1 **Vasoactive intestinal peptide (VIP) differentially affects inflammatory immune**
2 **responses in human monocytes infected with viable *Salmonella* or stimulated with LPS**

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## Summary

We compared the effect of VIP on human blood monocytes infected with Salmonella Typhimurium 4/74 or stimulated with LPS. VIP ($10^{-7}$ M) increased monocyte viability by 24% and 9% when cultured for 24h with 4/74 or *Salmonella* LPS (100 ng/ml) respectively. Significantly increased ($P< 0.05$) numbers of 4/74 were also recovered from monocytes co-cultured with VIP after 6h post-infection (pi) and this remained high after 24h pi. Both 4/74 and LPS increased ($P <0.05$) the concentration of TNF-α, IL-1β and IL-6 measured in monocyte supernatants. However, LPS induced this effect more rapidly while, with the exception of IL-6, 4/74 induced higher concentrations ($P< 0.05$). VIP significantly decreased ($P <0.05$) TNF-α and IL-1β production by 4/74-infected monocytes after 6 pi, but only after 24h in LPS-cultured monocytes. This trend was reversed for IL-6 production. However, TNF-α and IL-1β production by 4/74-infected monocytes, cultured with VIP, still remained higher ($P< 0.05$) than concentrations measured in supernatants cultured only with LPS. VIP also increased ($P< 0.05$) production of anti-inflammatory IL-10 in both 4/74 and LPS cultures after 24h. We also show a differential effect of VIP on the expression of TNFα and IL-6 receptors, since VIP was only able to decreased expression in LPS-stimulated monocytes but not in 4/74-infected monocytes.

In conclusion, we show a differential effect of VIP on human monocytes infected with virulent *Salmonella* or stimulated with LPS. Our study suggests that the use of VIP in bacteraemia and/or sepsis may be limited to an adjunctive therapy to antibiotic treatment.

46

47 ## Introduction

48 To date, most models of human sepsis involve murine studies or immortalised human
49 immune cells, such as monocytic THP1 cells, stimulated with Lipopolysaccharide (LPS).
50 However, the dynamic interaction of primary human blood monocyte with virulent Gram
51 negative bacteria is likely to be different to that observed in these models, which essentially
52 investigate endotoxaemia rather than bacteraemia. The general model of disease progression
53 in sepsis proposes two phases. The first phase is characterised by uncontrolled production of
54 inflammatory mediators leading to systemic inflammatory response syndrome (SIRS) (1-3)
55 which leads to acute sepsis and may lead to hypoperfusion and organ collapse (termed septic
56 shock). The second phase of disease is characterised by the production of a compensatory
57 anti-inflammatory response syndrome (CARS), required to restore homeostasis but which can
58 lead to secondary, nosocomial, infection (4-5, 3). The SIRS (acute) phase of sepsis is
59 associated with high systemic concentrations of pro-inflammatory cytokines released by
60 monocytes and macrophages, such as TNF-α, IL-1 and IL-6 (6) and acute sepsis is associated
61 with uncontrolled systemic inflammation. Thus, intervention which reduces the inflammatory
62 immune response has been proposed as a rational therapeutic avenue. However, clinical trials
63 in which this has been attempted have largely failed. One reason is that some agents used
64 may be neutralised by the immune system or degraded by enzymes (7). Trials investigating
65 inhibition of Tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β) were also
66 disappointing, probably due to high systemic concentrations of other cytokines and in some
67 cases it seems that the cytokine status of patients was not measured prior to administration of
68 specific cytokine inhibitors (8). Broad ranging anti-inflammatories such as glucocorticoids
69 are also widely used in the treatment of sepsis but their effect is debateable probably due to

timing of therapy, dosage and the development of ‘steroid resistance’ by glucocorticoid receptors (9).

Studies, to date, have suggested that VIP is an excellent therapeutic candidate against sepsis because (i) it is a natural product which does not induce an immune response, (ii) it is identical in all mammals apart from guinea pigs (10), (iii) it is easily synthesised in large quantities, (iv) in murine models of LPS-induced sepsis, low concentrations of VIP (<5 nmol) prevent mortality (11) and (v) VIP inhibits LPS-induced cytokine production in human THP-1 cells (12-14) indicating that results from animal models may translate into human medicine. VIP therefore has great potential as a cost effective therapeutic against Gram negative sepsis.

Monocytes respond quickly to bacterial infection via inflammatory chemokines released in infected peripheral tissues (15). After recruitment into infected tissue, monocytes are subjected to signals from Th1 cells that respond to pathogen in tissues. This encounter results in the conversion of the monocytes to activated macrophages that are more competent in killing the pathogen and to initiate the systemic inflammatory response in order to clear the infection (16). Monocytes were used extensively in the past to study sepsis (17) and LPS-induced disease (18). However, nothing has been reported on the effect of VIP on the production of inflammatory mediators by human monocytes (rather than monocyte-like THP-1 cells) infected with virulent Gram negative bacteria, nor how this may compare to the effect of VIP when these monocytes are stimulated with LPS, since a discrepancy between the effect of VIP on these may have a significant impact on the use of VIP as a therapeutic.

The aim of the work we describe was to compare the effect of VIP on the production of inflammatory mediators produced by human monocytes infected with *Salmonella Typhimurium* 4/74 or stimulated by LPS.

## Materials and methods

### *Salmonella* and LPS

*S*. Typhimurium 4/74 and LPS from *S*. Typhimurium SL1181 (Sigma-Aldrich, Poole, Dorset UK) were used to stimulate human monocytes in the study. In all experiments, S. Typhimurium 4/74 was cultured with monocytes at a multiplicity of infection of 10:1 (MOI =10). Monocytes were cultured with LPS at a concentration of 100 ng/ml, unless otherwise stated. Porcine VIP (95 % purity by HPLC) was obtained from Sigma-Aldrich, UK.

### Isolation of peripheral blood monocytes (PBM)

Human blood was purchased from the blood transfusion service (Sheffield, UK). The blood was diluted with sterile PBS then gently poured onto Histopaque-1077 (Sigm-Aldrich, UK) prior to isolation of the buffer coat, as standard procedure. After appropriate washing steps, buffy coat supernatants were resuspended with appropriate amounts of cold MACS buffer and anti-CD14 antibody coated micromagnetic beads (Miltenyi Biotech, Bisley, Surrey UK) according to manufacturer's instructions. The viability of isolated monocytes was assessed using Trypan blue (10% v/v) (Sigma) and was found to be > 90 % prior to use.

### *Salmonella* invasion and LPS stimulation assays

The cultured monocytes were firstly washed with sterile PBS then the bacteria were added to the cultured monocytes at MOI of 10:1 at 37°C and 5% $Co_2$ for 60 min. The cells were then washed and co-cultured with RPMI media contained 100µg/ml of gentamycin (Sigma-Aldrich, UK) with or without VIP (Sigma-Aldrich, UK) and placed in the incubator for a further 60 minutes. The cultured cells were washed again and the media was substituted with RPMI containing 25µg/ml of gentamycin with or without VIP ($10^{-7}$ M) for a further 6 or 24h.

118 The cells were then washed three times with PBS at room temperature and then lysed using
119 1% Triton X (Fisher Scientific LTD, Loughborough, UK) for 15 minutes at 37 $^{O}$C.
120 Intracellular bacterial counts were determined by serial dilution at different time points 2, 6,
121 24 hours post infection. Viable bacterial cells counts were measured as colony forming units
122 per ml (CFU/ml).
123 In other experiments monocytes were cultured with LPS, with or without VIP ($10^{-7}$ M), prior
124 to isolation of supernatants for cytokine measurement or cell harvesting, for analysis of
125 receptor expression. The dose effect of VIP on production of TNF-α and IL-10 by LPS-
126 cultured monocytes was also measured in monocyte supernatants following co-culture of
127 monocytes with LPS and different VIP concentrations ranging from $10^{-6}$-$10^{-8}$ M.

128 **Monocyte survival assay**

129 The uptake of the fluorescent restriction dye Propidium iodide (PI) was used to measure the
130 viability of cells under the experimental procedures described above. After 24h post-culture
131 monocytes were incubated in PBS containing PI (10µg/ml) for 10 min. The number of non-
132 viable cells (PI +) was assessed uaing FACSCanto II analyser (Becton Dickinson, USA).
133 Samples were acquired using BD FACSDiva™ (BD Biosciences, USA) and analysed using
134 CyFlogic 2.8 software, licensed to Nottingham University. Monocytes which had been
135 immersed in ice cold (-20 0C) methanol for 30 min were used as a positive control and
136 monocytes cultured in media only for 24h were used as a negative control. All experiments
137 were performed in triplicate on 3 separate occasions.

138

139 **Measurement of cytokine concentration in monocyte supernatants**

140 ELISA kits (R&D Systems Abingdon, Oxford, UK) were used to measure TNFα, IL-1β, IL-
141 6, IL-10 and IL-4 in the supernatants isolated from monocytes (2, 6 and 24h post-culture

142 infected with *S*. Typhimurium 4/74 or cultured with LPS, with or without co-culture with VIP
143 ($10^{-7}$ M). Supernatants isolated from monocytes cultured in media only were used as a
144 negative control. All experiments were performed in triplicate on 3 separate occasions.
145
146 **Expression of cytokine receptors on monocyte membranes**
147 FACS analyses were performed to determine the effect of *S*. Typhimurium 4/74 infection or
148 LPS culture on the expression of monocyte cytokine receptors and to determine whether, or
149 not, this was affected by VIP ($10^{-7}$ M). After 24h post-treatment, $1x10^6$ monocytes, from each
150 group, were washed 3 times in FACS buffer (EDTA 2mM; BSA 1% w/v) at 300g for 10 min
151 per wash step. The cell pellets were then incubated with human Trustain FcX (Biolegend, San
152 Diego, CA, USA) for 15 min to block FC receptors. After washing 3 times in FACS buffer,
153 the cell pellets were re-suspended in FACS buffer containing relevant cytokine receptor
154 antibodies for 45 min on ice in the dark. The antibodies used were as follows; Mouse anti-
155 human TNF-α receptor (anti-TNFR.1/CD120) and isotype control (Mouse IgG2b.FITC)
156 (eBioscience, Santa Clara, CA, USA), Rat anti-human IL-6 receptor (IL-6R/CD126) and
157 isotype control (Rat IgG2a.PE) (eBioscience, USA) and mouse anti-human IL-10 receptor
158 (IL-10R/CD210) and isotype control (Mouse IgG1.FITC) (Biolegend, USA). In addition to
159 this, some groups of monocytes were incubated with mouse anti-Human TLR4 antibody (or
160 mouse IgG1.FITC isotype control antibody) to ascertain the effect of bacteria and VIP on a
161 non-cytokine (but highly relevant) monocyte receptor.
162 The concentration of all antibodies and FC blocking reagent are shown in Table 1. After
163 antibody incubation, the cells were washed 3 times in FACS buffer and then re-suspended in
164 0.2 ml of FACS buffer prior to analysis. on a FACSCanto II analyser ( BD, USA). Samples
165 were acquired using the BD FACSDiva™ (BD Biosciences, USA) and analysed using the
166 CyFlogic 2.8 software.

## Statistical analysis

ANOVA analyses with one way classification were performed to determine significance between experimental groups. Tukey's post-hoc test was used to determine significant differences between groups at the 95% confidence limit ($P = 0.05$).

## Results

Our results show that after 24h post infection (pi) of human monocytes with *S*. Typhimurium 4/74, only 44% of cells remained viable, as measured by uptake of propidium iodide (Fig 1A) but this was significantly increased to 68% when co-cultured with VIP ($10^{-7}$ M) . In comparison, when monocytes were stimulated with *Salmonella* LPS (100 ng/ml) for 24h, viability remained high at 79% but was further increased to 88% when co-cultured with VIP (Fig 1A). VIP also significantly ($P$ <0.05) increased the numbers of bacteria recovered from monocytes at 6h pi by about 1.5 log and more were also recovered after 24h, but the difference was not significant (Fig 1B).

We next compared the effect of VIP on the secretion of pro and anti-inflammatory cytokines by monocytes infected with 4/74 or cultured with LPS. *S*. Typhimurium 4/74 and LPS both significantly ($P$ <0.05) increased the concentration of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) measured in monocyte supernatants. LPS had a much more rapid effect on monocytes and significantly increased cytokine levels within 2h, whereas similar increases were not measured in supernatants isolated from 4/74-infected monocytes at this time point (Fig 2A-E).

TNF-α and IL-1β concentrations induced by Salmonella infection (Fig 2A and C) were much greater than those induced by LPS culture (Fig 2B and D). Addition of VIP to 4/74-infected monocytes induced a significant decrease ($P$ <0.05) in TNF-α production (Fig 2A) and IL-1β

191 production (Fig 2C) at 6 and 24h pi but VIP only significantly decreased ($P$ <0.05) TNF-α
192 and IL-1β production after 24h pi in LPS-cultured monocytes (Fig 2B and D respectively).
193 However even when 4/74-infected monocytes were cultured with VIP, the concentration of
194 TNF-α or IL-1β remained at levels significantly greater (P <0.05) than those measured in
195 supernatants from monocytes stimulated with LPS which were not co-cultured in VIP.
196 However, LPS induced a greater and much more rapid IL-6 response after 6h (Fig 2F)
197 compared to IL-6 production by 4/74-infected monocytes, although this was equivalent after
198 24h (Fig 2E). Conversely, VIP induced a significant decrease in IL-6 production by
199 monocytes cultured with LPS after 6 and 24h (Fig 2F) but a significant reduction in IL-6
200 produced by 4/74-infected monocytes was only measured after 24h post-culture in (Fig 2E).
201 4/74 and LPS both significantly ($P$ <0.05) increased the concentration of IL-4 produced by
202 monocytes and this concentration was not significantly changed by the addition of VIP to the
203 culture media (Fig 3A and B). Similarly, and LPS both significantly ($P$ <0.05) increased the
204 concentration of IL-10 produced by monocytes. However, the addition of VIP to the media in
205 this case slightly increased IL-10 production by monocytes infected with 4/74, or cultured
206 with LPS, after 6h but after 24h the increase was significant ($P$ <0.05) with 4/74 inducing
207 much higher levels of IL-10 than that measured in the supernatants of LPS stimulated
208 monocytes (Fig 3C and D).

209 Since VIP had little effect on either LPS-induced TNF-α or IL-10 production after 6h, we
210 investigated whether a dose effect of VIP could be measured at this time point. VIP did have
211 a dose-dependent effect on LPS-stimulated production of inflammatory (TNF-α) and anti-
212 inflammatory (IL-10) cytokines. When added at concentration ranges from $10^{-6}$, $10^{-7}$ and $10^{-8}$
213 M, VIP increased the concentration of LPS-induced TNF-α (Fig 4B) but VIP only
214 significantly inhibited ( P < 0.05) LPS-induced TNF-α production by monocytes when added

215 at a concentration of $10^{-6}$ M (Fig 4A). We also observed a dose effect of VIP on IL-10
216 production but the dynamics of this were very different to those measured for TNF-α. In this
217 case, VIP at $10^{-7}$ or $10^{-8}$ M increased IL-10 concentration in the supernatants of LPS-
218 stimulated monocytes above that measured in supernatants isolated from monocytes cultured
219 only with LPS (Fig 4B) and at $10^{-8}$ M, this increase was significant ($P < 0.05$).

220 Finally we compared the effect of VIP on expression of cytokine receptor proteins on the
221 surface of monocytes infected with 4/74, or cultured with LPS for 6h. Our results show that
222 both TNFR.1 (Fig 5A) and IL-6R (Fig 5B) were increased on the surface of monocytes
223 infected with 4/74, or cultured with LPS for 6h. VIP had no effect on this expression by
224 Salmonella-infected monocytes but did decrease expression of both receptors on the surface
225 of monocytes cultured with LPS (Fig 5A and B). Expression of IL-10R did not increase
226 above isotype control levels on the surface of monocytes infected with *S*. Typhimurium 4/74
227 and this was not altered by VIP. However, LPS stimulated an increase in IL-10R expression
228 on the surface of monocytes and this upregulation of receptor was inhibited by VIP (Fig 5C).
229 We then investigated the effect of VIP on expression of TLR4 which is critical receptor in
230 LPS detection and innate immune response but is involved in LPS (rather than cytokine)
231 detection. In these experiments we found that both Salmonella infection or LPS culture
232 upregulated TLR4 expression by monocytes and in both cases this was reduced when the
233 cells were co-cultured with VIP (Fig 5D).

234

## 235 Discussion

236 To date, most studies which have investigated the effect of VIP on bacterial-induced
237 inflammatory pathways have involved LPS and either murine macrophages (19,20), murine

238 models of disease (11) or immortalised human THP-1 monocytes (12-14). Although LPS is a
239 very strong immunogen it lacks the genetic complexity of intact bacteria. For example,
240 bacteria express a number of other substances such as flagellin, CpG oligodeoxynucleotides,
241 peptidoglycans and lipoproteins which also initiate an inflammatory response. They also
242 utilse sophisticated secretion systems to invade and survive in cells and which encode
243 proteins that may down-regulate host immune responses. There are very few reported studies
244 which have investigated the effect of whole viable bacteria on human monocytes and the role
245 of VIP in this system as a potential therapeutic in sepsis.

246 In this current study we show that VIP increased the viability of monocytes infected with *S.*
247 Typhimurium 4/74 or exposed to LPS. The greatest effect of VIP on viability was when
248 monocytes were infected with *Salmonella*, with less than half remaining viable when cultured
249 with 4/74 for 24h, but in co-culture with VIP viability increased by >20%. VIP has
250 previously been reported to prevent LPS-induced cell death in rat neuronal mesenteric cells
251 (21) and our study is therefore in accordance with this. S. Typhimurium SL1344 has been
252 previously reported to induce apoptosis in human monocyte-derived macrophages (22) and
253 also in the human monocytic cell line, U937 (23). However, a positive correlation between
254 increased apoptosis of blood monocytes and increased survival of sepsis patients has been
255 previously reported (24) and elevated monocyte numbers has also been reported to correlate
256 with sepsis (25). It is possible, therefore, that the physiological response to sepsis is to reduce
257 some of the capacity of the immune system (monocytes) to produce inflammatory mediators
258 by inducing apoptosis in these cells. If this is the case, then administration of VIP to patients
259 who have Gram negative bacteraemia may have a detrimental effect. It is probably also the
260 case that the increase in *Salmonella* survival associated with VIP that we have shown is due
261 to the increased survival of monocytes. However, addition of VIP to culture media was

262 associated with decreased production of inflammatory cytokines (TNFα, IL-1β and IL-6) and
263 an increase in anti-inflammatory IL-10. Early studies have shown that TNFα, IL-1β were
264 upregulated in the blood of volunteers in which LPS was intravenously administered (26-27).
265 Following meta-analysis, Lv et al., (28) reported that TNFα may be a useful biomarker of
266 neontal sepsis. Meta-analyses have also indicated that TNF-α therapy may reduce mortality if
267 adminiistered prior to shock and may also have therapeutic benefit in patients with high IL-6
268 titres (29) and that in paediatric sepsis, TNFα levels could be used as a biomarker as it
269 strongly correlates with Gram negative culture from patients (30). This latter study may be
270 highly relevant to ours since we show that VIP reduces TNFα production by 4/74-infected
271 monocytes, although it would also suggest that TNFα production is a double-edged sword in
272 that it probably is needed to kill bacteria also. As such, VIP may be a useful adjunctive
273 therapy to antiobiotic treatment. A study by Gogos *et al*., (31) reported that IL-10
274 concentration was postively correlated with the onset of sepsis and that high IL-10/TNF- α
275 ratio indicated a poor prognosis. High circulating levels of IL-6 and IL-10 have also been
276 associated with mortaility in other studies (32, 25). Since we show that VIP inhibits IL-6 but
277 increases IL-10 production by monocytes it is difficult to say what effect this may have *in*
278 *vivo* and it is also possible that this could be beneficial or detrimental depending on the phase
279 of disease (SIRS or CARS). In a study of 178 biomarkers for sepsis, it was reported that only
280 5 had a specificity and sensitivity > 90% (33) and none of these biomarkers were cytokines
281 we report here. However, the cytokines we have studied are known to stimulate production of
282 most of these biomarkers and so inhibiting cytokines during ongoing bacterial infection may
283 have a therapeutic, down-stream, effect.

284 We also studied the effect of VIP on the expression of important monocyte receptors.
285 Surprisingly, we found that VIP was unable to suppress the increased expression of TNFR.1,

286 or IL-6R on the surface of monocytes infected with 4/74 but was able to do so when
287 monocytes were stimulated with LPS. We also show a very different response in expression
288 of IL-10R, on the surface of monocytes, when comparing *S*. Typhimurium 4/74 infection
289 with LPS culture. In this case, 4/74-infected monocytes were unable to upregulate IL-10R
290 and this was not altered by co-culture with VIP. However, LPS increased IL-10R expression
291 and this was decreased by co-culture with VIP. Therefore, the effect of VIP differs greatly in
292 respect to expression of cytokine receptors depending on whether human monocytes are
293 infected with virulent Gram negative bacteria or stimulated with LPS. This suggests that
294 bacterial factors, other than LPS, may prevent the inhibitory effect of VIP on expression of
295 these receptors and may indicate that administration of VIP, during sepsis, could be less
296 effective if the patient has ongoing Gram negative infection. TLR4 activation of NFKB has
297 long been associated with the development of sepsis (34-35) and it is perhaps significant that
298 we show that VIP inhibits TLR4 expression in monocytes stimulated with LPS but also
299 following infection by 4/74. Previously this effect has only been reported in human
300 monocytic THP-1 cells stimulated with LPS from *Escherichia Coli* or *Porphyromonas*
301 *Gingivalis* (14), While VIP also inhibits TLR4 mRNA (although surface protein was not
302 measured) in murine macrophages stimulated with LPS (36). Therefore, we show that VIP
303 inhibits the initial detection and down-stream transcriptional response to both virulent
304 *Salmonella* and LPS and is most likely the reason why VIP inhibits production of the
305 inflammatory cytokines we have measured in this study.

306 In conclusion our study suggests that VIP may have therapeutic value in human sepsis by
307 inhibiting the production of inflammatory cytokines and cytokine receptors by blood
308 monocytes during endotoxaemia, although the timing of VIP intervention (during the SIRS or
309 CARS phase of disease) may be critical. However, we show that VIP does not down-regulate

310 some important cytokine receptors when monocytes are infected with virulent *Salmonella*
311 and, although it decreases the production of inflammatory cytokines by these cells, the level
312 of cytokines still remain higher than that induced by LPS alone. Furthermore, VIP increases
313 the viability of infected monocytes which may have an overall detrimental effect during
314 bacteraemia/sepsis. Therefore, we suggest that VIP would probably need to be administered
315 as an adjunctive therapy to antibiotic treatment rather than a 'stand-alone' therapy as
316 suggested by earlier murine studies using LPS.

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321

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430 **Figure Legends**

431

432 **Figure 1. VIP increases the viability of both human monocytes and *S*. Typhimurium**

433 **4/74 after 24h post-infection**

434 (A); FACS analysis was performed to show that VIP (10-7 M) increased monocyte survival

435 after 24 pi with *S*. Typhimurium 4/74 (MOI = 10) or following culture with Salmonella LPS

436 (100 ng/ml) for 24h. * = significant increase ($P < 0.05$) in the numbers of viable monocytes

437 co-cultured with VIP and *S*. Typhimurium or LPS. Positive control = monocytes cultured

438 with methanol. Negative control = monocytes cultured in media only over the same time

439 period and , as an additional control VIP (10-7M) alone was added to monocytes cultures but

440 this had no effect on monocytes survival (data not shown). Monocytes were cultured with the

441 fluorescent restriction dye Propidium iodide (1 μg/ml) to assess cell death.

442 (B); Shows the number of viable *S*. Typhimurium 4/74 recovered from monocytes after 2-24h

443 pi, with or without VIP ($10^{-7}$ M). * = significant increase ($P < 0.05$) in the number of S.

444 Typhimurium 4/74 recovered from infected monocytes co-cultured with VIP after 24h.

445 Histograms show data of mean values calculated from triplicate experiments performed on

446 three separate occasions. Error bars show standard deviation (SD) from the mean.

447

448 **Figure 2. VIP inhibits the production of TNF-α and IL-β by human monocytes infected**

449 **with *S*. Typhimurium 4/74 but the concentration of both remain significantly higher**

450 **than TNF-α and IL-β stimulated by LPS**

451 ELISA analyses showing that VIP ($10^{-7}$ M) significantly inhibited ($P$ <0.05) TNF-α (A) and

452 IL-1β (C) production by human monocytes infected with *S*. Typhimurium 4/74 (MOI =10) at

453 6 and 24h pi. VIP also significantly inhibited (*P* <0.05) TNF-α (B) and IL-1β (D) production
454 by human monocytes following culture with LPS (100 ng/ml) for 24h. VIP significantly (*P* <
455 0.05) inhibited production of IL-6 by monocytes infected with *S*. Typhimurium 4/74 at 24h pi
456 (E) but VIP inhibited IL-6 production by monocytes cultured with LPS for both 6 and 24h
457 (F). In 4/74-infected monocytes cultured with VIP, TNF-α and IL-1β (A and C)
458 concentrations remain significantly higher (P <0.05) than those measured in monocyte
459 supernatants cultured only with LPS (B and D). Negative controls = monocytes cultured in
460 media only over the same time periods and, as an additional control, VIP (10-7M) alone was
461 added to monocytes cultures but this had no effect on cytokine production (data not shown).
462 * = significant decrease (*P* < 0.05). Histograms show data of mean values calculated from
463 triplicate experiments performed on three separate occasions. Error bars show standard
464 deviation (SD) from the mean.

465

466 **Figure 3. VIP has no effect on IL-4 production by human monocytes infected with *S*.**
467 **Typhimurium 4/74 or cultured with LPS but increases production of IL-10 following**
468 **either treatment**.

469 ELISA analyses showing that VIP ($10^{-7}$ M) had no effect on IL-4 production by human
470 monocytes infected with S. Typhimurium 4/74 (MOI =10) (A) or following culture with
471 Salmonella LPS (100 ng/ml) (B) for 2-24h. VIP significantly increased (*P* <0.05) IL-10
472 production by monocytes infected with *S*. Typhimurium 4/74 after 24h pi. (C). VIP also
473 increased IL-10 production by monocytes cultured with LPS for 6 and 24h and after 24h this
474 increase was significant (*P* <0.05) (D). Negative controls = monocytes cultured in media only
475 over the same time periods and, as an additional control, VIP (10-7M) alone was added to
476 monocytes cultures but this had no effect on cytokine production (data not shown). * =

477 significant decrease ($P < 0.05$). Histograms show data of mean values calculated from

478 triplicate experiments performed on three separate occasions. Error bars show standard

479 deviation (SD) from the mean.

480

481 **Figure 4. VIP dose dependently changes TNF-α and IL-10 production by LPS-cultured**

482 **cells.**

483 ( (A); ELISA analyses showing that decreased TNFα production by monocytes, infected with

484 *S*. Typhimurium 4/74 for 6h pi, was correlated with increased VIP concentration ($10^{-6}$-$10^{-8}$

485 M). * = significant decrease in TNFα ($P < 0.05$) compared with TNFα production by

486 monocytes cultured with LPS alone. (B); Decreased IL-10 production by monocytes infected

487 with *S*. Typhimurium 4/74 for 6h pi was correlated with increased VIP concentration ($10^{-6}$-

488 $10^{-8}$ M). * = significant increase in IL-10 ($P < 0.05$) compared with IL-10 production by

489 monocytes cultured with LPS alone. Negative controls = monocytes cultured in media only

490 over the same time periods and, as an additional control, VIP (10-7M) alone was added to

491 monocytes cultures but this had no effect on cytokine production (data not shown).

492 Histograms show data of mean values calculated from triplicate experiments performed on

493 three separate occasions. Error bars show standard deviation (SD) from the mean.

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498 **Figure 5. VIP inhibits expression of cytokine receptors on the surface of human**
499 **monocytes cultured with LPS but not following infection with *S*. Typhimurium 4/74.**

500 FACS analyses showed that VIP (10-7 M) had no effect on the expression of TNFR.1 (A);
501 IL-6R (C) or IL-10R (E) on the surface of human monocytes infected with *S*. Typhimurium
502 4/74 (MOI =10) after 6h pi. However, VIP inhibited expression of TNFR.1 (B); IL-6R (D) or
503 IL-10R (F) on the surface of human monocytes cultured with Salmonella LPS (100 ng/ml)
504 after 6h pi. Conversely VIP (10-7 M) inhibited expression of TLR4 on the surface of
505 monocytes infected with *S*. Typhimurium 4/74 (G) or following culture with LPS (H) for 6h.

506 = Isotype control; = Monocytes cultured with *S*. Typhimurium 4/74 or LPS;

507 = Monocytes cultured with *S*. Typhimurium 4/74 or LPS and VIP. FACS histograms
508 are representative of data obtained from triplicate experiments performed on three separate
509 occasions.

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511 **Table 1. The concentration of antibodies and isotype controls used in cytokine receptor**
512 **FACS analyses.**

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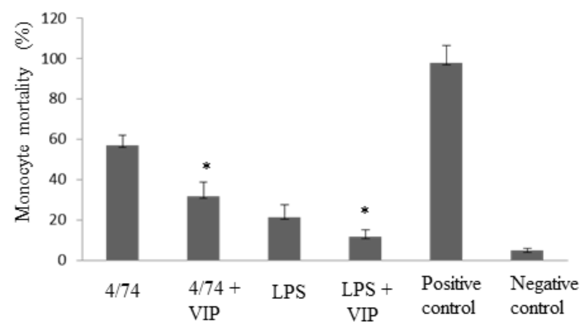
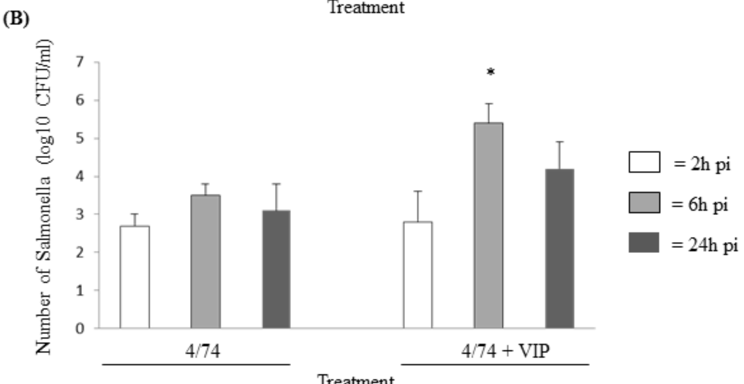
Figure 1 Askar et al

(A)




(B)

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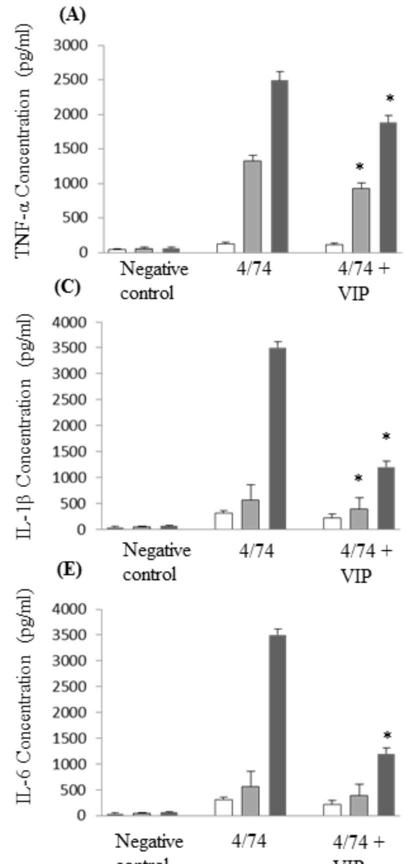


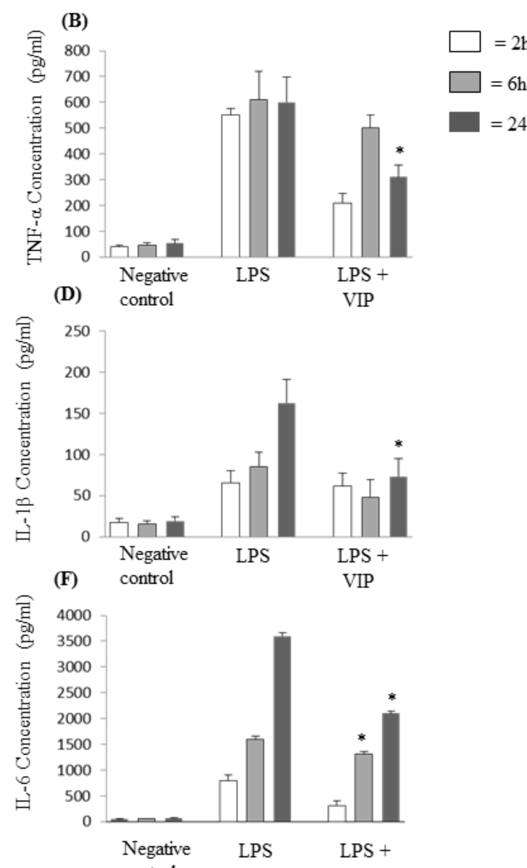


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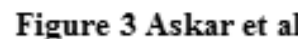
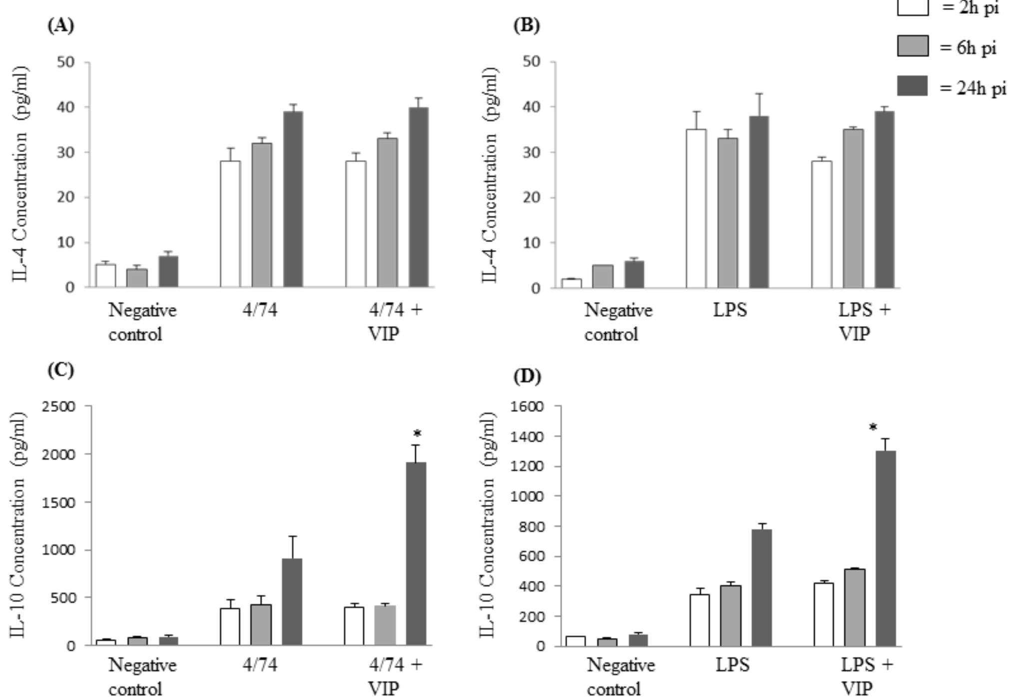


Figure 3 Askar et al

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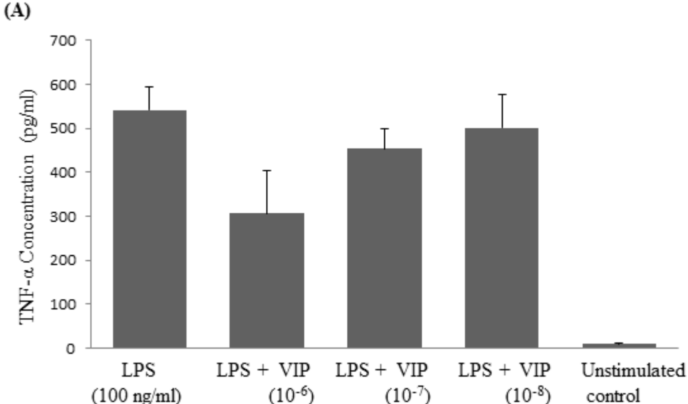


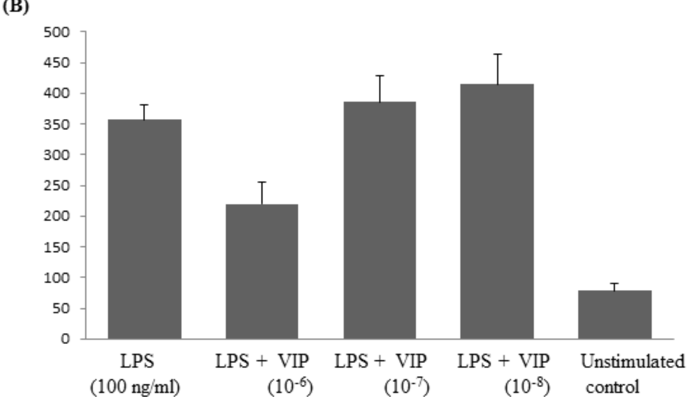


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**Figure 5 Askar et al**

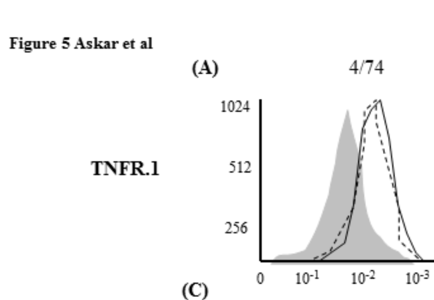
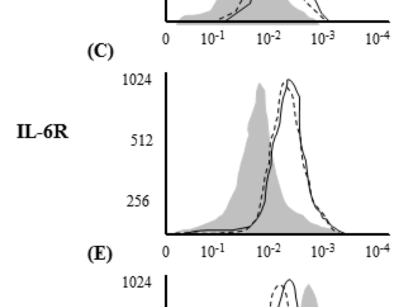
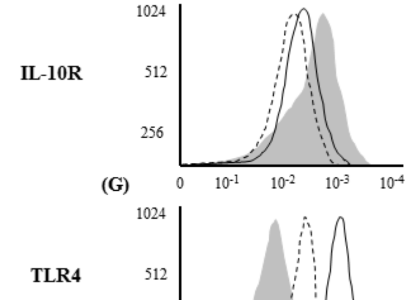
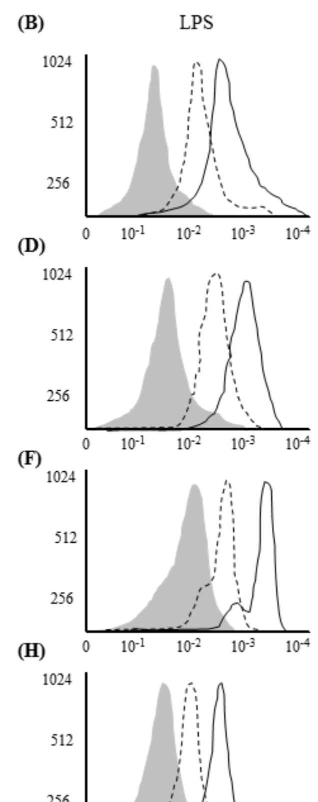


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Table 1 Askar et al

| Antibodies | Concentration | Isotype control antibodies |
| --- | --- | --- |
| Mouse anti-human TFN-R clone H398. FITC. | 0.5 µg | Anti-Mouse IgG2b.FITC. |
| Rat anti-human CD210 (IL-10R) PE. | 0.5 µg | Anti-rat IgG2a. PE. |
| Mouse anti-human CD126 /IL-6 receptor alpha. PerCP. | 0.06 µg (5µl) | Anti-mouse IgG1.PerCP. |
| Human Trustain FcX (FcReceptor Blocking solution. | 0.5 µg | N/A. |
| Mouse anti-human TLR4.FITC | 0.5 µg | Anti-Mouse IgG1.FITC |

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