, *Tg(mpeg1:mCherry-F)ump2Tg*,  
139 *Tg(mpeg1:mCherryCAAX)sh378*, *Tg(lyz:Ds-RED2)nz50* *TgBAC(il-1 $\beta$ :eGFP)sh445* and  
140 *Tg(phd3:EGFP)i144* (31, 32, 35–37).

141

#### 142 **Tailfin injury**

143 Inflammation was induced in zebrafish embryos by tail transection as previously  
144 described (38). Embryos were anaesthetised at 2 days post fertilisation (dpf) by immersion  
145 in 0.168mg/ml Tricaine, and transection of the tail was performed using a scalpel blade.  
146 Larvae were imaged by confocal microscopy at 16 hour post wound (hpw) on a Leica TCS-  
147 SPE confocal on an inverted Leica DMI8 base and imaged using 20x or 40x objective  
148 lenses.

149

#### 150 ***Mycobacterium marinum***

151 Mm infection experiments were performed using *M. marinum* M (ATCC #BAA-535),  
152 containing a psMT3-mCherry or psMT3 mCrimson vector (39). Injection inoculum was  
153 prepared from an overnight liquid culture in the log-phase of growth resuspended in 2%  
154 polyvinylpyrrolidone40 (PVP40) solution (CalBiochem) as previously described (40). 100 or  
155 150 colony forming units (CFU) were injected into the caudal vein at 28-30hpf as previously  
156 described (41).

157

#### 158 **Confocal microscopy of transgenic larvae**

159 1dpi and 4dpi transgenic zebrafish larvae infected with fluorescent Mm strains were  
160 mounted in 0.8-1% low melting point agarose (Sigma-Aldrich) and imaged on a Leica TCS-  
161 SPE confocal on an inverted Leica DMI8 base and imaged using 20x or 40x objective  
162 lenses.

163 For quantification purposes acquisition settings and area of imaging were kept the  
164 same across groups. Corrected total cell fluorescence was calculated for each cell using  
165 Image J as previously described (42, 43).

166

### 167 **RNA injections**

168 Embryos were injected with dominant *hif-1ab* (ZFIN: *hif1ab*) variant RNA at the one  
169 cell stage as previously described (44). *hif-1a* variants used were dominant active (DA) and  
170 dominant negative (DN) *hif-1a*. Phenol red (PR) (Sigma Aldrich) was used as a vehicle  
171 control.

172

### 173 **Pathway inhibitors**

174 Unless otherwise stated, embryos were treated from 4 hours pre-Mm infection to 24  
175 hours post infection (hpi) by addition to the embryo water and DMSO was used as a  
176 negative solvent control. The pan hydroxylase inhibitor, DMOG (dimethylloxaloylglycine,  
177 Enzo Life Sciences), was used at a 100 $\mu$ M concentration by incubation in E3 embryo media  
178 as previously described (44). The selective PHD inhibitors JNJ-402041935 (Cayman  
179 Chemicals) and FG-4592 (Selleckchem) were used at 100 $\mu$ M and 5 $\mu$ M respectively (45, 46).  
180 The selective inhibitor of COX-1, SC-560 (Cayman Chemicals), was used at 30 $\mu$ M and the  
181 selective inhibitor of COX-2, NS-398 (Cayman Chemicals), was used at 15 $\mu$ M by incubation  
182 in E3 embryo media as previously described (47). The 15-LOX inhibitor PD146176 (Tocris)  
183 was microinjected (1nl of 40nM) at 1 hour post infection (hpi). The leukotriene B4 receptor 1  
184 (BLTR1) inhibitor, U-75302 (Abcam), was used at 30mM by incubation in E3 embryo media  
185 as previously described (48) together with the BLTR2 inhibitor, LY255283 (Abcam), which  
186 was used at 1mM. Exogenous prostaglandin E2 (Cayman Chemicals) and 15-keto-  
187 prostaglandin E2 (Cayman Chemicals) were added by incubation in E3 at a concentration of  
188 1 $\mu$ M (49).

189

### 190 **Hypoxia**

191 Embryos were incubated in 5% oxygen (with 5% carbon dioxide) in a hypoxia hood  
192 (Baker-Ruskinn Sci-tive UM-027) from 32hpi for 6 hours and were imaged at 2dpf. Embryos  
193 from the same clutch kept in incubated normoxic room air were used as controls (33).

194

#### 195 **Bacterial pixel count**

196 Mm mCherry infected zebrafish larvae were imaged at 4dpi on an inverted Leica  
197 DMI8 with a 2.5x objective lens. Brightfield and fluorescent images were captured using a  
198 Hamamatsu OrcaV4 camera. Bacterial burden was assessed using dedicated pixel  
199 counting software as previously described (50).

200

#### 201 **Human cells**

202 Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque PLUS  
203 (GE Healthcare) density centrifugation of whole blood from healthy donors (National  
204 Research Ethics Service reference 07/Q2305/7). PBMC were seeded at  $2 \times 10^6$ /ml in 24-well  
205 tissue culture plates in RPMI 1640 media (Gibco) containing 2mmol/L L-glutamine (Lonza)  
206 and 10% newborn calf serum (Gibco). Cells were cultured in 5% CO<sub>2</sub> at 37°C and non-  
207 adherent cells were removed after 24 hours. Remaining adherent cells were cultured for 14  
208 days, with twice weekly media exchange, in RPMI 1640 supplemented with 2mmol/L L-  
209 glutamine and 10% heat-inactivated fetal bovine serum (FBS) (PAN Biotech) (51).

210 For experiments RPMI 1640 + 25mM HEPES (Gibco) containing 2mmol/L L-  
211 glutamine and 10% heat-inactivated FBS and drugs were pre-equilibrated in normoxia or  
212 hypoxia (0.8% O<sub>2</sub>, 5% CO<sub>2</sub>, 70% humidity at 37°C) for 24 hours prior to the experiment (52).

213 Cells were introduced to the hypoxic workstation (Baker-Ruskinn Sci-Tive UM-027)  
214 where culture media was removed and replaced with hypoxia-equilibrated media as detailed  
215 above. Cells were treated in duplicate or triplicate wells with 100µg/ml LPS (InvivoGen),  
216 DMSO (Sigma-Aldrich), or 15mM COX-2 inhibitor (NS398) (Cayman Chemical) and  
217 incubated for 18 hours. For normoxic controls, cells were treated identically within a class-2  
218 tissue culture hood and transferred to a normoxic tissue-culture incubator.



219 Cell supernatants were collected following 18 hours incubation in normoxia or  
220 hypoxia and were frozen for subsequent analysis. Culture supernatants were assayed in  
221 duplicate or triplicate using a human TNF ELISA MAX (Biolegend). In samples where  
222 undiluted supernatants fell outside the standard curve, samples were re-run following 1:10  
223 dilution in assay buffer.

224

## 225 **Statistical analysis**

226 All data were analysed (Prism 7.0, GraphPad Software) using unpaired, two-tailed t-  
227 tests for comparisons between two groups and one-way ANOVA (with Bonferonni post-test  
228 adjustment) for other data. P values shown are: \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ .

229

## 230 **Results**

231

### 232 ***tnfa*:GFP expression is upregulated by injury, infection and Hif-1 $\alpha$ stabilisation**

233 TNF is a central regulator of the M1 inflammatory response to inflammation and  
234 pathogen challenge. Here, we use two transgenic zebrafish lines that express GFP via a *tnfa*  
235 promoter region; *TgBAC(tnfa:GFP)pd1028* and *Tg(tnfa:eGFP-F)ump5Tg* (31, 32). The  
236 *TgBAC(tnfa:GFP)pd1028* was made using BAC (bacterial artificial chromosome)  
237 transgenesis and contains 50kb of promoter region (32). The *Tg(tnfa:eGFP-F)ump5Tg* line  
238 has a smaller promoter region (3.8kb) than the BAC line (31) and has previously been  
239 demonstrated to be upregulated in macrophages after tailfin injury in a zebrafish model (31).  
240 Here, we demonstrate that this injury induced upregulation of macrophage *tnfa*:GFP is also  
241 found in the BAC transgenic line (*TgBAC(tnfa:GFP)pd1028* crossed to  
242 *Tg(mpeg1:mCherryCAAX)sh378*) shown 16 hours post wound (hpw) (Figure 1A). TNF has  
243 been implicated in all stages of TB infection, however the cell types involved *in vivo* have  
244 been difficult to observe (53). We used the *tnfa* BAC promoter GFP line to establish the  
245 transcriptional regulation of *tnfa* during pre- and early- larval Mm granuloma formation  
246 stages. *tnfa*:GFP expression was induced in larvae following Mm infection before granuloma

247 onset (at 1 day post infection, dpi), and after granuloma formation (at 4dpi) (Figure 1B).  
248 *tnfa:GFP* expression was predominantly found in macrophages with internalised Mm, shown  
249 by co-localisation of fluorescence with *mpeg-1:mCherry* expressing macrophages and  
250 mCrimson expressing Mm (Figure 1C). Macrophage expression was confirmed using the  
251 other *tnfa* promoter driven line, (*Tg(tnfa:eGFP-F)ump5Tg*, crossed to *Tg(mpeg1:mCherry-*  
252 *F)ump2Tg*) (Figure S1). Our data demonstrate that injury and Mm induced *tnfa* expression  
253 occurs in macrophages as part of an early M1 response.

254 Hif-1 $\alpha$  can be stabilised in zebrafish both genetically and pharmacologically using  
255 dominant active constructs and hydroxylase inhibitors respectively (29, 44). We tested  
256 whether Hif-1 $\alpha$  promotes M1 polarisation using the *tnfa:GFP* BAC transgenic line as a  
257 readout of M1 macrophages (31, 32). Hif-1 $\alpha$  was stabilised genetically in larvae using DA  
258 Hif-1 $\alpha$  RNA (44), which resulted in upregulation of *tnfa:GFP* expression (Figure 1D-F)  
259 indicating a shift of macrophage phenotype towards an activated M1 response. Dominant  
260 negative (DN) Hif-1 $\alpha$  caused no upregulation of *tnfa:GFP* expression (Figure 1E-F). To  
261 demonstrate that it was also possible to boost the Hif-1 $\alpha$  M1 pro-inflammatory response  
262 pharmacologically, the hypoxia mimetic DMOG (dimethyloxaloylglycine, a pan-hydroxylase  
263 inhibitor that stabilises Hif-1 $\alpha$  via inactivation of regulatory prolyl hydroxylase enzymes) was  
264 used to stabilise endogenous levels of Hif-1 $\alpha$  (57). DMOG treatment upregulated expression  
265 of *tnfa:GFP* compared to DMSO solvent controls, phenocopying the DA-Hif-1 $\alpha$  response  
266 (Figure 1G). Recently described hypoxia mimetics with reportedly greater prolyl hydroxylase  
267 selectively, JNJ-42041935 and FG4592, had similar effects to DMOG (Figure 1H and I) (33,  
268 45, 46). During both homeostasis and disease physiology, Hif-1 $\alpha$  stability is regulated by  
269 microenvironmental hypoxia. To simulate this we subjected the *tnfa:GFP* line to 5% oxygen  
270 for 6 hours at 32hpf and looked for GFP expression at 48hpf. This level of hypoxia was  
271 sufficient to switch on GFP expression in the *Tg(phd3:GFP)i144* hypoxia reporter zebrafish  
272 (Figure S2) (33). Six hours of 5% oxygen increased *tnfa:GFP* expression at 48hpf,  
273 compared to normoxic controls, to a similar level as that observed with genetic or

274 pharmacological Hif-1 $\alpha$  stabilisation (Figure 1J). Together, these data indicate that *tnfa*  
275 expression is part of a pro-inflammatory M1 macrophage response to hypoxia and stabilised  
276 Hif-1 $\alpha$ , a response that is targetable by pharmacological agents and has the potential to aid  
277 the host response to bacterial challenge.

278

### 279 **Hif-1 $\alpha$ dependent *tnfa:GFP* transcription requires cyclooxygenase**

280 Eicosanoids are lipid signalling molecules produced by macrophages during early  
281 microbial infection as part of an M1 response (22, 23). We tested whether cyclooxygenase  
282 inhibition affected Hif-1 $\alpha$  mediated *tnfa:GFP* production. At basal levels, *tnfa:GFP* was not  
283 altered by the Cox-1 inhibitor SC560 or the Cox-2 inhibitor NS398 compared to DMSO  
284 treated negative controls (Figure 2A-B). Strikingly, DA Hif-1 $\alpha$ -induced *tnfa:GFP* expression  
285 was significantly abrogated to basal levels by both SC560 and NS398 (Figure 2A-B). We  
286 have previously shown that DA Hif-1 $\alpha$  induces the expression of another important pro-  
287 inflammatory cytokine, *il-1 $\beta$* , shown by an *il-1 $\beta$ :GFP* transgenic line (Figure S3A) (33). Cox-1  
288 inhibition by SC560 did not abrogate Hif-1 $\alpha$  induced *il-1 $\beta$ :GFP*, while Cox-2 inhibition with  
289 NS398 caused a small decrease that did not reach basal levels (Figure S3B-C). These data  
290 indicate that Hif-1 $\alpha$ -induced *tnfa* expression is caused by a product of the cyclooxygenase  
291 arm of the arachidonic acid pathway via a novel macrophage HIF/COX/TNF axis (Figure  
292 2C), while Hif-1 $\alpha$  induced *il-1 $\beta$*  does not fully act via this pathway suggestive of complex  
293 regulation of M1 pro-inflammatory cytokines by Hif-1 $\alpha$  stabilisation.

294 We next tested whether injury- and infection-induced *tnfa* are cyclooxygenase  
295 dependent processes. Macrophage *tnfa:GFP* expression induced after injury was not  
296 abrogated by cyclooxygenase inhibition using NS398 (Figure 2D-E). Similarly,  
297 cyclooxygenase inhibition using either SC560 or NS398 did not alter the expression of Mm-  
298 induced *tnfa:GFP* (Figure 2F-G). Our data indicate that macrophage *tnfa* expression can be  
299 driven by the presence of DAMPS (injury) or PAMPS (Mm) and that these are  
300 cyclooxygenase independent mechanisms (Figure 2H).

301 We have previously shown that Hif-1 $\alpha$  stabilisation is host-protective in the zebrafish  
302 Mm model, an effect that is dependent on macrophage expression of proinflammatory *il-1 $\beta$*   
303 (33). We therefore hypothesised that priming macrophages with increased *tnfa* via Hif-1 $\alpha$   
304 stabilisation may be protective against subsequent Mm infection. Neither genetic nor  
305 pharmacological Hif-1 $\alpha$  stabilisation were additive to the *tnfa:GFP* expression caused by  
306 either injury or Mm infection (Figure 2C-F and S4A-D). To test whether DA Hif-1 $\alpha$  priming of  
307 macrophage *tnfa* had an effect on the outcome of Mm infection, bacterial burden was  
308 quantified in DA Hif-1 $\alpha$ -injected embryos with cyclooxygenase inhibition (having  
309 demonstrated that Hif-1 $\alpha$ -induced *tnfa* requires cyclooxygenase activity in Figure 2A-C).  
310 Neither SC560 nor NS398 mediated inhibition of Hif-1 $\alpha$ -induced *tnfa* expression diminished  
311 the host-protective effect of DA Hif-1 $\alpha$  (Figure S5A-C) or DMOG treated larvae (Figure S5D-  
312 F). Taken together these data indicate that injury- and Mm- induced *tnfa* is not further  
313 increased by Hif-1 $\alpha$  stabilisation and that cyclooxygenase mediated early priming of *tnfa* in  
314 macrophages is not required for the Hif-1 $\alpha$  mediated reduction of bacterial burden.

315

316 **Blocking cyclooxygenase independent arachidonic acid pathways does not abrogate**  
317 **Hif-1 $\alpha$  upregulation of *tnfa:GFP***

318 To investigate whether the effect of the cyclooxygenase inhibitors on *tnfa* was  
319 specific to the prostaglandin path of the arachidonic acid pathway, we targeted the lipoxin  
320 and leukotriene producing arms using the 15-Lipoxygenase inhibitor PD146176 and  
321 leukotriene B4 receptor antagonists. The 15-Lipoxygenase inhibitor PD146176 did not block  
322 the *tnfa:GFP* induced by DA Hif-1 $\alpha$  (Figure 3A-B). Furthermore, PD146176 increased  
323 *tnfa:GFP* expression on its own in the absence of infection, although not to the same extent  
324 as DA Hif-1 $\alpha$  (Figure 3A-B). PD146176 also did not affect *tnfa:GFP* expression after Mm  
325 infection (Figure 3A-B), nor did it block the protective effect of DA Hif-1 $\alpha$ . Treatment with  
326 PD146176 was sufficient to decrease Mm burden, but not to the same extent as DA Hif-1 $\alpha$   
327 (Figure 3C). Leukotriene B4 inhibition, using the BLTR1/2 antagonists U75302 and

328 LY255283, did not increase *tnfa:GFP* levels and also did not block DA Hif-1 $\alpha$  induced  
329 *tnfa:GFP* (Figure 3D-E). These data indicate that blocking components of the lipoygenase  
330 dependent arms of the arachidonic acid pathway do not block the Hif-1 $\alpha$  effect on *tnfa*  
331 expression and do not replicate the effects observed by blocking the cyclooxygenase  
332 dependent arm (Figure 3F).

333

### 334 **Hif-1 $\alpha$ -induced *tnfa:GFP* requires active prostaglandin E2**

335 A key family of immune regulators downstream of arachidonic acid and cyclooxygenases are  
336 prostaglandins. The best characterised of these as a regulator of macrophage function is  
337 prostaglandin E2 (PGE2). We tested whether PGE2 was a mediator in the HIF/COX/TNF  
338 pathway by addition of exogenous PGE2 to DA Hif-1 $\alpha$  larvae. Exogenous PGE2 had no  
339 effect on *tnfa:GFP* expression in the absence or presence of Mm infection (Figure 4A-D).  
340 However, PGE2 was able to rescue the decrease in *tnfa:GFP* expression after Cox-1 (Figure  
341 4A and C) or Cox-2 (Figure 4B and D) inhibition in the DA Hif-1 $\alpha$  larvae. Furthermore,  
342 addition of PGE2 alone led to a significant increase in the DA Hif-1 $\alpha$ -induced *tnfa:GFP*  
343 expression (Figure 4A-D). These rescuing effects were not observed by addition of  
344 exogenous 15-keto prostaglandin E2, an immunologically inactive degradation product of  
345 PGE2 (Figure 4E-F) (49, 58, 59). These data indicate that Hif-1 $\alpha$ -induced *tnfa:GFP* requires  
346 active prostaglandin E2 (Figure 4G).

347

### 348 **The HIF-COX-TNF axis is conserved in human macrophages**

349 To translate our findings from zebrafish to humans we tested whether HIF-1 $\alpha$   
350 stabilisation in human macrophages induces TNF expression. We found that human  
351 monocyte derived macrophages (hMDMs) produced higher levels of TNF protein in hypoxia  
352 (0.8% oxygen) than those in normoxia, measured by an anti-human TNF ELISA (Figure 5A).  
353 Furthermore, treatment with the COX-2 inhibitor, NS398, reduced this hypoxia-induced TNF  
354 back to the equivalent levels found in normoxia (Figure 5A). This was replicated when HIF-

355  $1\alpha$  was stabilised in hHDMs using the hypoxia mimetic FG4592 (Figure 5B). These data  
356 indicate that the HIF/COX/TNF axis is a conserved mechanism in human macrophages and  
357 could be important in human disease.

358

## 359 **Discussion**

360 Control of macrophage function during homeostasis and infection are critical for  
361 healthy tissues and must integrate changes in the local microenvironment with cytokine  
362 signalling. Understanding of signalling pathways that link the microenvironment with  
363 macrophage phenotypic outcomes will identify potential novel therapeutic avenues for  
364 control of macrophages during disease. Currently, the mechanisms and molecular cues  
365 leading to different macrophage phenotypes are not well-defined *in vivo*.

366 Here we show that a disease relevant microenvironmental cue, hypoxia signalling via  
367 Hif-1 $\alpha$ , upregulates macrophage *tnfa* expression in a cyclooxygenase dependent manner *in*  
368 *vivo*. TNF regulation by hypoxia has been shown in a range of mammalian cells, and its  
369 promoter region contains HIF responsive elements (HREs), resulting in some level of direct  
370 regulation by HIF-1 $\alpha$  (60–63). We observed an M1 pro-inflammatory *tnfa* response with  
371 stabilised Hif-1 $\alpha$ , demonstrating that hypoxia signalling alone can lead to a switch of  
372 macrophage polarisation to an M1 phenotype, consistent with our previous observations  
373 concerning *il-1 $\beta$*  activation (33). Not only could *tnfa* activation be achieved by genetic  
374 stabilisation of Hif-1 $\alpha$ , but also using hypoxia mimetics and physiological hypoxia. These  
375 findings indicate that the Hif-1 $\alpha$  pro-inflammatory switch is targetable by pharmaceuticals  
376 and could be druggable during disease. Hypoxic regulation of TNF via COX has previously  
377 been demonstrated in mammalian osteoblasts, however, little is known about this interaction  
378 (26). Our data demonstrate that Hif-1 $\alpha$  upregulation of macrophage *tnfa* is dependent on  
379 cyclooxygenase and further shows that the mechanism is likely to be via the production of  
380 PGE2. The degradation metabolite 15-keto-PGE2 did not rescue the loss of *tnfa* expression  
381 after cyclooxygenase inhibition, consistent with previous reports that 15-keto-PGE2 is

382 unable to bind the prostaglandin E2 EP receptors, demonstrating a requirement for active  
383 PGE2 (64).

384 Mycobacteria mediated macrophage polarisation is complex, with M1 and M2  
385 phenotypes observed during pathogenesis, however, the mechanisms determining  
386 macrophage phenotype are not fully characterised (56). The Mtb/Mm granuloma has been  
387 widely studied in human, mice and zebrafish and is rich in pro-inflammatory cytokine  
388 production (32, 33, 65–69). Here, we observed that an M1 pro-inflammatory *tnfa* response  
389 was induced at pre-granuloma stages as well as during granuloma formation *in vivo*. A key  
390 advantage of using fluorescent transgenic zebrafish lines, such as *tnfa:GFP*, is that they  
391 enable identification of the cell type producing the transgene in an intact organism. This is  
392 especially important when studying early innate immune cell interactions with pathogens, as  
393 it eliminates the risk of activation during a sorting process. TNF is required for the control of  
394 initial TB infection in mice and also of latent TB in humans (68, 70–73). Patients on anti-TNF  
395 therapies for immune diseases such as Crohn's disease and rheumatoid arthritis, which  
396 have proved effective treatments for these debilitating illnesses, have an associated  
397 increased risk of TB reactivation and infectious disease (72, 74). In the zebrafish Mm model  
398 it has been widely demonstrated that *Tnfa* is required for control of early infection, with  
399 perturbation of *Tnfa* signalling leading to high infectious burdens (75). When *Hif-1 $\alpha$*  driven  
400 *tnfa* was downregulated by cyclooxygenase inhibition, there was no effect on the decrease  
401 in bacterial burden resulting from stabilisation of *Hif-1 $\alpha$* . Furthermore, none of the  
402 arachidonic acid pathway inhibitors used in our study had any effect on *Tnfa* production after  
403 Mm infection. These findings are consistent with Mm induced macrophage *Tnfa* production  
404 being independent of arachidonic acid, and is most likely TLR mediated as has been  
405 reported elsewhere (43, 56). Our data indicate that there is no additional protective effect of  
406 priming macrophages with increased levels of *Tnfa* before infection onset. Interestingly, this  
407 is not the case for another important M1 cytokine, *Il-1 $\beta$* , where *Hif-1 $\alpha$*  stabilisation fails to  
408 reduce bacterial burden if *Il-1 $\beta$*  is blocked early in infection, (33). Our findings indicate that



409 different M1 cytokines induced by Hif-1 $\alpha$  stabilisation can have differential effects on the  
410 outcome of infection, and highlight that, although TNF and IL-1 $\beta$  are often used as markers  
411 of M1 polarisation, their roles in the pro-inflammatory macrophage are distinct.

412 Blocking cyclooxygenase independent arms of the arachidonic acid pathway did not  
413 inhibit Hif-1 $\alpha$ -induced macrophage *tnfa* transcription in the same way as blocking the  
414 cyclooxygenase/PGE2 arm. Inhibition of the 15-lipoxygenase arm was sufficient to induce  
415 macrophage *tnfa* transcription which was host protective and decreased Mm burden. These  
416 findings indicate a potential shift towards the COX/TNF dependent axis when 15-  
417 lipoxygenase is blocked and are consistent with previous observations suggesting that  
418 inhibition of 15-Lipoxygenase can have immune activating effects (48).

419 Our data reveal a novel mechanism of TNF activation in macrophages via a Hif-1 $\alpha$ /  
420 cyclooxygenase/PGE2 axis. Importantly, this pathway is conserved in human macrophages,  
421 meaning that it could be targetable for pharmaceutical intervention to promote macrophage  
422 M1-type polarisation in human disease. Although the effect of the HIF/COX/TNF axis in early  
423 Mm infection was negligible, this pathway is likely to have important roles in numerous  
424 disease situations. Multiple disease pathologies have hypoxia as a features of the tissue  
425 microenvironment, concurrent with macrophage influx. Hif-1 $\alpha$  mediated M1 switching and  
426 TNF activation may be pertinent in later TB infection situations where granulomas have  
427 stabilised Hif-1 $\alpha$  due to necrotic and hypoxic centres (76, 77). It is not only bacterial  
428 infections such as TB that might be influenced by a HIF-1 $\alpha$  mediated M1/TNF switch.  
429 Hypoxia is a key hallmark of cancers with high levels of HIF-1 $\alpha$  widely found in those that  
430 produce large tumours where the centre is hypoxic (78). This is also true of  
431 cyclooxygenase/PGE2 production, where these important immunomodulatory signals are  
432 required for cellular adaption to the tumour microenvironment (79). Certain types of cancer  
433 induce inflammatory microenvironments, where cytokines and other immunomodulators can  
434 contribute to the progression of cancers (80). Macrophages play central roles in cancer-  
435 inflammation that could be exploited utilising pharmaceutical control of this novel



436 HIF/COX/TNF mechanism. The zebrafish larvae is small enough to be fully oxygenated at  
437 the 2-5dpf stages of this study (81). Further investigation of the HIF/COX/TNF axis in models  
438 where hypoxia is a key hallmark of disease pathology is required to uncover the full  
439 therapeutic relevance of this potentially important novel macrophage pathway.

440 In conclusion, we have identified a novel mechanism for macrophage *tnfa*  
441 transcription via Hif-1 $\alpha$  and cyclooxygenase that is conserved from zebrafish to humans,  
442 highlighting a druggable mechanism to induce an M1-macrophage response. Importantly,  
443 this axis links a microenvironmental cue, hypoxia, to a macrophage pro-inflammatory M1  
444 cytokine, *tnfa* expression. We provide strong evidence to show that this response acts via  
445 the cyclooxygenase/PGE2 arm of the arachidonic pathway. Due to the central roles of these  
446 modulators in disease microenvironments we anticipate that this HIF/COX/TNF pathway  
447 may have important implications in conditions such as inflammation, infection and cancer.

448

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### 670 **Author Contributions**

671 Conceived and designed the experiments: PME, AL. Performed the experiments: PME, AL.  
672 Analysed the data: PME, AL. Wrote the paper: PME.

673

### 674 **Figure Legends**

675

### 676 **Figure 1. Macrophage *tnfa:GFP* is upregulated by injury, Mm infection and Hif-1 $\alpha$** 677 **stabilisation**

678 (A) Fluorescent confocal micrographs of 3 days post fertilisation larvae with or without tailfin  
679 wound (induced 16 hours previously) with example macrophages at the tailfin site. *tnfa*  
680 expression was detected by GFP levels, in green, using the *TgBAC(tnfa:GFP)pd1028*  
681 transgenic line. Macrophages are shown in red using a *Tg(mpeg1:mCherryCAAX)sh378*  
682 line. The asterisk in the not wounded control indicates a neuromast that is fluorescent in all  
683 channels as a marker of position.

684 (B) Fluorescent confocal micrographs of 1dpi Mm infected larvae, prior to granuloma  
685 formation (left panels), and 4 dpi, early granuloma (right panels) stages of infection. *tnfa*  
686 expression was detected by GFP levels, in green, using the *TgBAC(tnfa:GFP)pd1028*

687 transgenic line. Mm mCrimson is shown in the far-red (cyan) channel. Increased levels of  
688 *tnfa:GFP* expression were detectable in cells associated with infection.

689 (C) Fluorescent confocal micrographs of 1 day post infection (dpi) caudal vein region of  
690 infection. *tnfa* expression was detected by GFP levels, in green, using the  
691 *TgBAC(tnfa:GFP)pd1028* transgenic line. Macrophages are shown in red using a  
692 *Tg(mpeg1:mCherryCAAX)sh378* line. Mm Crimson is shown in the far-red channel (cyan,  
693 right panels) with a PVP control (left panels). Without infection there is little detectable  
694 *tnfa:GFP* expression, while infected and uninfected macrophages have higher levels of  
695 *tnfa:GFP* in the Mm group. Dotted lines indicate the yolk extension of the larvae where there  
696 is non-specific fluorescence.

697 (D) Fluorescent confocal micrographs of 2 days post fertilisation larvae in the caudal vein  
698 region. *tnfa* expression was detected by GFP levels, in green, using the  
699 *TgBAC(tnfa:GFP)pd1028* transgenic line. Macrophages are shown in red using a  
700 *Tg(mpeg1:mCherryCAAX)sh378* line. Larvae were injected at the 1 cell stage with dominant  
701 active (DA) Hif-1 $\alpha$  or phenol red (PR) control.

702 (E) Fluorescent confocal micrographs of 2dpf zebrafish imaged around the caudal vein  
703 region. *tnfa* expression was detected by GFP levels, in green, using the  
704 *TgBAC(tnfa:GFP)pd1028* transgenic line. Larvae were injected at the 1 cell stage with  
705 dominant negative (DN) or dominant active (DA) Hif-1 $\alpha$  or phenol red (PR) control. Dotted  
706 lines indicate the yolk extension of the larvae where there is non-specific fluorescence.

707 (F) Graph shows corrected fluorescence intensity levels of *tnfa:GFP*. Dominant active Hif-1 $\alpha$   
708 (DA1, filled bar) had significantly increased *tnfa:GFP* levels compared to phenol red (PR)  
709 and dominant negative Hif-1 $\alpha$  (DN1) injected controls (white bars). Data shown are mean  $\pm$   
710 SEM, n=66 cells from 12 embryos accumulated from 3 independent experiments and  
711 corresponds to images in (E).

712 (G) Fluorescent confocal micrographs of 2dpf *TgBAC(tnfa:GFP)pd1028* transgenic larvae  
713 treated with DMOG or DMSO control. Dotted lines indicate the yolk extension of the larvae

714 where there is non-specific fluorescence. Graph shows corrected fluorescence intensity  
715 levels of *tnfa:GFP* confocal z-stacks in larvae. DMOG treated larvae (filled bars) had  
716 significantly increased *tnfa:GFP* levels compared to DMSO controls (white bars). Data  
717 shown are mean  $\pm$  SEM, n=24 cells from 6 embryos representative of 2 independent  
718 experiments.

719 (G) Fluorescent confocal micrographs of 2dpf *TgBAC(tnfa:GFP)pd1028* transgenic larvae  
720 treated with the PHD inhibitor JNJ-42041935 or a DMSO solvent control. Dotted lines  
721 indicate the yolk extension of the larvae where there is non-specific fluorescence. Graph  
722 shows corrected fluorescence intensity levels of *tnf-a:GFP* confocal z-stacks at 2dpf treated  
723 with JNJ-42041935 (filled bars) or DMSO controls (white bars). Data shown are mean  $\pm$   
724 SEM, n=48 cells from 8 embryos accumulated from 2 independent experiments.

725 (H) Fluorescent confocal micrographs of 2dpf *TgBAC(tnfa:GFP)pd1028* transgenic larvae  
726 treated with the PHD inhibitor FG4592 or a DMSO solvent control. Dotted lines indicate the  
727 yolk extension of the larvae where there is non-specific fluorescence. Graph shows  
728 corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in larvae at 2dpf  
729 treated with FG4592 (filled bars) or DMSO controls (white bars). Data shown are mean  $\pm$   
730 SEM, n=54 cells from 9 embryos accumulated from 3 independent experiments.

731 (I) Fluorescent confocal micrographs of 2dpf *TgBAC(tnfa:GFP)pd1028* transgenic larvae  
732 incubated in room normoxia or 5% oxygen (hypoxia) for 6 hours between 32-38hpf. Dotted  
733 lines indicate the yolk extension of the larvae where there is non-specific fluorescence.  
734 Graph shows corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in larvae  
735 at 2dpf incubated in 5% oxygen (filled bars) or normoxia controls (white bars). Data shown  
736 are mean  $\pm$  SEM, n=72 cells from 12 embryos accumulated from 3 independent  
737 experiments.

738

739 **Figure 2. Hif-1 $\alpha$ -activated *tnfa:GFP* is cyclooxygenase dependent while injury and**  
740 **infection induced *tnfa:GFP* is cyclooxygenase independent**

741 (A) Fluorescent confocal micrographs of 2dpf caudal vein region of larvae. *tnfa* expression  
742 was detected by GFP levels, in green, using the *TgBAC(tnfa:GFP)pd1028* transgenic line.  
743 Phenol red (PR) and dominant active Hif-1 $\alpha$  (DA1) injected larvae were treated with DMSO,  
744 SC560 (Cox-1 inhibitor) and NS398 (Cox-2 inhibitor). Dotted lines indicate the yolk extension  
745 of the larvae where there is non-specific fluorescence.

746 (B) Corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in PR (white bars)  
747 and DA1 (filled bars) injected larvae at 2dpf treated with Cox inhibitors. Data shown are  
748 mean  $\pm$  SEM, n=36 cells from 6 embryos representative of 3 independent experiments.

749 (C) Schematic of the arachidonic pathway indicating where stabilising Hif-1 $\alpha$  upregulates  
750 *tnfa* via cyclooxygenase, an effect that is blocked using the Cox1/2 inhibitors SC560/NS398.

751 (D) Fluorescent confocal micrographs of 3 days post fertilisation larvae with or without tailfin  
752 wound (induced 16 hours previously) in phenol red (PR) or dominant active Hif-1 $\alpha$  embryos.  
753 *tnfa* expression was detected by GFP levels, in green, using the *TgBAC(tnfa:GFP)pd1028*  
754 transgenic line. Embryos were treated with DMSO or NS398 (Cox-2 inhibitor).

755 (E) Corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in PR (white bars)  
756 and DA1 (filled bars) injected larvae at 2dpf treated with Cox inhibitors. Data shown are  
757 mean  $\pm$  SEM, n=24 (in not wounded) or n=36 (in wounded) cells from 6 embryos  
758 accumulated from 2 independent experiments. Not-wounded fish had fewer macrophages at  
759 the tailfin site, hence the lower cell number in these groups.

760 (F) Fluorescent confocal micrographs of 1dpi caudal vein region of Mm Crimson infected  
761 larvae. *tnfa* expression was detected by GFP levels, in green, using the  
762 *TgBAC(tnfa:GFP)pd1028* transgenic line. Phenol red (PR) and dominant active Hif-1 $\alpha$  (DA1)  
763 injected larvae were treated with DMSO, SC560 (Cox-1 inhibitor) and NS398 (Cox-2  
764 inhibitor) and infected with Mm Crimson. Dotted lines indicate the yolk extension of the  
765 larvae where there is non-specific fluorescence.

766 (G) Corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in PR (white bars)  
767 and DA1 (filled bars) Mm Crimson infected larvae at 1dpi treated with Cox inhibitors. NB,

768 DMSO NoMm is a no Mm control taken from figure 2B for comparison with Mm infected data  
769 (data is from the same experiment). Data shown are mean  $\pm$  SEM, n=36 cells from 6  
770 embryos representative of 3 independent experiments.

771 (H) Schematic showing that injury and infection upregulate *tnfa* independently of  
772 cyclooxygenase, an effect that is not blocked by the Cox1/2 inhibitors SC560/NS398.

773

774 **Figure 3. Blocking 15-lipoxygenase or leukotriene B4 receptors does not abrogate**  
775 **DA-Hif-1 $\alpha$ -upregulation of *tnfa:GFP***

776 (A) Fluorescent confocal micrographs of 1dpi caudal vein region of Mm and PVP infected  
777 larvae. *tnfa* expression was detected by GFP levels, in green, using the  
778 *TgBAC(tnfa:GFP)pd1028* transgenic line. Phenol red (PR) and dominant active Hif-1 $\alpha$  (DA1)  
779 injected larvae were treated with DMSO or PD146176 (15-Lipoxygenase inhibitor). Non-  
780 infected larvae are in the left panels (PVP) and Mm Crimson infected larvae are in the right  
781 panels (Mm). Dotted lines indicate the yolk extension of the larvae where there is non-  
782 specific fluorescence.

783 (B) Corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in uninfected  
784 larvae (PVP, empty bars) and infected larvae (Mm, filled bars) at 1dpi of data shown in (A)  
785 after treatment with DMSO or PD146176 (15-Lipoxygenase inhibitor). Data shown are mean  
786  $\pm$  SEM, n=42 cells from 7 embryos accumulated from 3 independent experiments.

787 (C) Bacterial burden at 4dpi after injection of DA Hif-1 $\alpha$  (DA1) and 24 hours of the 15-  
788 lipoxygenase inhibitor PD146176, using DMSO as a negative solvent control. Data shown  
789 are mean  $\pm$  SEM, n=48-50 as accumulated from 3 independent experiments.

790 (D) Fluorescent confocal micrographs of 1dpi caudal vein region of Mm and PVP infected  
791 larvae shown in A. *tnfa* expression was detected by GFP levels, in green, using the  
792 *TgBAC(tnfa:GFP)pd1028* transgenic line. Phenol red (PR) and dominant active Hif-1 $\alpha$  (DA1)  
793 injected larvae were treated with DMSO or U75302/LY255283 (BLTR1/2 inhibitors). Non-  
794 infected larvae are in the left panels (PVP) and Mm Crimson infected larvae are in the right

795 panels (Mm). Dotted lines indicate the yolk extension of the larvae where there is non-  
796 specific fluorescence.

797 (E) Corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in uninfected  
798 larvae (PVP, empty bars) and infected larvae (Mm, filled bars) at 1dpi of data shown in (D)  
799 after treatment with DMSO or U75302/LY255283 (BLTR1/2 inhibitors). Data shown are  
800 mean  $\pm$  SEM, n=30 cells from 5 embryos accumulated from 2 independent experiments.

801 (F) Schematic of the arachidonic pathway indicating where stabilising Hif-1 $\alpha$  upregulates  
802 *tnfa* via cyclooxygenase, an effect that is not blocked using the 15-lipoxygenase inhibitor  
803 PD146176 or BLTR1/2 inhibitors U75302/LY255283.

804

805 **Figure 4. Hif-1 $\alpha$ -induced *tnfa:GFP* requires active prostaglandin E2**

806 (A) Fluorescent confocal micrographs of 1dpi caudal vein region of Mm. *tnfa* expression was  
807 detected by GFP levels, in green, using the *TgBAC(tnfa:GFP)pd1028* transgenic line.  
808 Phenol red (PR) and dominant active Hif-1 $\alpha$  (DA1) injected larvae were treated with DMSO  
809 or SC560 (Cox-1 inhibitor) in the presence or absence of endogenous prostaglandin E2  
810 (PGE2). All larvae are PVP injected. Dotted lines indicate the yolk extension of the larvae  
811 where there is non-specific fluorescence.

812 (B) Fluorescent confocal micrographs of 1dpi caudal vein region. *tnfa* expression was  
813 detected by GFP levels, in green, using the *TgBAC(tnfa:GFP)pd1028* transgenic line.  
814 Phenol red (PR) and dominant active Hif-1 $\alpha$  (DA1) injected larvae were treated with DMSO  
815 or NS398 (Cox-2 inhibitor) in the presence or absence of endogenous prostaglandin E2  
816 (PGE2). All larvae are PVP injected. Dotted lines indicate the yolk extension of the larvae  
817 where there is non-specific fluorescence.

818 (C) Corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in PVP injected  
819 phenol red (PR, empty bars) and dominant active Hif-1 $\alpha$  (DA1, filled bars) larvae at 1dpi of  
820 data shown in (B) after treatment with DMSO/NS398/PGE2. Data shown are mean  $\pm$  SEM,  
821 n=54 cells from 9 embryos accumulated from 3 independent experiments.

822 (D) Corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in PVP injected  
823 phenol red (PR, empty bars) and dominant active Hif-1 $\alpha$  (DA1, filled bars) larvae at 1dpi of  
824 data shown in (B) after treatment with DMSO/NS398/PGE2. Data shown are mean  $\pm$  SEM,  
825 n=54 cells from 9 embryos accumulated from 3 independent experiments.

826 (E) Fluorescent confocal micrographs of 1dpi caudal vein region. *tnf-a* expression was  
827 detected by GFP levels, in green, using the *TgBAC(tnfa:GFP)pd1028* transgenic line.  
828 Phenol red (PR) and dominant active Hif-1 $\alpha$  (DA1) injected larvae were treated with DMSO  
829 or NS398 (Cox-2 inhibitor) in the presence or absence of endogenous 15-keto prostaglandin  
830 E2 (15K). All larvae are PVP injected. Dotted lines indicate the yolk extension of the larvae  
831 where there is non-specific fluorescence.

832 (F) Corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in PVP injected  
833 phenol red (PR, empty bars) and dominant active Hif-1 $\alpha$  (DA1, filled bars) larvae at 1dpi of  
834 data shown in (E) after treatment with DMSO/NS398/15-keto PGE2. Data shown are mean  $\pm$   
835 SEM, n=24 cells from 4 embryos accumulated from 2 independent experiments.

836 (G) Schematic of the arachidonic pathway indicating where blocking cyclooxygenase  
837 prevents Hif-1 $\alpha$  upregulation of *tnfa*, an effect rescued by endogenous PGE2.

838

839 **Figure 5. Hypoxia induces TNF in human MDMs in a COX-2 dependent manner**

840 (A) TNF ELISA of human monocyte derived macrophages incubated in normoxia or 0.8%  
841 hypoxia with or without treatment with NS398. Data shown are mean  $\pm$  SEM, n=5-8  
842 biological repeats from 3-4 donors.

843 (B) TNF ELISA of human monocyte derived macrophages incubated in normoxia treated  
844 with FG4592 with or without treatment with NS398. Data shown are mean  $\pm$  SEM, n=6  
845 biological repeats from 3 donors.

846

847 **Figure S1. Mm infection induced *tnfa:GFP* in macrophages in a second transgenic**  
848 **line.**

849 Fluorescent confocal micrographs of 1dpi caudal vein region of infection. *tnfa* expression  
850 was detected by GFP levels, in green, using the *Tg(tnfa:eGFP-F)ump5Tg* transgenic line.  
851 Macrophages are shown in red using a *Tg(mpeg1:mCherry-F)ump2Tg* line. Mm Crimson is  
852 shown in the blue channel. Dotted lines indicate the yolk extension of the larvae where there  
853 is non-specific fluorescence.

854

855 **Figure S2. 5% oxygen induces expression of the *phd3:GFP* hypoxia transgene.**

856 Fluorescent micrographs of 48hpf *Tg(phd3:eGFP)i144* hypoxia reporter zebrafish after  
857 incubated in 6 hours of 5% oxygen from 32-38hpf, or normoxic controls.

858

859 **Figure S3. DA-Hif-1 $\alpha$  induced *il-1 $\beta$ :GFP* is not altered by Cox-1 inhibition and  
860 decreased by Cox-2 inhibition.**

861 (A) Fluorescent confocal micrographs of 2dpf caudal vein region. *il-1 $\beta$*  expression was  
862 detected by GFP levels, in green, using the *TgBAC(il-1 $\beta$ :GFP)SH445* transgenic line.  
863 Macrophages are shown in red using a *Tg(mpeg1:mCherryCAAX)sh378* line. Without  
864 infection there is little detectable *il-1 $\beta$ :GFP* expression in phenol red (PR) controls, while DA-  
865 Hif-1 $\alpha$  macrophages have higher levels of *il-1 $\beta$ :GFP* in the Mm group.

866 (B) Fluorescent confocal micrographs of 1dpi caudal vein region of PVP injected larvae. *il-1 $\beta$*   
867 expression was detected by GFP levels. Phenol red (PR) and dominant active Hif-1 $\alpha$  (DA1)  
868 injected larvae were treated with DMSO, SC560 (COX-1 inhibitor) and NS398 (COX-2  
869 inhibitor).

870 (C) Corrected fluorescence intensity levels of *il-1 $\beta$ :GFP* confocal z-stacks in PVP injected  
871 larvae at 1dpi from (B). Data shown are mean  $\pm$  SEM, n=36 cells from 6 embryos  
872 representative of 3 independent experiments.

873

874 **Figure S4. Mm infection induced *tnfa:GFP* is not altered by Hif-1 $\alpha$  stabilisation**



875 (A) Fluorescent confocal micrographs of Mm Crimson infected 1dpi zebrafish imaged around  
876 the caudal vein region. *tnfa* expression was detected by GFP levels, in green, using the  
877 *TgBAC(tnfa:GFP)pd1028* transgenic line. Larvae were injected at the 1 cell stage with  
878 dominant negative (DN) or dominant active (DA) Hif-1 $\alpha$  or phenol red (PR) control. Dotted  
879 lines indicate the yolk extension of the larvae where there is non-specific fluorescence.

880 (B) Graph shows corrected fluorescence intensity levels of *tnfa:GFP*. Dominant active Hif-1 $\alpha$   
881 (DA1, filled bar) showed no change in *tnfa:GFP* levels in the presence of Mm bacterial  
882 challenge compared to phenol red (PR) and dominant negative Hif-1 $\alpha$  (DN1) injected  
883 controls (white bars). NB, PR NoMm is a no Mm control taken from figure 1A for comparison  
884 with Mm infected data, the data for which is from the same experiment as this one. Data  
885 shown are mean  $\pm$  SEM, n=66 cells from 12 embryos accumulated from 3 independent  
886 experiments.

887 (C) Fluorescent confocal micrographs of 2dpf Mm infected *TgBAC(tnfa:GFP)pd1028*  
888 transgenic larvae treated with DMOG or DMSO control. Dotted lines indicate the yolk  
889 extension of the larvae where there is non-specific fluorescence.

890 (D) Graph shows corrected fluorescence intensity levels of *tnfa:GFP* confocal z-stacks in  
891 larvae. DMOG treated larvae (filled bars) had significantly increased *tnfa:GFP* levels  
892 compared to DMSO controls (white bars). Data shown are mean  $\pm$  SEM, n=24 cells from 6  
893 embryos representative of 2 independent experiments.

894

895 **Figure S5. Cyclooxygenase inhibition does not block the protective effect of Hif-1 $\alpha$**   
896 **stabilisation in Mm infection.**

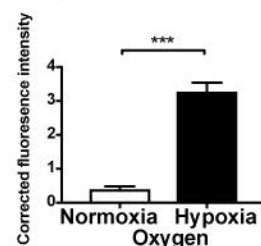
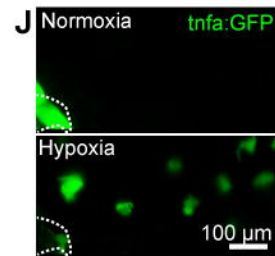
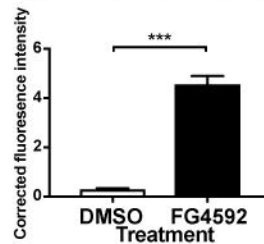
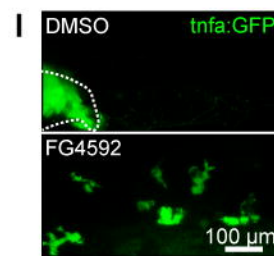
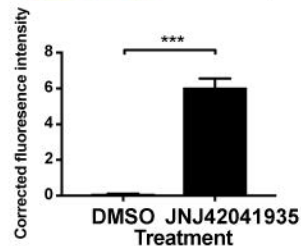
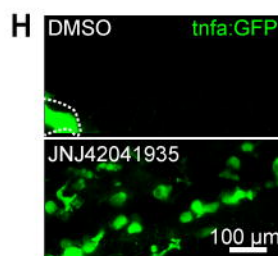
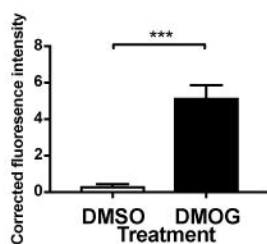
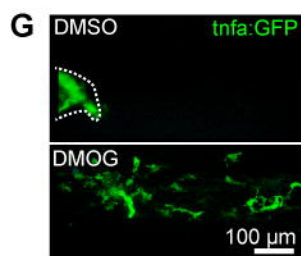
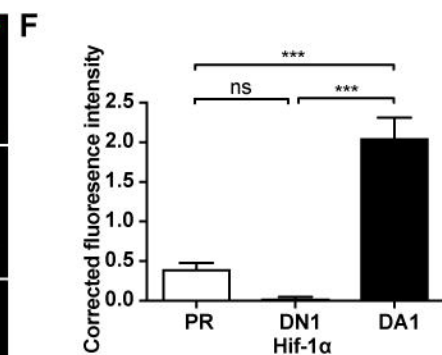
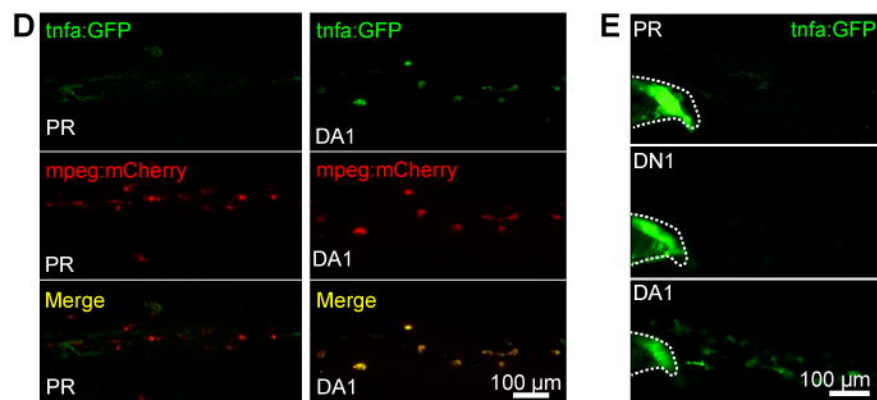
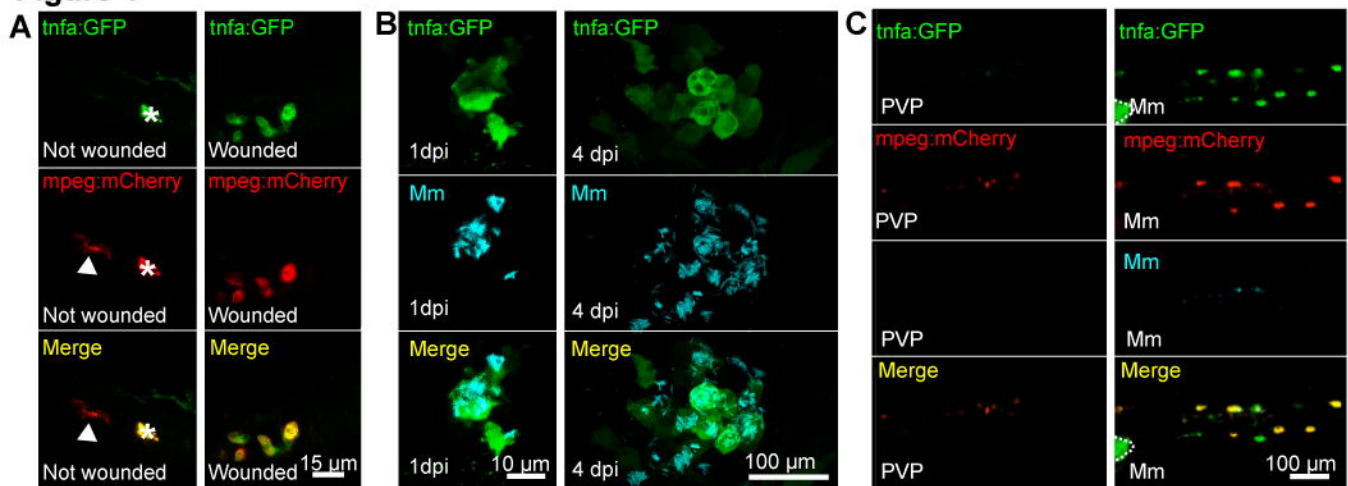
897 (A) Bacterial burden of SC560 treated larvae at 4dpi after injection with phenol red (PR) or  
898 dominant active Hif-1 $\alpha$  (DA1). Data shown are mean  $\pm$  SEM, n=35 as accumulated from 3  
899 independent experiments.

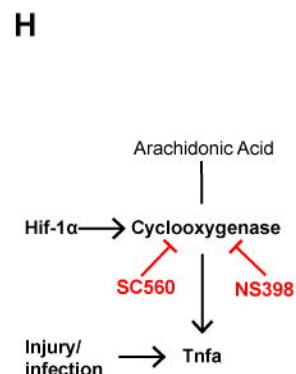
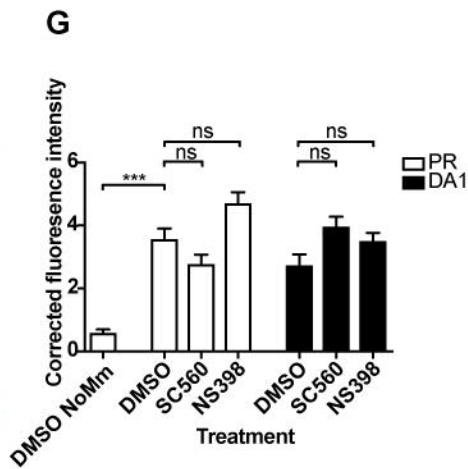
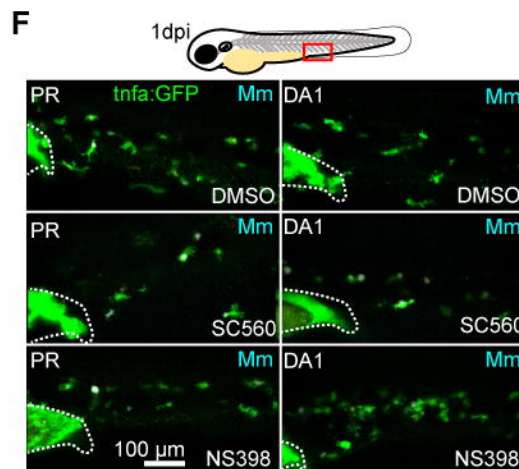
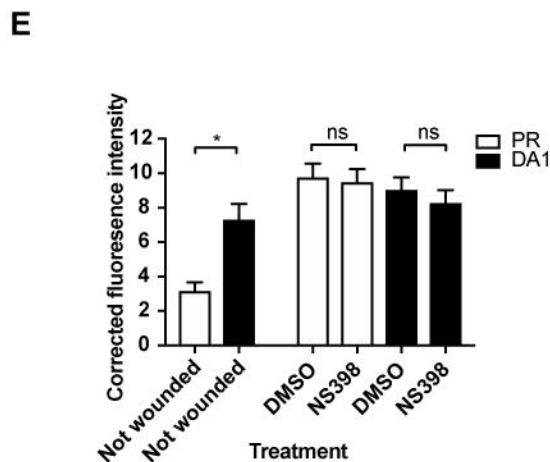
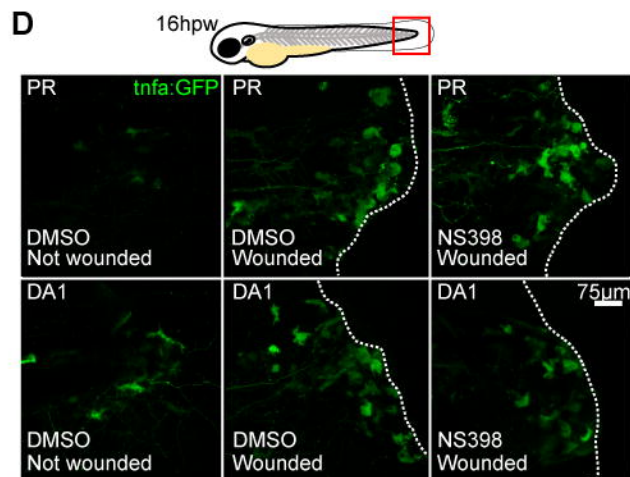
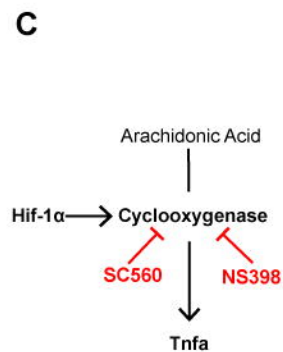
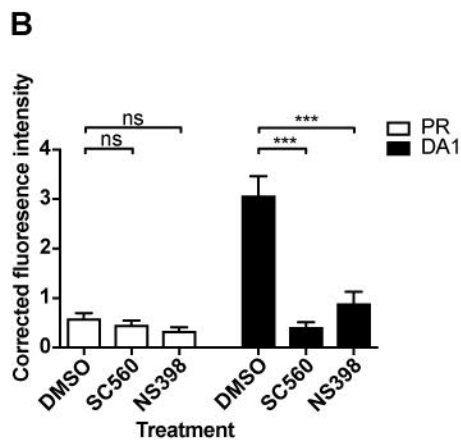
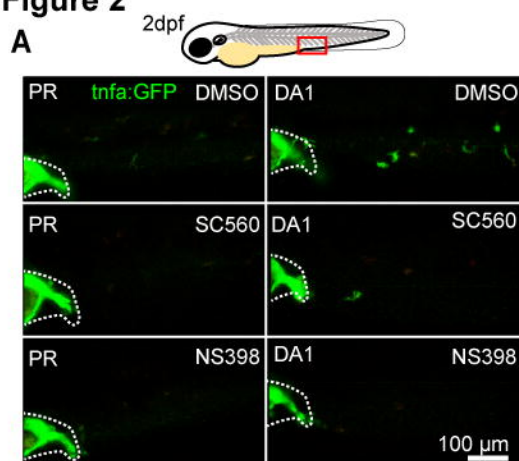
900 (B) Bacterial burden of NS398 treated larvae at 4dpi after injection with phenol red (PR) or  
901 dominant active Hif-1 $\alpha$  (DA1). Data shown are mean  $\pm$  SEM, n=35 as accumulated from 3  
902 independent experiments.

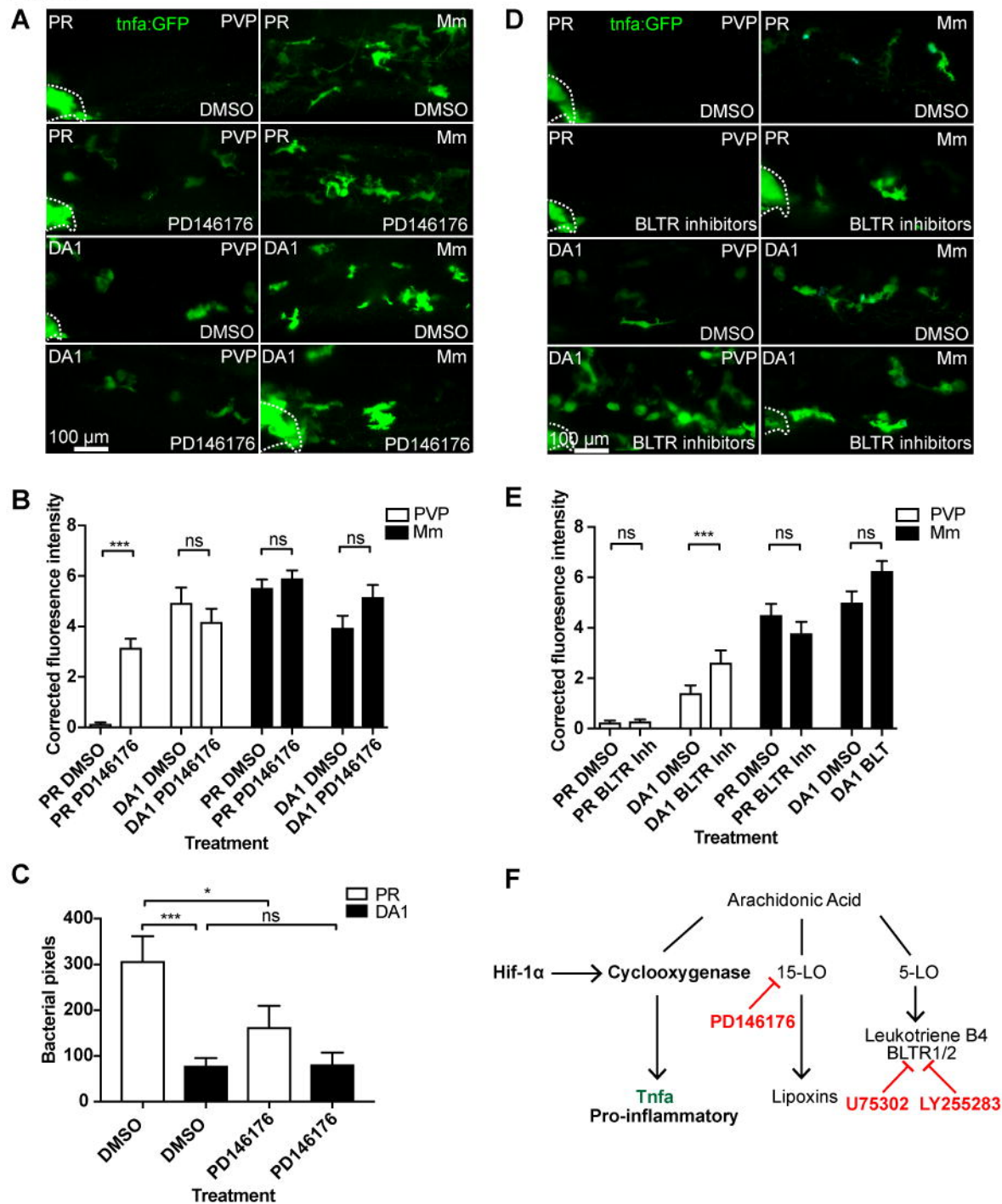
903 (C) Bacterial burden of DMSO treated larvae at 4dpi after co-treatment with DMSO or  
904 DMOG. Data shown are mean  $\pm$  SEM, n=33-36 as accumulated from 3 independent  
905 experiments.

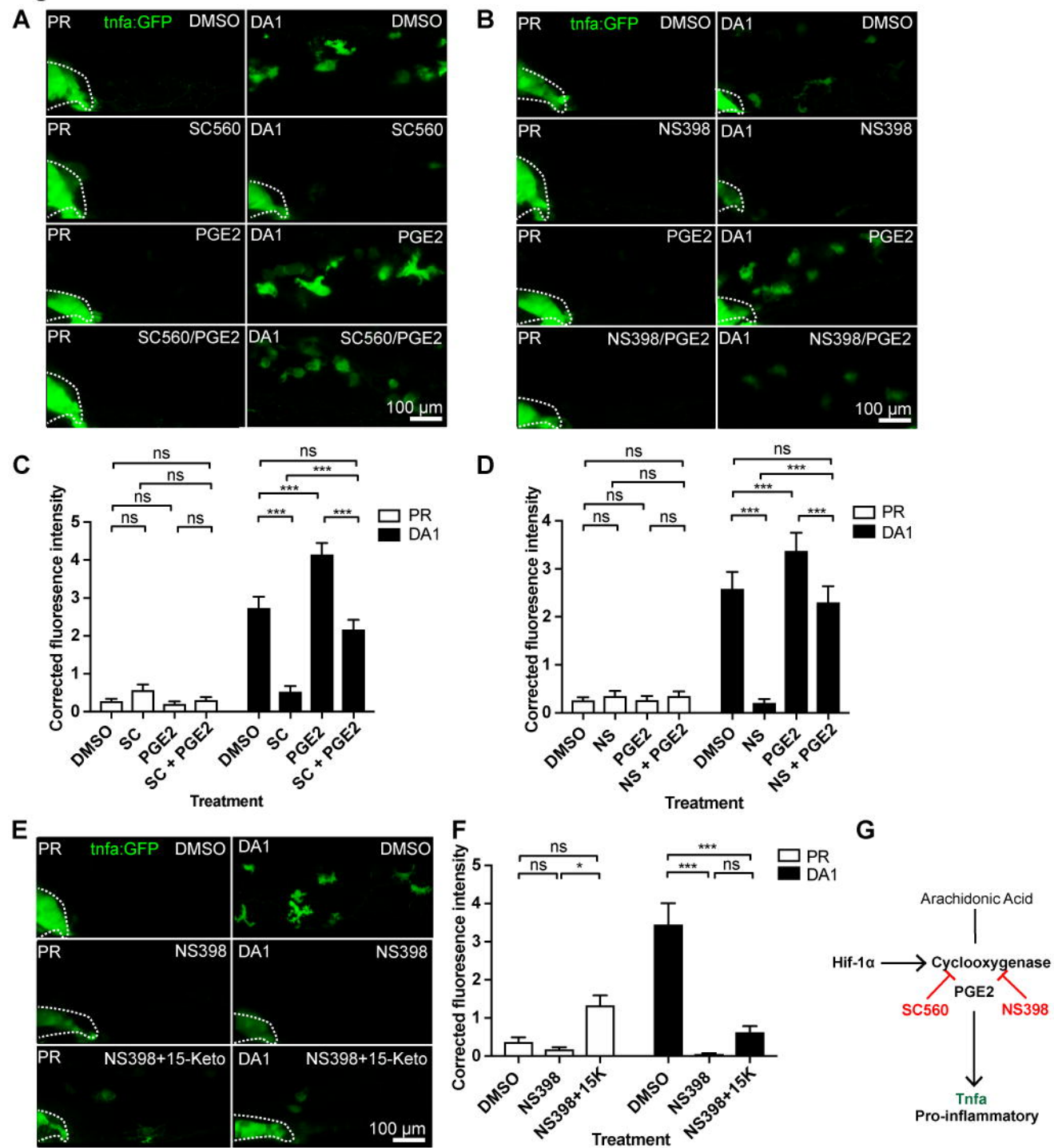
906 (D) Bacterial burden of SC560 treated larvae at 4dpi after co-treatment with DMSO or  
907 DMOG. Data shown are mean  $\pm$  SEM, n=33 as accumulated from 3 independent  
908 experiments.

909 (E) Bacterial burden of NS398 treated larvae at 4dpi after co-treatment with DMSO or  
910 DMOG. Data shown are mean  $\pm$  SEM, n=33 as accumulated from 3 independent  
911 experiments.

**Figure 1**

**Figure 2**

**Figure 3**

**Figure 4**



**Figure 5**

