



Askar, Basim Ali and Ibrahim, Hiba and Barrow, Paul and Foster, Neil (2015) Vasoactive intestinal peptide (VIP) differentially affects inflammatory immune responses in human monocytes infected with viable Salmonella or stimulated with LPS. *Peptides*, 71 . pp. 188-195. ISSN 1873-5169

Access from the University of Nottingham repository:

<http://eprints.nottingham.ac.uk/41584/1/Askar%20et%20al%20Peptides.pdf>

Copyright and reuse:

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

This article is made available under the Creative Commons Attribution Non-commercial No Derivatives licence and may be reused according to the conditions of the licence. For more details see: <http://creativecommons.org/licenses/by-nc-nd/2.5/>

A note on versions:

The version presented here may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the repository url above for details on accessing the published version and note that access may require a subscription.

For more information, please contact eprints@nottingham.ac.uk

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

3

4 Basim Askar, Hiba Ibrahim, Paul Barrow and *Neil Foster

5 School of Veterinary Medicine and Science, University of Nottingham, Nottingham UK

6

7 *Corresponding author

8 Neil Foster

9 School of Veterinary Medicine and Science

10 University of Nottingham

11 Sutton bonington campus

12 Sutton Bonington

13 Leicestershire

14 NG7 2NR

15 Telephone: 0115 9516433

16 Email: n.foster@nottingham.ac.uk

17 Fax: +44 (0) 115 951 6415

18

19

20

21

22

23 **Summary**

24 We compared the effect of VIP on human blood monocytes infected with *Salmonella*
25 Typhimurium 4/74 or stimulated with LPS. VIP (10^{-7} M) increased monocyte viability by
26 24% and 9% when cultured for 24h with 4/74 or *Salmonella* LPS (100 ng/ml) respectively.
27 Significantly increased ($P < 0.05$) numbers of 4/74 were also recovered from monocytes co-
28 cultured with VIP after 6h post-infection (pi) and this remained high after 24h pi. Both 4/74
29 and LPS increased ($P < 0.05$) the concentration of TNF- α , IL-1 β and IL-6 measured in
30 monocyte supernatants. However, LPS induced this effect more rapidly while, with the
31 exception of IL-6, 4/74 induced higher concentrations ($P < 0.05$). VIP significantly decreased
32 ($P < 0.05$) TNF- α and IL-1 β production by 4/74-infected monocytes after 6 pi, but only after
33 24h in LPS-cultured monocytes. This trend was reversed for IL-6 production. However,
34 TNF- α and IL-1 β production by 4/74-infected monocytes, cultured with VIP, still remained
35 higher ($P < 0.05$) than concentrations measured in supernatants cultured only with LPS. VIP
36 also increased ($P < 0.05$) production of anti-inflammatory IL-10 in both 4/74 and LPS cultures
37 after 24h. We also show a differential effect of VIP on the expression of TNF α and IL-6
38 receptors, since VIP was only able to decreased expression in LPS-stimulated monocytes but
39 not in 4/74-infected monocytes.

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

43

44

45

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 1 β (IL-1 β) 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

70 timing of therapy, dosage and the development of 'steroid resistance' by glucocorticoid
71 receptors (9).

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

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

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

94 **Materials and methods**

95 ***Salmonella* and LPS**

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

101

102 **Isolation of peripheral blood monocytes (PBM)**

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

110

111 ***Salmonella* invasion and LPS stimulation assays**

112 The cultured monocytes were firstly washed with sterile PBS then the bacteria were added to
113 the cultured monocytes at MOI of 10:1 at 37°C and 5% CO₂ for 60 min. The cells were then
114 washed and co-cultured with RPMI media contained 100µg/ml of gentamycin (Sigma-
115 Aldrich, UK) with or without VIP (Sigma-Aldrich, UK) and placed in the incubator for a
116 further 60 minutes. The cultured cells were washed again and the media was substituted with
117 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 °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 (10 μ g/ml) for 10 min. The number of non-
132 viable cells (PI +) was assessed using 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 °C) 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 TNF α , IL-1 β , 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, 1×10^6 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.

167 **Statistical analysis**

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

171

172 **Results**

173 Our results show that after 24h post infection (pi) of human monocytes with *S. Typhimurium*
174 4/74, only 44% of cells remained viable, as measured by uptake of propidium iodide (Fig 1A)
175 but this was significantly increased to 68% when co-cultured with VIP (10^{-7} M) . In
176 comparison, when monocytes were stimulated with *Salmonella* LPS (100 ng/ml) for 24h,
177 viability remained high at 79% but was further increased to 88% when co-cultured with VIP
178 (Fig 1A). VIP also significantly ($P < 0.05$) increased the numbers of bacteria recovered from
179 monocytes at 6h pi by about 1.5 log and more were also recovered after 24h, but the
180 difference was not significant (Fig 1B).

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

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

191 production (Fig 2C) at 6 and 24h pi but VIP only significantly decreased ($P < 0.05$) TNF- α
192 and IL-1 β 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-1 β 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 utilise 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 (TNF α , IL-1 β and IL-6) and
263 an increase in anti-inflammatory IL-10. Early studies have shown that TNF α , IL-1 β 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 TNF α may be a useful biomarker of
266 neonatal sepsis. Meta-analyses have also indicated that TNF- α therapy may reduce mortality if
267 administered prior to shock and may also have therapeutic benefit in patients with high IL-6
268 titres (29) and that in paediatric sepsis, TNF α 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 TNF α production by 4/74-infected
271 monocytes, although it would also suggest that TNF α 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 antibiotic treatment. A study by Gogos *et al.*, (31) reported that IL-10
274 concentration was positively 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 mortality 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.

317 **Acknowledgements**

318 The work was funded by the Ministry of Higher Education and Scientific Research,
319 Kurdistan–Iraq (awarded to BA) and Sudan university for science and technology and
320 Faculty for the future, Gordon Memorial college scholarship (awarded to HI).

321

322 **References**

- 323 1. Annane D, Bellissant E, Cavaillon JM. Septic shock. *Lancet* 2005; **365**: 63-78.
- 324 2. Davis BH. 2005. Improved diagnostic approaches to infection/sepsis detection. *Expert Rev*
325 *Mol Diagn* 2005; **5**: 193-207.
- 326 3. Ward NS, Casserly B, Ayala A. The compensatory anti-inflammatory response syndrome
327 (CARS) in critically ill patients. *Clin Chest Med* 2008; **29**: 617-625.
- 328 4. Wolk K, W. Docke W, von Baehr V, Volk H, Sabati R. Comparison of monocyte functions
329 after LPS- or IL-10-induced reorientation: importance in clinical immunoparalysis.
330 *Pathobiology* 1999; **67**: 253-256.

- 331 5. Gullo A, Iscra F, Di Capua G, Berlot G, Lucangelo U, Chierago ML, Ristagno G,
332 Peratoner A, Fasiolo S, Consales C, De Martino G, Tufano R. Sepsis and organ dysfunction:
333 an ongoing challenge. *Minerva Anesthesiol* 2005; **71**: 671-699.
- 334 6. Danikas DD, Karakantza M, Theodorou GL, Sakellaropoulos GC, Gogos CA. Prognostic
335 value of phagocytic activity of neutrophils and monocytes in sepsis. Correlation to CD64 and
336 CD14 antigen expression. *Clin Exp Immunol* 2008; **154**: 87-97.
- 337 7. Svenson S, Tomalia DA. Dendrimers in biomedical applications--reflections on the field.
338 *Adv Drug Deliv Rev* 2005; **15**: 2106-2129.
- 339 8. Cohen J. Sepsis and septic shock: inching forwards. *Clin Med* 2009; **9**: 256-257.
- 340 9. Antonucci E, Fiaccadori E, Taccone FS, Vincent JL. Glucocorticoid administration in
341 sepsis and septic shock: time for a paradigm change? *Minerva Anesthesiol* 2014; **80**: 1058-
342 1062.
- 343 10. Du BH, Eng J, Hulmes JD, Chang M, Pan YC, Yalow RS. Guinea pig has a unique
344 mammalian VIP. *Biochem Biophys Res Commun* 1985; **128**: 1093-1098.
- 345 11. Delgado M, Martinez C, Pozo D, Calvo JR, Leceta J, Ganea D, Gomariz RP. Vasoactive
346 intestinal peptide (VIP) and pituitary adenylate cyclase-activation polypeptide (PACAP)
347 protect mice from lethal endotoxemia through the inhibition of TNF- α and IL-6. *J. Immunol*
348 1999; **162**: 1200-1205.
- 349 12. Foster N, Cheetham J, Taylor JJ, Preshaw PM. VIP Inhibits Porphyromonas gingivalis
350 LPS-induced immune responses in human monocytes. *J. Dent Res* 2005; **84**: 999-1004.
- 351 13. Foster N, Andreadou K, Jamieson L, Preshaw PM, Taylor J.J. VIP inhibits P. gingivalis
352 LPS-induced IL-18 and IL-18BP α in monocytes. *J. Dent Res* 2007; **86**: 883-887.
- 353 14. Foster N, Lea SR, Preshaw PM, Taylor JJ. Pivotal advance: vasoactive intestinal peptide
354 inhibits up-regulation of human monocyte TLR2 and TLR4 by LPS and differentiation of
355 monocytes to macrophages. *J. Leuko Biol* 2007; **81**: 893-903.

- 356 15. Faix JD. Biomarkers of sepsis. *Crit Rev Clin Lab Sci* 2013; **50**: 23-36.
- 357 16. Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. *J.*
358 *Immunol* 2008; **181**: 3733-3739.
- 359 17. Xiu F, Jeschke MG. Perturbed mononuclear phagocyte system in severely burned and
360 septic patients. *Shock* 2013; **40**: 81-88.
- 361 18. Lyakh LA, Koski GK, Telford W, Gress RE, Cohen PA, Rice NR. Bacterial
362 lipopolysaccharide, TNF- α , and calcium ionophore under serum-free conditions promote
363 rapid dendritic cell-like differentiation in CD14⁺ monocytes through distinct pathways that
364 activate NF- κ B. *J. Immunol* 2000; **165**: 3647-3655.
- 365 19. Delgado M, Ganea, D. Inhibition of endotoxin-induced macrophage chemokine
366 production by vasoactive intestinal peptide and pituitary adenylate cyclase-activating
367 polypeptide in vitro and in vivo. *J. Immunol* 2001; **167**: 966-975.
- 368 20. Delgado M, Pozo D, Martinez C, Leceta J, Calvo JR, Ganea D, Gomariz RP. Vasoactive
369 intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit endotoxin-
370 induced TNF-alpha production by macrophages: in vitro and in vivo studies. *J. Immunol*
371 1999; **162**: 2358-2367.
- 372 21. Arciszewski MB, Sand E, Ekblad E. Vasoactive intestinal peptide rescues cultured rat
373 myenteric neurons from lipopolysaccharide induced cell death. *Regul Pept* 2008; **146**: 218-
374 223.
- 375 22. Zhou X, Mantis N, Zhang XR, Potoka DA, Watkins SC, Ford HR. Salmonella
376 typhimurium induces apoptosis in human monocyte-derived macrophages.
377 *Microbiol Immunol* 2000; **44**: 987-995.
- 378 23. Forsberg M, Blomgran R, Lerm M, Särndahl E, Sebti SM, Hamilton A, Stendahl O,
379 Zheng L. Differential effects of invasion by and phagocytosis of *Salmonella typhimurium* on

380 apoptosis in human macrophages: potential role of Rho-GTPases and Akt. *J. Leuko Biol*
381 2003; **74**: 620-629.

382 24. Giamarellos-Bourboulis EJ, Routsis C, Plachouras D, Markaki V, Raftogiannis M,
383 Zervakis D, Koussoulas V, Orfanos S, Kotanidou A, Armaganidis A, Roussos C,
384 Giamarellou H. Early apoptosis of blood monocytes in the septic host: is it a mechanism of
385 protection in the event of septic shock? *Crit Care* 2006; **10**: R76.

386 25. Zhou J, Chaudhry H, Zhong Y, Ali MM, Perkins LA, Owens WB, Morales JE, McGuire
387 FR, Zumbun EE, Zhang J, Nagarkatti PS, Nagarkatti M. Dysregulation in microRNA
388 expression in peripheral blood mononuclear cells of sepsis patients is associated with
389 immunopathology. *Cytokine* 2014; **71**: 89-100

390 26. Hesse DG, Tracey KJ, Fong Y, Manogue KR, Palladino MA Jr, Cerami A, Shires GT,
391 Lowry SF. Cytokine appearance in human endotoxemia and primate bacteremia. *Surg*
392 *Gynecol and Obstet* 1988; **166**: 147-153.

393 27. Cannon JG, Tompkins RG, Gelfand JA, Michie HR, Stanford GG, van der Meer JW,
394 Endres S, Lonnemann G, Corsetti J, Chernow B, Wilmore DW, Wolff SM, Burke JF,
395 Dinarello CA. Circulating interleukin-1 and tumor necrosis factor in septic shock and
396 experimental endotoxin fever. *J.Infect Dis* 1990; **161**: 79-84.

397 28. Lv B, Huang J, Yuan H, Yan W, Hu G, Wang J. Tumor Necrosis Factor- α as a
398 Diagnostic Marker for Neonatal Sepsis: A Meta-Analysis. *Sci World J* 2014; **article ID**
399 **471463**,

400 29. Lv S, Han M, Yi R, Kwon S, Dai C, Wang R. Anti-TNF- α therapy for patients with
401 sepsis: a systematic meta-analysis. *Int J Clin Pract* 2014; **68**: 520-528.

402 30. Kumar S, Rizvi M. Prognostic serum tumor necrosis factor-alpha in paediatric patients
403 with sepsis. *J. Infect Dev Ctries* 2009; **3**: 437-441.

404 31. Gogos CA, Drosou E, Bassaris HP, Skoutelis A. Pro- versus anti-inflammatory cytokine
405 profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J.*
406 *Infect Dis* 1990; **16**: 982-987.

407 32. Hong T-H, Chang C-H, Ko W-J, Lin C-F, Liu H-H, Chow L-P, Huang C-T, Yu S-L,
408 Chen Y-S. 2014. Biomarkers of early sepsis may be correlated with outcome. *J. Trans Med*
409 2014; **12**: 146.

410 33. Pierrakos C, Vincent J-L. Sepsis biomarkers: a review. *Crit. Care* 2010; **14**: R15.

411 34. Opal SM, Huber CE. Bench-to-bedside review: Toll-like receptors and their role in septic
412 shock. *Crit. Care* 2002; **6**: 125–136.

413 35. Lakhani SA, Bogue CW. Toll-like receptor signaling in sepsis. *Curr. Opin. Pediatr* 2003;
414 **15**: 278–282.

415 (36) Arranz A, Androulidaki A, Zacharioudaki V, Martinez C, Margioris AN, Gomariz RP,
416 Tsatsanis C. Vasoactive intestinal peptide suppresses toll-like receptor 4 expression in
417 macrophages via Akt1 reducing their responsiveness to lipopolysaccharide. *Mol Immunol*
418 2008; **45**: 2970-2980.

419
420
421
422
423
424
425
426
427
428
429

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^{-7} M) 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 μ g/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-1 β (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-1 β (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-1 β (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 TNF α 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 TNF α ($P < 0.05$) compared with TNF α 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^{-7} M) 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.

494

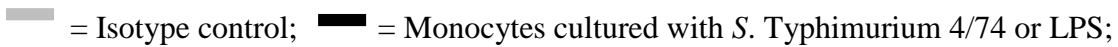

495


496

497

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.

510

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

513

514

515

516

517

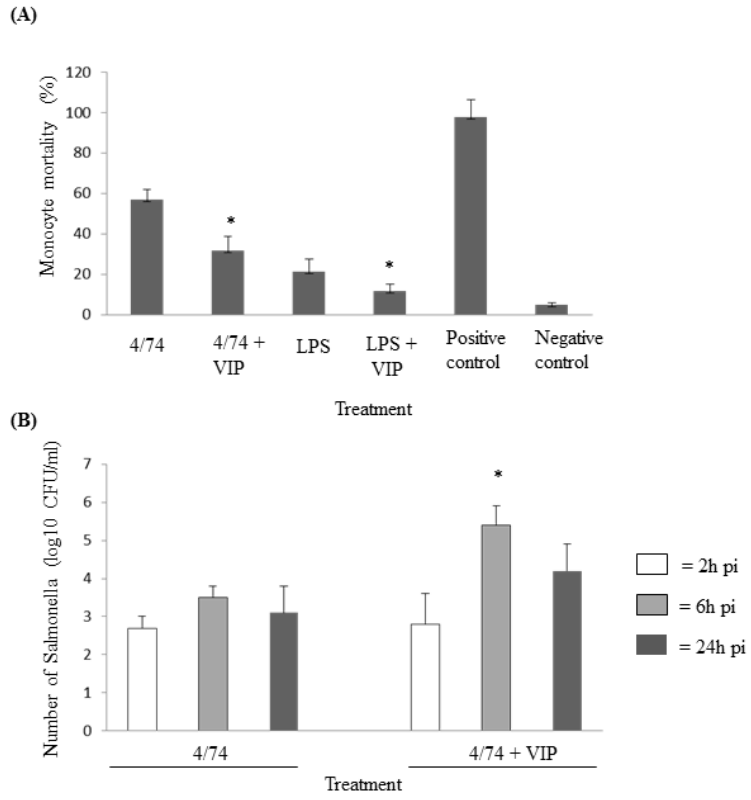
518

519

520

521

Figure 1 Askar et al



522

523

524

525

526

527

528

529

530

531

532

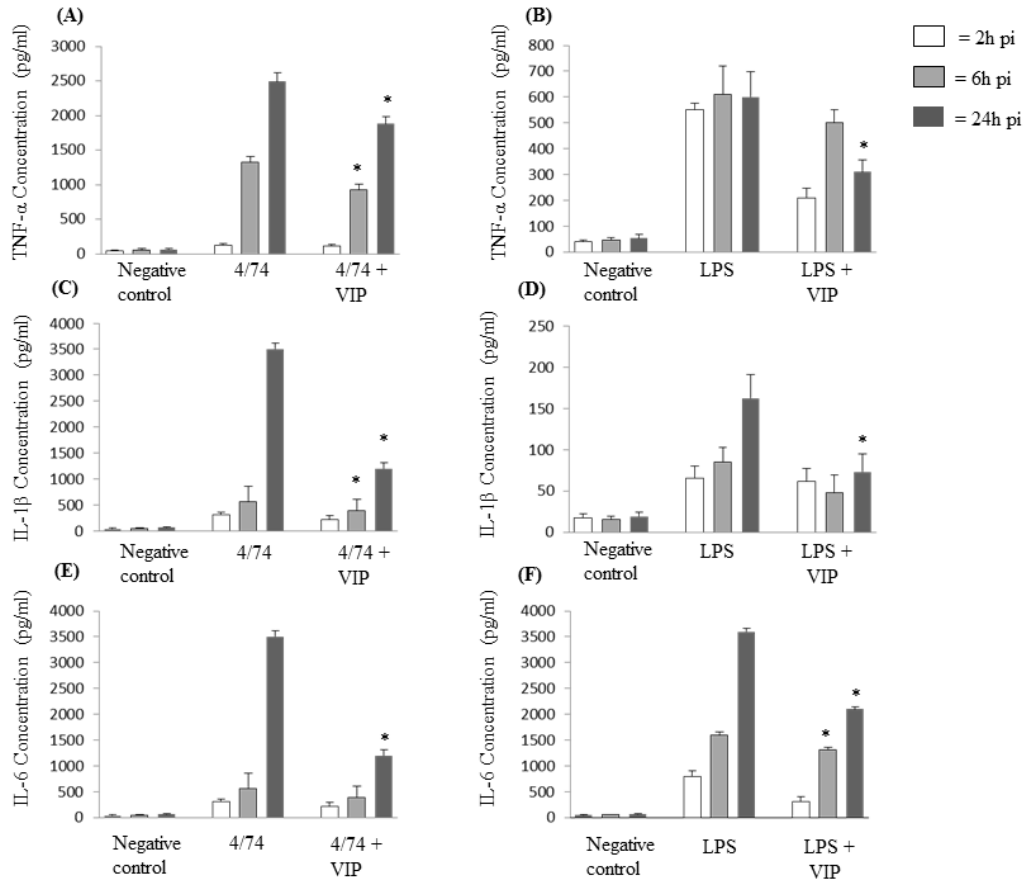
533

534

535

536

Figure 2 Askar et al



537

538

539

540

541

542

543

544

545

546

547

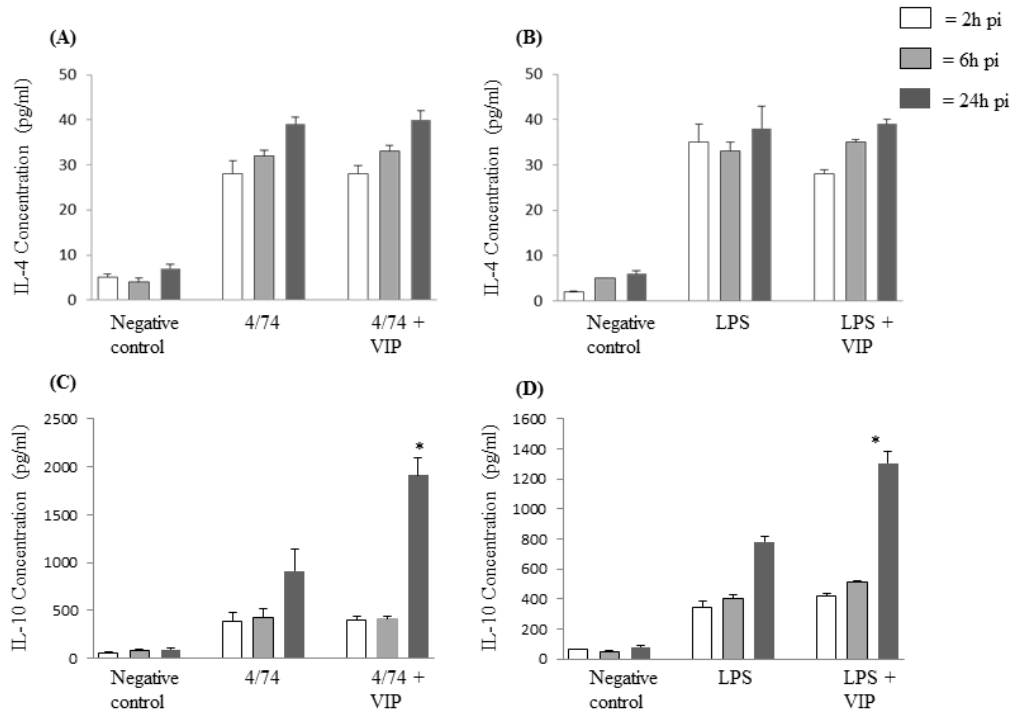
548

549

550

551

Figure 3 Askar et al



552

553

554

555

556

557

558

559

560

561

562

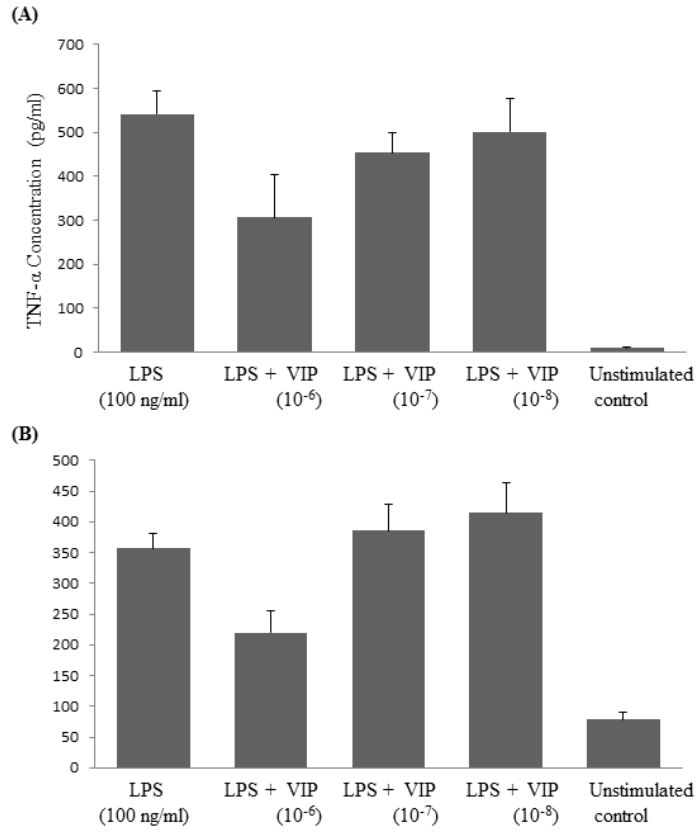
563

564

565

566

Figure 4 Askar et al



567

568

569

570

571

572

573

574

575

576

577

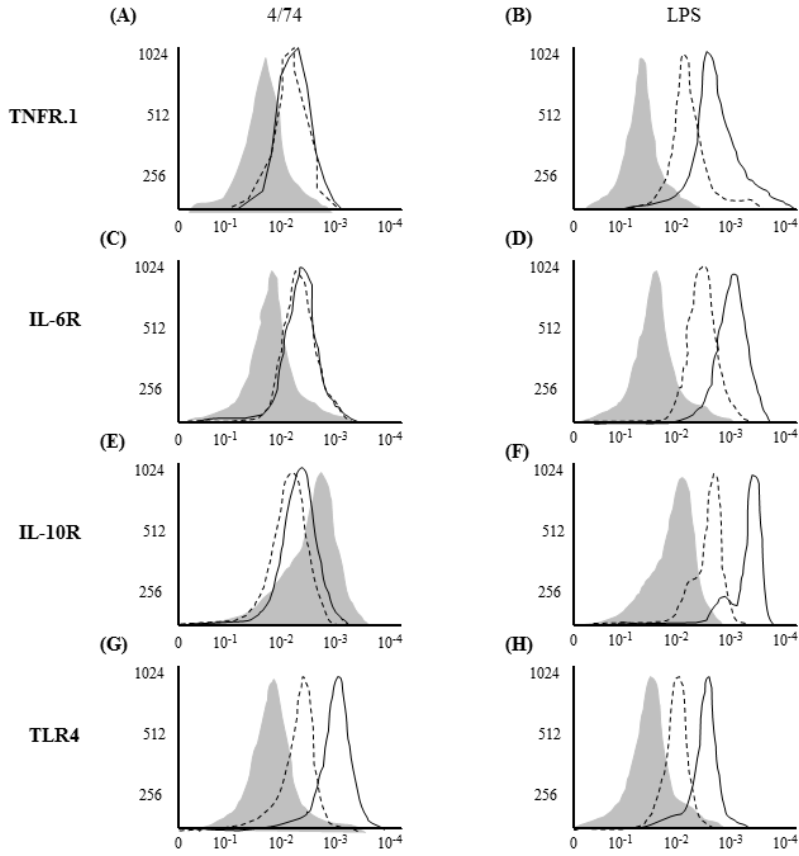
578

579

580

581

Figure 5 Askar et al



582

583

584

585

586

587

588

589

590

591

592

593

594

595

596

Table 1 Askar et al

Antibodies	Concentration	Isotype control antibodies
Mouse anti-human TNF-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

597
 598
 599
 600
 601
 602
 603
 604
 605
 606
 607
 608
 609
 610
 611