

1 **Immunomodulation by vasoactive intestinal peptide is associated with increased survival**
2 **and growth of Salmonella Typhimurium in mice.**

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22 **Running Title:** Salmonella exploits host VPAC1

23 **Key Words:** Salmonella VIP immunomodulation

24

25 **Abstract**

26 Studies have shown that administration of vasoactive intestinal peptide (VIP) in mice rescues
27 them from lethal endotoxaemia and that this is correlated with decreased concentration of
28 inflammatory cytokines. VIP has, therefore, been proposed as a novel anti-inflammatory which
29 could be used in the treatment of Gram negative sepsis. However, the effect of VIP has not been
30 reported in mice infected with viable Gram negative bacteria.

31 Here, we show that *Salmonella enterica* serovar Typhimurium 4/74 significantly increased
32 expression of mRNA of a type 1 receptor (VPAC1) for anti-inflammatory vasoactive intestinal
33 peptide (VIP) in murine ileum and mesenteric lymph nodes at day 6 post-infection (d6 pi) and in
34 the spleen at d3 pi. When VIP (5 nmol/ml) was administered to *S. Typhimurium*-infected mice,
35 there was a significant increase in the number of *S. Typhimurium* cultured from murine faeces
36 and ileum at d3 and 6 pi and in MLN and spleen at d3 dpi, compared to faeces and tissues
37 examined from mice infected with *S. Typhimurium* (without VIP administration). Administration
38 of VIP to *S. Typhimurium*-infected mice also altered the splenic architecture, resulting in a lack
39 of discernable periarterial lymphoid sheaths or marginal zones at d6 pi but liver histology
40 appeared similar on both d3 and d6 pi. The effects of VIP administration were correlated with a
41 significant decrease in expression of inflammatory cytokine mRNA, associated with systemic
42 inflammatory response syndrome (SIRS) of bacteraemia and acute sepsis.

43 We conclude that VIP inhibits expression of diagnostic/prognostic cytokine biomarkers of sepsis
44 in *S. Typhimurium*-infected mice. However, this is occurred with a concomitant increase in
45 *Salmonella* growth in tissues and increased bacterial shedding in faeces. Thus, VIP may have
46 potential as an adjunctive therapy to antibiotics in sepsis.

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49 **1.0 Introduction**

50 The bi-phasic model for human sepsis proposes a phase of uncontrolled production of
51 inflammatory mediators, which leads to systemic inflammatory response syndrome (SIRS) (1-2),
52 followed by a compensatory anti-inflammatory response syndrome (CARS) (3). The SIRS
53 (acute) phase of sepsis is associated with high systemic concentrations of pro-inflammatory
54 cytokines released by monocytes and macrophages, such as TNF- α , IL-1 and IL-6 (4).

55 Intervention with anti-inflammatories has been proposed as a rational therapeutic avenue during
56 SIRS but specific inhibition of IL-1 β or TNF- α has failed (5), while broad ranging anti-
57 inflammatories, such as glucocorticoids, are also widely used in the treatment of sepsis but their
58 effect is debateable probably due to timing of therapy, dosage and the development of steroid
59 resistance by glucocorticoid receptors (6).

60 In murine models of LPS-induced Gram negative sepsis, intra-peritoneal administration of low
61 concentrations of VIP (5 nmol) prevented mortality and this was associated with inhibition of
62 inflammatory cytokines (7). In this regard, VIP may have potential in the treatment of Gram
63 negative sepsis. The amino acid structure of VIP is highly conserved throughout the vertebrates
64 and is identical in all mammals apart from guinea pigs (8). VIP exerts its biological effect via
65 three G-protein coupled receptors; VIP receptor 1 (VPAC1), VPAC2 and a receptor which is
66 also activated via the pituitary adenylate-cyclase activating polypeptide (PAC1). However, the
67 immunosuppressive action of VIP on LPS-stimulated murine macrophages occurs via VPAC1
68 (9) and therefore the interaction of VIP with VPAC1 during infection is worth further
69 investigation. Increased VPAC1 expression was also reported in human peripheral blood
70 monocytes following intravenous administration of LPS and this was correlated with increased
71 VIP concentration in sera (10).

72 However, using virulent *S. Typhimurium* (rather than LPS) we have shown that VIP promotes
73 survival of *S. Typhimurium* within human monocytes, which is associated with inhibition of
74 proinflammatory cytokines and increased survival of the infected cells (11). This may indicate
75 that *S. Typhimurium* increases receptivity of monocytes to VIP, specifically to inhibit production
76 of inflammatory mediators, thus gaining survival advantage

77 The aim of this current study was to; (1) investigate the effect of a previously reported
78 therapeutic concentration of VIP (5 nmol/ml) in mice infected with viable *Salmonella* rather than
79 LPS. (2) investigate expression of the immunosuppressive VPAC1 receptor during infection; to
80 examine the hypothesis that increased VPAC1 and VIP administration may correlate with
81 decreased inflammatory cytokine expression and increased *Salmonella* survival

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83 **2.0 Material and methods**

84 **2.1 Reagents**

85 Unless otherwise stated all reagents were purchased from Sigma-Aldrich, Poole, Dorset UK.

86 PCR and microarray reagents were purchased from Qiagen, Manchester, UK.

87 **2.2 Bioethics**

88 All studies were conducted following approval by local ethics committees and under UK Home

89 Office license.

90 **2.3 Bacterial culture and strains.**

91 *S. Typhimurium* 4/74 were grown in Luria-Bertani (LB) broth (Life Technologies Ltd., Paisley,

92 United Kingdom) for 18 h at 37°C under agitation. The bacteria were then sub-cultured in fresh

93 LB broth for 4 h to late log phase (established by conventional counts of CFU). Prior to

94 infection, bacteria were adjusted to a multiplicity of infection (MOI) of 10.

95 **2.4 Murine studies**

96 Female BALB/c mice, 6-10 weeks old, were divided into 3 groups as follows: Group 1, 12 mice
97 were orally gavaged with 100 µl PBS and intra-peritoneally (i.p) injected with 50 µl of PBS 1h
98 later. Group 2, 20 mice were orally gavaged with 1×10^4 *S. Typhimurium* 4/74 in 100 µl PBS
99 (sub-lethal dose) and injected i.p. with 50 µl of PBS 1h later. Group 3. 20 mice were orally
100 gavaged with 100 µl PBS and injected i.p. with VIP (5 nmol in 50 µl PBS) 1h later. Half of
101 each group of mice were euthanised by cervical dislocation on either day 3 (d3) or day 6 (6d)
102 post-infection (pi). This experiment was performed in duplicate (N= 24 uninfected control, 40 *S.*
103 *Typhimurium* infected and 40 *S. Typhimurium* infected/VIP. Spleen, ilea, mesenteric MLN and
104 livers were aseptically removed and fixed in 10 % neutral buffered formalin (NBS) (histological
105 analysis), or RNAlater (PCR analysis) or homogenised in sterile PBS using Griffith's tubes for
106 bacterial enumeration. In addition, litter trays were changed daily and faecal pellets on 3 and 6
107 dpi were collected for enumeration of *S. Typhimurium*.

108 **2.5 Histological studies**

109 Histological analysis of tissues was performed using standard techniques. Briefly, tissue fixed in
110 NBS was washed in 70% ethanol prior to clearing and finally embedding in paraffin blocks. The
111 blocks were sectioned using a microtome and stained by routine haematoxylin and eosin
112 methods prior to examination using a conventional light microscope. 5 sections were examined
113 per organ per mouse.

114 **2.6 Quantitative PCR analysis**

115 To investigate expression of VPAC1 and inflammatory cytokines in murine tissues, quantitative
116 PCR was used. RNA was purified from murine tissue using Rneasy plus kits (Qiagen, Hilden,
117 Germany). RNA quantity and quality were measured using a NanoDrop 8000 spectrophotometer

118 (Thermo Scientific, Warrington, UK) and was converted into complimentary DNA (cDNA)
119 using a SuperScript first strand DNA synthesis kit (Invitrogen, Carlsbad, CA, USA). For
120 quantitative PCR analysis, primers and probes were designed using a universal probe library
121 (Roche Diagnostics, Mannheim, Germany). A PCR reaction volume (20 μ l) consisting of 10 μ l
122 Light Cycler 480 Probes Master (Roche, Germany), 1 μ m of each forward and reverse primer
123 (Eurofins MWG, Operon, Germany) and 0.2 μ l, labelled with Fluorescein and dark quencher
124 dye. The total volume was adjusted to 20 μ l using PCR water. In negative control wells, PCR
125 water was added instead of cDNA. Standard curves of target and reference genes were
126 performed at dilution ranges between 1:10- 10⁵. Thermal cycles consisted of denaturing at 95 °C
127 for 10 min and 40 cycles of sample amplification at 95 °C for 10 secs, 60 °C for 30 secs, 72 °C
128 for 1 min and cooling at 40 °C for 30 secs, performed using a Roche applied sciences light cycler
129 480 (Roche, Germany). All data was normalised to expression in uninfected mouse tissues and
130 quantification was determined by comparison to the reference gene GAPDH, using the Pfaffl
131 method (12). All primers pairs used in the PCRs are shown in Table 1.

132 Table 1. Forward and reverse primers and probes used in PCR reactions.

133 **2.7 Statistical Analysis**

134 Two-tailed unpaired student's *t* test or one-way ANOVA were performed to determine
135 significant differences between different groups (control negative, *S. Typhimurium* infected,
136 with or without VIP) using Graph Pad Prism software. Bonferroni's multiple comparisons test
137 was applied to examine significant difference between the means of more than two groups,
138 following ANOVA. Significance values were determined at the 95% confidence limit ($P < 0.05$).

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142 **3.0 Results**

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144 **3.1 *S. Typhimurium* increases VPAC1 expression in murine tissues**

145 VPAC1 mRNA expression was significantly increased in murine tissue following *S.*
146 *Typhimurium* infection at d3 and 6 pi. In the ileum VPAC1 expression was significantly
147 increased at d3 pi ($P < 0.05$) above control level. Administration of VIP also significantly
148 increased VPAC1 expression ($P < 0.05$) at d6 post-administration. A significant increase (P
149 < 0.05) in VPAC1 expression in *S. Typhimurium*-infected ileal tissue compared to ileal tissue
150 from mice in which VIP was administered was measured at d3 pi only (Fig 1A). In the MLN, *S.*
151 *Typhimurium* infection induced a significant increase in VPAC1 expression on both d3 and 6 pi
152 above control expression ($P < 0.05$) and on both days VPAC1 expression in infected MLN was
153 significantly higher than VPAC1 expression in MLN of VIP administered mice ($P < 0.05$), in
154 which VPAC1 was not differentially expressed. *S. Typhimurium* also significantly increased
155 VPAC1 expression in the spleens of mice at both d3 and 6 pi compared to VPAC1 expression in
156 controls ($P < 0.05$) (Fig 1B). Administration of VIP also significantly increased VPAC1
157 expression in the spleen on both days 3 and 6 compared to VPAC1 expression in controls (P
158 < 0.05). However, a significant increase ($P < 0.05$) in VPAC1 expression in *S. Typhimurium*-
159 infected spleen compared to spleens from mice in which VIP was administered was measured at
160 6 dpi (Fig 1C).

161 **3.2 VPAC1 expression correlates with the numbers of *S. Typhimurium* recovered from** 162 **tissues following VIP administration**

163 This trend in VPAC1 expression correlated with the numbers of *S. Typhimurium* recovered from
164 tissues following VIP administration. Therefore, following VIP administration, significantly
165 more *S. Typhimurium* were isolated from the faeces (Fig 2A) and ileum (Fig 2B) of infected

166 mice on both d3 and 6 pi compared to *S. Typhimurium* infected mice which had not been
167 injected with VIP ($P < 0.05$). A significantly greater number of *S. Typhimurium* were also
168 recovered from the MLN (Fig 2C) and spleen (Fig 2D) at 6 dpi following VIP administration,
169 compared to *S. Typhimurium*-infected mice which had not been injected with VIP ($P < 0.05$).

170 **3.3 VIP induces histological changes in the spleens of mice infected with *S. Typhimurium***

171 On d3 pi, splenic architecture was similar in *S. Typhimurium* infected mice, *S. Typhimurium*
172 infected mice in which VIP was administered and uninfected mice, with tightly packed
173 periarteriolar sheaths (PALS) and clear marginal zones (Fig 3A). However, administration of
174 VIP to *S. Typhimurium*-infected mice altered the splenic architecture, resulting in a lack of
175 discernable periarterial lymphoid sheaths or marginal zones by d6 pi (Fig 3B). On both d3 and 6
176 pi, liver histology appeared similar in *S. Typhimurium* infected mice and in infected mice
177 administered with VIP, with immune cell infiltrates of similar size and number (Fig 3C and D).
178 The nature of the cellular infiltrate was also lymphocyte/neutrophil in infected tissues with or
179 without VIP.

180 **3.4 VIP decreases cytokine mRNA expression in *S. Typhimurium*-infected tissues**

181 We studied the expression of prognostic cytokine markers of bacteraemia/sepsis in *S.*
182 *Typhimurium*-infected mice and upregulation of these cytokines was mostly inhibited by VIP in
183 tissue.

184 Generally, administration of VIP alone significantly increased expression of IL-1 β and IL-6
185 above control levels in the murine tissues we examined, and this was also generally the case
186 when mice were infected with *S. Typhimurium* (Fig 4A-F). However, administration of VIP to *S.*
187 *Typhimurium*-infected mice significantly inhibited upregulation of IL-1 β in the ileum at d6 pi (P
188 < 0.01) (Fig 4A); in the MLN at both d3 ($P < 0.01$) and 6 pi ($P < 0.05$) (Fig 4B) and in the spleen

189 at d3 pi ($P < 0.01$) (Fig 4C). The same trend in IL-6 expression was observed; therefore VIP
190 significantly inhibited IL-6 in the ileum at 6 dpi ($P < 0.01$) (Fig 4D); in the MLN at both d3 (P
191 < 0.01) and 6 pi ($P < 0.05$) (Fig 4E) and in the spleen at d3 pi ($P < 0.01$) (Fig 4F).

192 Administration of VIP alone did not increase TNF- α expression above control levels in the
193 ileum or MLN on either d3 or 6 post-administration but significantly increased TNF- α
194 expression in the spleen at d6 ($P < 0.05$) (Fig 5A-C). TNF- α expression was significantly
195 increased ($P < 0.05$) in the ilea of *S. Typhimurium*-infected mice at d6 pi above control levels (P
196 < 0.05) but no significant difference ($P > 0.05$) in TNF- α expression was detected in the ilea of *S.*
197 *Typhimurium*-infected mice in which VIP was administered (Fig 5A). *S. Typhimurium*
198 significantly increased TNF- α expression in the ilea ($P > 0.01$) and spleens ($P > 0.05$) of mice at
199 both d3 and 6 pi (Fig 5B and C) and VIP administration in *S. Typhimurium*-infected mice
200 significantly decreased TNF- α expression in the MLN on both d3 ($P < 0.01$) and 6 pi ($P < 0.01$)
201 (Fig 5B) and in the spleen at d3 pi ($P > 0.01$) (Fig 5C).

202 Administration of VIP alone significantly increased expression of IFN- γ above control levels in
203 the ileum, MLN and spleen at d6 post-administration ($P < 0.05$) (Fig 5D-F). *S. Typhimurium*
204 infection induced significantly increased expression of IFN- γ at d6 pi in the ileum ($P < 0.01$) (Fig
205 5D) and MLN ($P < 0.01$) (Fig 5E) and at d3 and 6 pi in the spleen ($P < 0.05$) (Fig 5F). VIP
206 significantly decreased IFN- γ expression in the ileum ($P < 0.01$) (Fig 5D) and MLN ($P < 0.01$)
207 (Fig 5E) at d6 pi and spleen in the spleen at both d3 and 6 pi ($P < 0.05$) (Fig 5F).

208 Administration of VIP alone significantly increased IL-10 mRNA expression above control
209 levels only on d6 post-administration and only in splenic tissue ($P < 0.05$) (Fig 6C). *S.*
210 *Typhimurium* infection induced significantly increased IL-10 expression in the ileum at both d3
211 and 6 pi ($P < 0.01$) (Fig 6A); in the MLN at d6 pi ($P < 0.01$) (Fig 6B) and in the spleen at both 3

212 and d6 pi ($P < 0.05$) (Fig 6C). VIP significantly decreased IL-10 upregulation in the ilea of *S.*
213 Typhimurium-infected mice at both d3 and 6 pi ($P < 0.01$) (Fig 6A); in the MLN at d6 pi (P
214 < 0.05) (Fig 6B) and in the spleen at both d3 and 6 pi ($P < 0.05$) (Fig 6C).

215

216 **4.0 Discussion**

217 An original study by Delgado et al (1999) reported that administration of 5 nmol of VIP
218 significantly decreased mortality in mice in which Gram negative sepsis was induced by LPS and
219 that this was associated with significant reduction in inflammatory cytokine production (7).
220 Thus, VIP may have potential as a novel therapeutic in Gram negative bacteraemia and sepsis. In
221 accordance with this, we show that VIP administration in *S.* Typhimurium-infected mice also
222 inhibited upregulation of cytokines used as prognostic markers of sepsis (IL-1 β , IL-6, TNF- α ,
223 IFN- γ and IL-10). However, in addition to this we show that *S.* Typhimurium 4/74 infection
224 significantly increased VPAC1 expression in murine ileum, MLN and spleen and this was
225 associated with a concurrent and significant increase in the number of *S.* Typhimurium isolated
226 from these organs and in faeces, following VIP administration. This may indicate that *S.*
227 Typhimurium could utilise the VPAC1/VIP axis to increase survival by down-regulation of
228 inflammatory mediators. Delgado et al., (2000) (9) reported that a VPAC1 antagonist was more
229 efficient than a VPAC2 antagonist at inhibiting LPS-induced inflammatory mediators from
230 murine macrophages. In our study, neither VPAC2 nor PAC1 were differentially expressed in
231 any of the tissues we examined following infection (data not shown), which further supports the
232 hypothesis that VPAC1 is the dominant immunosuppressive receptor that VIP acts upon.
233 Storka et al., (2013) (10) also reported a $>30\%$ increase in VPAC1 expression in monocytes
234 isolated from human volunteers 24h after infusion of LPS and this was correlated with an

235 increased concentration of VIP in plasma. In our previous studies using viable *S. Typhimurium*,
236 rather than LPS, we have shown that VIP down-regulates inflammatory mediators and increases
237 *Salmonella* survival in human monocytes (11, 13) and murine macrophages (14); which may
238 suggest that *S. Typhimurium* utilises the VIP/VPAC1 axis to increase survival in humans as well
239 as mice. We have shown that although LPS does increase cellular expression of VPAC1, this is
240 much lower when compared to *S. Typhimurium*-infected cells (data not supplied). However,
241 *Salmonella* have evolved complex gene networks (pathogenicity islands) which are involved in
242 virulence and it is likely that some of these may influence VPAC1 expression.

243 All of the cytokines we have studied in this work have been shown to be expressed during *S.*
244 *Typhimurium* infection in vitro and in vivo and have been proposed as diagnostic and/or
245 prognostic biomarkers of sepsis. The SIRS (acute) phase of sepsis is associated with high
246 systemic concentrations of pro-inflammatory cytokines released by monocytes and macrophages,
247 such as TNF- α , IL-1 and IL-6 (4) and a study by Gogos et al. (2000) (15) reported that IL-10
248 concentration was positively correlated with the onset of sepsis and that high IL-10/TNF- α ratio
249 indicated a poor prognosis. High circulating levels of IL-6 and IL-10 have also been associated
250 with mortality in other studies (16, 17). However, we show that during virulent infection, IL-10
251 expression is increased by *S. Typhimurium* but this is not synergistically increased by VIP. This
252 is in contrast to a study by Delgado et al., (1999) (18) who reported a synergistic effect in mice,
253 in vivo and in vitro, following LPS stimulation. The difference we show is likely due to the use
254 of viable bacteria and once again highlights the limitations in using LPS only.

255 In the context of VIP, IFN- γ is the most studied of all. In murine J774 macrophages, VIP inhibits
256 IFN- γ -induced reactive oxygen species (14) which leads to intracellular survival of wild type and
257 *Phop* mutant *S. Typhimurium*. Normally, *S. Typhimurium* with a mutation of the *Phop/PhoQ*

258 regulon are highly attenuated in macrophages (19) and are unable to survive in mice (20). A
259 more recent study has also shown that VIP inhibited expression of IFN- γ and IFN- γ receptor
260 genes, and upregulated expression of suppressor of cytokine signalling (SOCS) 1 and 2, in
261 human monocytes infected with *S. Typhimurium* 4/74 (13). Furthermore, in post-operative
262 human sepsis, mortality has been associated with monocyte resistance to IFN- γ , which fails to
263 increase the release of crucial monocyte cytokines (21) and pneumonia-induced sepsis has been
264 reported to be associated with SNPs in IFN- γ genes (22). It is, therefore, likely that
265 *S. Typhimurium*-induced inhibition of inflammatory cytokines (such as IFN- γ), via the
266 VPAC1/VIP axis in murine tissues, had a profound effect on *S. Typhimurium* survival in vivo.

267 In conclusion, VIP decreases expression of cytokines which are hyper-expressed in sepsis.
268 Therefore, VIP may have some therapeutic value. However, the decrease in cytokine expression
269 is associated with an increase in the numbers of *S. Typhimurium* in tissue and shed in faeces; and
270 *S. Typhimurium* may increase VPAC1 expression in tissue for this purpose. The therapeutic use
271 of VIP would therefore need to be as an adjunctive therapy together with antibiotics and it is
272 possible that intravenous infusion of VIP could reduce the prolonged hyper-expression of
273 cytokines, which remain after antibiotic therapy has cleared the bacterial infection. The
274 longevity and concentration of VIP required for therapeutic effect needs further elucidation.

275

276 **Funding support:** The work was funded by a University of Duhok / Kurdistan Region of Iraq
277 scholarship awarded to BA.

278

279 **Acknowledgments**

280 We would like to thank Mr Scott Hulme for assistance with murine infection studies.

281 **Disclosures**

282 None to declare.

283

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350 **Figure Legends**

351

352 **Figure 1. *S. Typhimurium* 4/74 infection increases VPAC1 expression *in vivo***

353 *S. Typhimurium* infection significantly increased VPAC1 mRNA expression, above control
354 expression, in the ileum at d3 pi (A); in the mesenteric lymph nodes (MLN) (B) and spleen (C) at
355 both d3 and d6 pi. Administration of VIP (5 nmol) significantly increased VPAC1 mRNA
356 expression, above control expression, in the ileum after d3 (A) and in the spleen (C) at both d3
357 and d6 post-administration. *S. Typhimurium* infection also significantly increased VPAC1
358 expression above expression measured in mice in which VIP was measured in the ileum (3d pi; P
359 <0.05); MLN (d3 and d6 pi; $P <0.05$) and in the spleen (d6 pi; $P <0.05$). Values shown are
360 means obtained from duplicate experiments containing 12 uninfected (control) mice; 6
361 euthanised on day 3 and 6 on day 6. Mean values are also shown for a second group of 20 mice
362 infected with *S. Typhimurium*; 10 euthanised on day 3 and 10 on day 6 and a third group which
363 were infected with *S. Typhimurium* and injected with VIP (5 nMol). $N = 24$ control, 40 infected
364 and 40 infected/VIP. Fold changes are expressed as a comparison with mRNA expression
365 measured in uninfected (control) mice, assigned an arbitrary value of 1. Error bars show standard
366 deviation from the mean (SD). Linkage bars show significant differences in VPAC1 expression
367 ($P = 0.05$) between murine tissues infected with *S. Typhimurium* or following VIP administration
368 alone. Asterisk shows significant difference at $P = 0.05$ (*) between VPAC1 expression in
369 murine tissue infected with *S. Typhimurium* or following VIP administration alone compared to
370 unstimulated controls. Grey bar = VPAC1 expression in uninfected control mice; Black bar =
371 VPAC1 expression following *S. Typhimurium* infection or VIP administration for 3 days. White
372 bar = VPAC1 expression following *S. Typhimurium* infection or VIP administration for 6 days.

373 **Figure 2. VIP administration increases *S. Typhimurium* colonisation following**
374 **administration of VIP**

375 Administration of VIP (5 nmol) to infected mice significantly increased the number of *S.*
376 *Typhimurium* shed in feces (A) and isolated from the ileum (B) at d3 and d6 pi and also the
377 number of *S. Typhimurium* isolated from the mesenteric lymph nodes (MLN) (C) and spleen (D)
378 at d6 pi. Values shown are means of colony forming units per ml (CFU/ml). Error bars show
379 standard deviation from the mean (SD). Linkage bars show significant differences at $P = 0.05$
380 between mice infected with *S. Typhimurium* and *S. Typhimurium*-infected mice in which VIP
381 was administered. Black bar = d3 pi; White bar = d6 pi.

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384 **Figure 3. VIP alters the splenic architecture of *S. Typhimurium* infected mice**

385 (A) = Histological section of murine uninfected (control) spleens and d3 and 6 pi with *S.*
386 *Typhimurium* or *S. Typhimurium*-infected/ VIP administered. Closed arrows highlight tightly
387 packed periarteriolar sheaths (PALS) with clear marginal zones. Closed arrows show dispersed
388 PALS in *S. Typhimurium* infected mice (6d pi) in which VIP was administered. (B) =
389 Histological section of murine uninfected (control) livers at d3 and 6 pi with *S. Typhimurium* or
390 *S. Typhimurium*-infected/VIP administered. Closed arrows show similar cell infiltrate at d3 and
391 6 pi in mice infected with *S. Typhimurium* and in *S. Typhimurium*-infected mice in which VIP
392 was administered. All sections were stained with haematoxylin and Eosin. Images are
393 representative of 5 sections analysed per organ per mouse. Scale bar (bottom left) = 50 μm .

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396 **Figure 4. VIP inhibits expression of IL-1 β and IL-6 mRNA in *S. Typhimurium* infected**
397 **tissue**

398 Administration of VIP (5 nmol) alone significantly increased IL-1 β and IL-6 mRNA expression
399 above uninfected (control) levels in the ileum, MLN and spleen at d6 post-administration (A-F),
400 with the only exception being IL-6 in the MLN (E). *S. Typhimurium* infection significantly
401 increased expression of IL-1 β and IL-6 mRNA above uninfected (control) levels in the ileum,
402 MLN and spleen at d3 and/or 6 pi and this was inhibited when VIP was administered to *S.*
403 *Typhimurium*-infected mice (A-F). Fold changes are expressed as a comparison with mRNA
404 expression measured in uninfected (control) mice, assigned an arbitrary value of 1. Error bars
405 show standard deviation from the mean (SD). Linkage bars show significant differences (P =
406 0.05) between IL-1 β and IL-6 mRNA expression in tissue from mice in infected with *S.*
407 *Typhimurium* and *S. Typhimurium*-infected mice in which VIP was administered. Asterisk shows
408 significant difference at P =0.05 (*) or P =0.01 (**). Linkage bars show significant differences (P =
409 0.05) between IL-1 β and IL-6 mRNA expression in tissue from mice in which VIP alone was administered, in mice infected with *S. Typhimurium*
410 and *S. Typhimurium*-infected mice in which VIP was administered, compared to
411 uninfected/unstimulated (control) mice. Grey bar = IL-1 β or IL-6 mRNA expression in
412 uninfected control mice; Black bar = IL-1 β or IL-6 mRNA expression following *S. Typhimurium*
413 infection or VIP administration for 3 days. White bar = IL-1 β or IL-6 mRNA expression
414 following *S. Typhimurium* infection or VIP administration for 6 days.

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418 **Figure 5. VIP inhibits expression of TNF α and IFN- γ mRNA in *S. Typhimurium* infected**
419 **tissue**

420 Administration of VIP (5 nmol) alone significantly increased TNF α mRNA expression only in
421 the spleen at d6 post-administration (C) but significantly increased IFN- γ mRNA expression in
422 the ileum, MLN and spleen at d6 pi (D-F). *S. Typhimurium* infection significantly increased
423 expression of TNF α and IFN- γ mRNA above uninfected (control) levels in the ileum, MLN and
424 spleen at d3 and/or d6 pi and this was inhibited when VIP was administered to *S. Typhimurium*-
425 infected mice (A-F).

426 Fold changes are expressed as a comparison with mRNA expression measured in uninfected
427 (control) mice, assigned an arbitrary value of 1. Error bars show standard deviation from the
428 mean (SD). Linkage bars show significant differences ($P = 0.05$) between IL-1 β and IL-6
429 mRNA expression in tissue from mice in infected with *S. Typhimurium* and *S. Typhimurium*-
430 infected mice in which VIP was administered. Asterisk shows significant difference at $P = 0.05$
431 (*) or $P = 0.01$ (**) between IL-1 β and IL-6 mRNA expression in tissue from mice in which VIP
432 alone was administered, in mice infected with *S. Typhimurium* and *S. Typhimurium*-infected
433 mice in which VIP was administered, compared to uninfected/unstimulated (control) mice. Grey
434 bar = IL-1 β or IL-6 mRNA expression in uninfected control mice; Black bar = IL-1 β or IL-6
435 mRNA expression following *S. Typhimurium* infection or VIP administration for 3 days. White
436 bar = IL-1 β or IL-6 mRNA expression following *S. Typhimurium* infection or VIP administration
437 for 6 days.

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441 **Figure 6. *S. Typhimurium* increases IL-10 expression but is not synergistically increased by**
442 **VIP**

443 Administration of VIP (5 nmol) alone significantly increased TNF α mRNA expression only in
444 the spleen at d6 post-administration (C). *S. Typhimurium* infection significantly increased
445 expression of IL-10 mRNA above uninfected (control) levels in the ileum, MLN and spleen at 3
446 and/or 6 dpi and this was inhibited when VIP was administered to *S. Typhimurium*-infected mice
447 (A-F). Fold changes are expressed as a comparison with mRNA expression measured in
448 uninfected (control) mice, assigned an arbitrary value of 1. Error bars show standard deviation
449 from the mean (SD). Linkage bars show significant differences ($P = 0.05$) between IL-10
450 expression in tissue from mice in infected with *S. Typhimurium* and *S. Typhimurium*-infected
451 mice in which VIP was administered. Asterisk shows significant difference at $P = 0.05$ (*) or P
452 $= 0.01$ (**) between IL-10 expression in tissue from mice in which VIP alone was administered,
453 in mice infected with *S. Typhimurium* and *S. Typhimurium*-infected mice in which VIP was
454 administered, compared to uninfected/unstimulated (control) mice. Grey bar = IL-10 mRNA
455 expression in uninfected control mice; Black bar = IL-10 mRNA expression following *S.*
456 *Typhimurium* infection or VIP administration for 3 days. White bar = IL-10 expression following
457 *S. Typhimurium* infection or VIP administration for 6 days.

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464 **Table 1. Primer pairs used in qPCR analysis**

465 Table shows the primer pairs (forward and reverse sequences) used to determine expression of
466 cytokines, VPAC1 and GAPDH (reference gene) in murine tissues.

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Fig 1. Askar et al

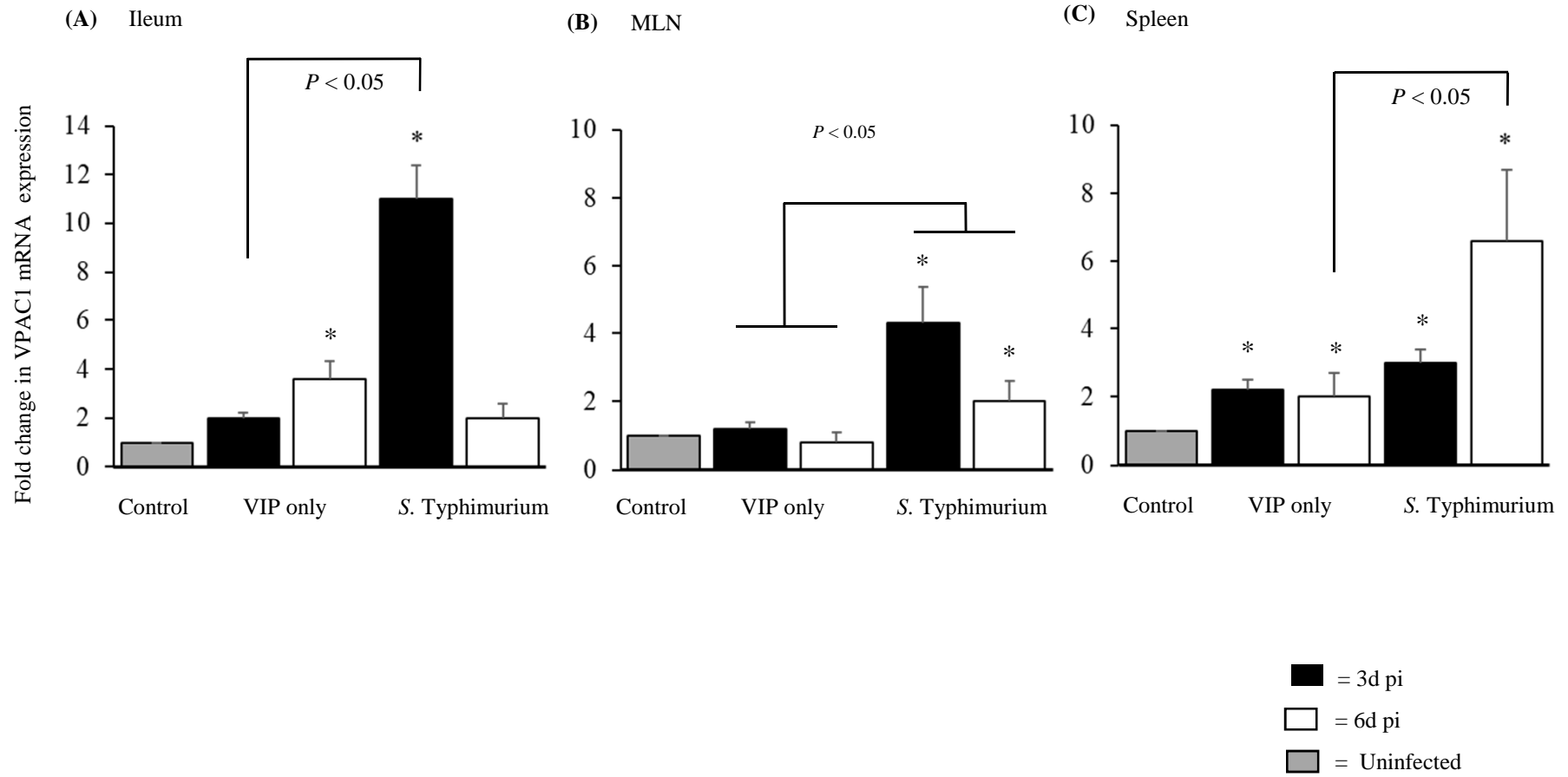


Fig 2. Askar et al

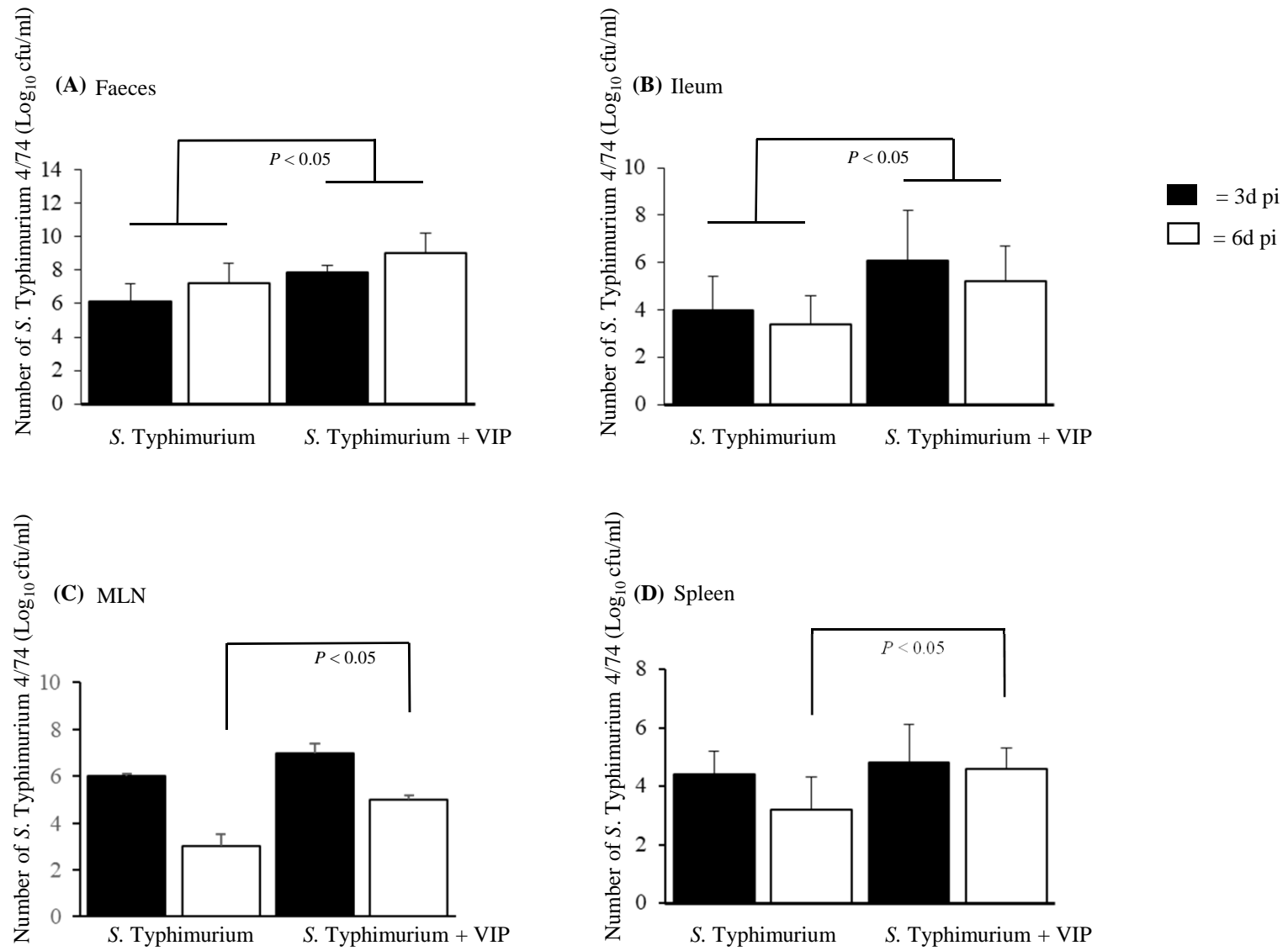


Fig 3. Askar et al

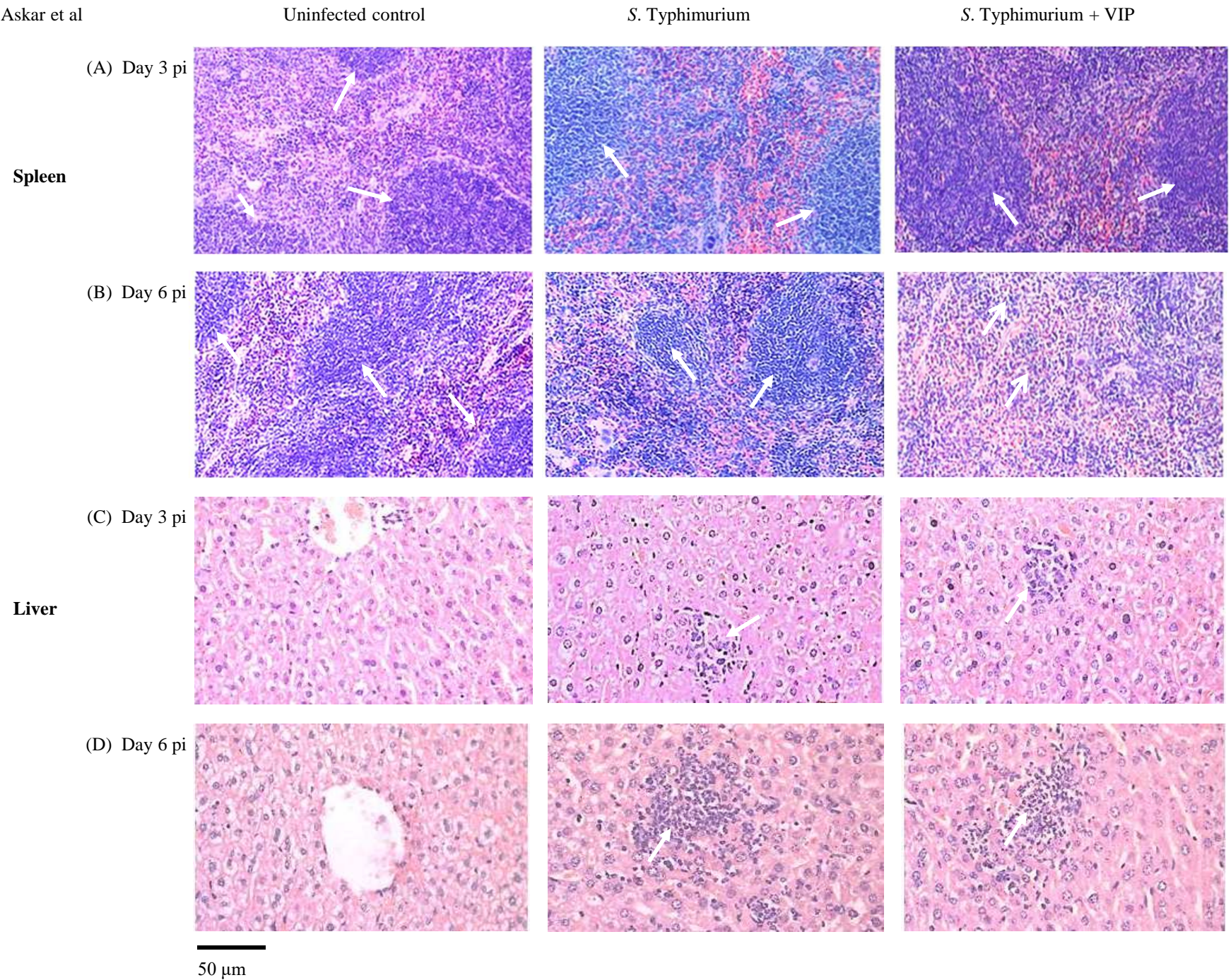


Fig 4. Askar et al

■ = 3d pi
□ = 6d pi

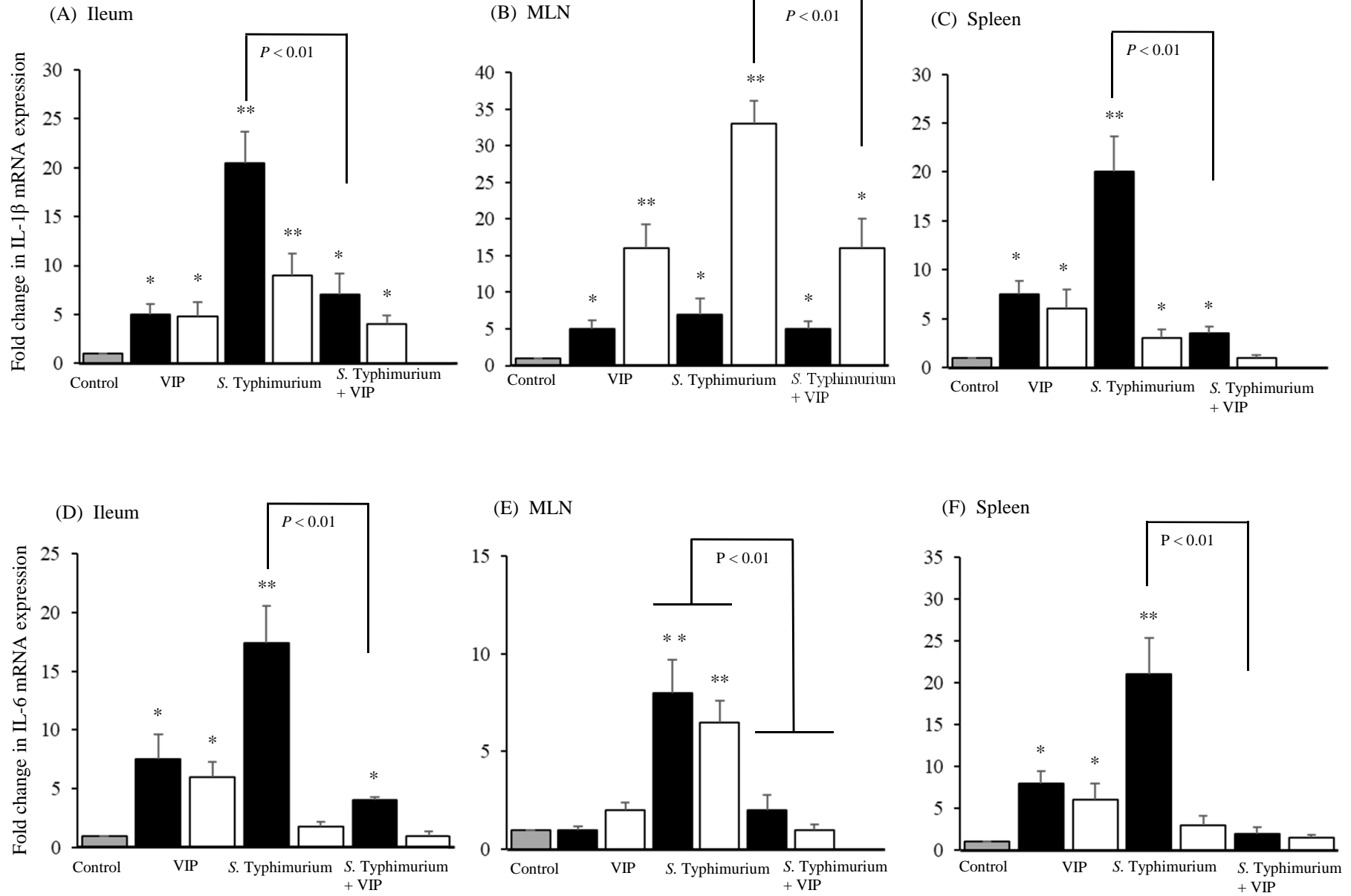


Fig 5. Askar et al

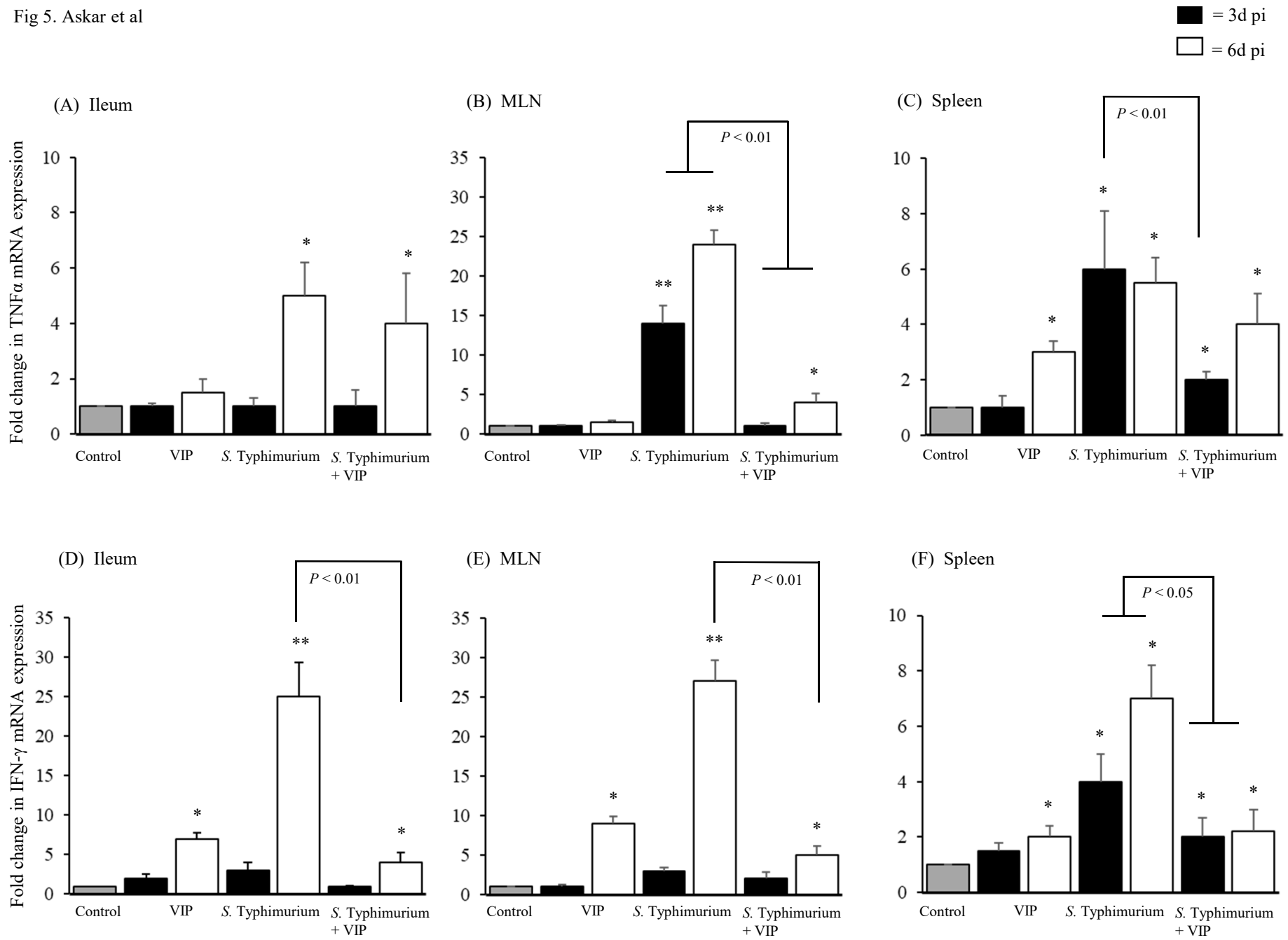


Fig 6. Askar et al

■ = 3d pi
□ = 6d pi

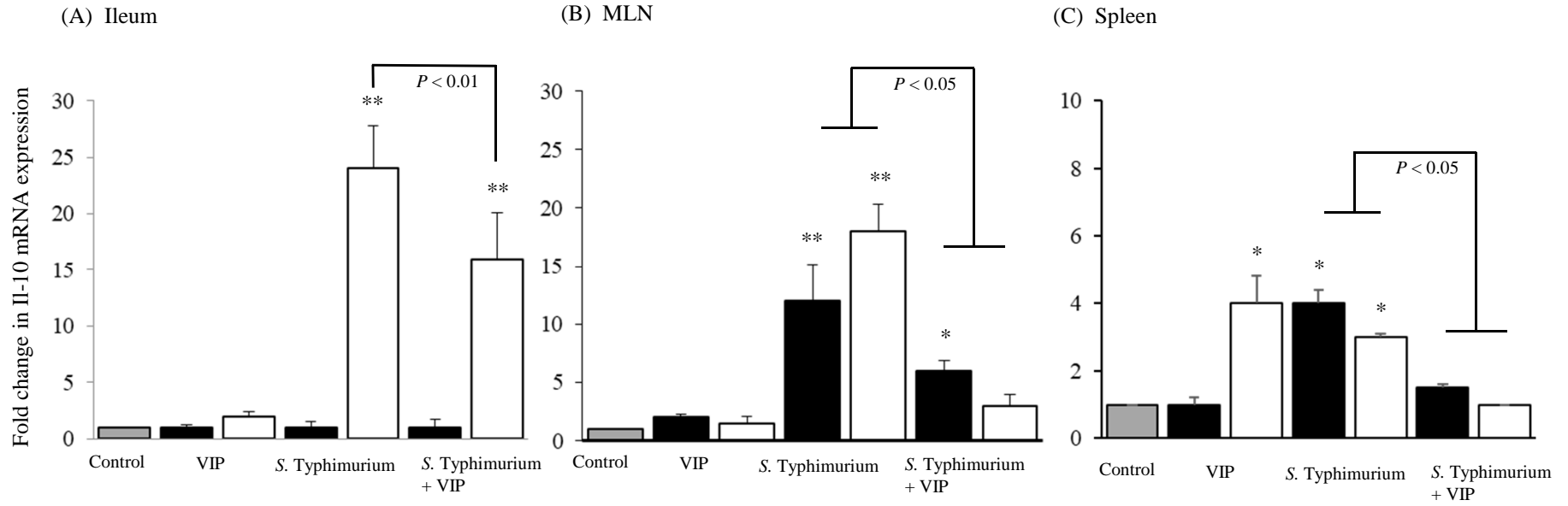


Table 1. Askar et al

Cytokine	Forward primer	Reverse primer
IL-6	5'-CAC AAG TCC GGA GAG GAG AC-3'	5'-CAG AAT TGC CAT TGC ACA AC-3'
TNF-α	5'-ACC CTC ACA CTC AGA TCA TCTT-3'	5'-GGT-TGT CTT TGA GAT CCA TGC-3'
IFN-γ	5'-CAG AGC CAG ATT ATC TCT TTC TAC CTC AGA C-3'	5'-CTT TTT CGC CTT GCT GTT GCT GAA G-3'
IL-1β	5'-CGC AGC AGC ACA TCA ACA AGA GC-3'	5'-TGT CCT CAT CCT GGA AGG TCC ACG-3'
IL-10	5'-CAA CAT ACT GCT AAC CGA CTC CT-3'	5'-TGA GGG TCT TCA GCT TCT CAC-3'
VPAC1	5'-GAT GTG GGA CAA CCT CAC CTG-3'	5'-TAG CCG TGA ATG GGG GAA AAC-3'
GAPDH	5'-ACA ATG AAT ACG GCT ACAG-3'	5'-GGT CCA GGG TTT CTT ACT-3'