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

Basim Askar[1], John Higgins, Paul Barrow and *Neil Foster

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

[1]Current address. The University of Duhok, Duhok – Kurdistan Region – Iraq P.O Box 78.

*Corresponding author:

Neil Foster

School of Veterinary Medicine and Science

Sutton Bonington campus

University of Nottingham

Nottingham

NG7 2NR

Tel: 0115 9516433

Email: n.foster@nottingham.ac.uk

Running Title: Salmonella exploits host VPAC1

Key Words: Salmonella VIP immunomodulation

Abstract

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

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

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

1.0 Introduction

The bi-phasic model for human sepsis proposes a phase of uncontrolled production of inflammatory mediators, which leads to systemic inflammatory response syndrome (SIRS) (1-2), followed by a compensatory anti-inflammatory response syndrome (CARS) (3). The SIRS (acute) phase of sepsis is associated with high systemic concentrations of pro-inflammatory cytokines released by monocytes and macrophages, such as TNF-α, IL-1 and IL-6 (4). Intervention with anti-inflammatories has been proposed as a rational therapeutic avenue during SIRS but specific inhibition of IL-1β or TNF-α has failed (5), while broad ranging anti-inflammatories, such as glucocorticoids, 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 (6).

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

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

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

2.0 Material and methods

2.1 Reagents

Unless otherwise stated all reagents were purchased from Sigma-Aldrich, Poole, Dorset UK. PCR and microarray reagents were purchased from Qiagen, Manchester, UK.

2.2 Bioethics

All studies were conducted following approval by local ethics committees and under UK Home Office license.

2.3 Bacterial culture and strains.

S. Typhimurium 4/74 were grown in Luria-Bertani (LB) broth (Life Technologies Ltd., Paisley, United Kingdom) for 18 h at 37°C under agitation. The bacteria were then sub-cultured in fresh LB broth for 4 h to late log phase (established by conventional counts of CFU). Prior to infection, bacteria were adjusted to a multiplicity of infection (MOI) of 10.

2.4 Murine studies

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

2.5 Histological studies

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

2.6 Quantitative PCR analysis

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

(Thermo Scientific, Warrington, UK) and was converted into complimentary DNA (cDNA) using a SuperScipt first strand DNA synthesis kit (Invitrogen, Carlbad, CA, USA). For quantitative PCR analysis, primers and probes were designed using a universal probe library (Roche Diagnostics, Mannheim, Germany). A PCR reaction volume (20 µl) consisting of 10 µl Light Cycler 480 Probes Master (Roche, Germany), 1 µm of each forward and reverse primer (Eurofins MWG, Operon, Germany) and 0.2 µl, labelled with Fluorescein and dark quencher dye. The total volume was adjusted to 20 µl using PCR water. In negative control wells, PCR water was added instead of cDNA. Standard curves of target and reference genes were performed at dilution ranges between 1:10- 10^5. Thermal cycles consisted of denaturing at 95 ^{0}C for 10 min and 40 cycles of sample amplification at 95 ^{0}C for 10 secs, 60 ^{0}C for 30 secs, 72 ^{0}C for 1 min and cooling at 40 ^{0}C for 30 secs, performed using a Roche applied sciences light cycler 480 (Roche, Germany). All data was normalised to expression in uninfected mouse tissues and quantification was determined by comparison to the reference gene GAPDH, using the Pfaffl method (12). All primers pairs used in the PCRs are shown in Table 1.

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

2.7 Statistical Analysis

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

3.0 Results

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

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

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

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

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

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

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

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

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

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

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

4.0 Discussion

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

Storka et al., (2013) (10) also reported a >30% increase in VPAC1 expression in monocytes isolated from human volunteers 24h after infusion of LPS and this was correlated with an

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

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

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

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

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

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

Acknowledgments

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

Disclosures

None to declare.

References

1. Annane, D., E. Bellissant, and J.M. Cavaillon. 2005. Septic shock. *Lancet*. 365:63-78.

2. Davis, B.H. 2005. Improved diagnostic approaches to infection/sepsis detection. *Expert Rev Mol* Diagn. 5:193-207.

3. Ward, N.S., B. Casserly, and A. Ayala. 2008. The compensatory anti-inflammatory response syndrome (CARS) in critically ill patients. *Clin Chest Med*. 29:617-625.

4. Danikas, D.D., M. Karakantza, G.L. Theodorou, G.C. Sakellaropoulos, and C.A. Gogos. 2008. Prognostic value of phagocytic activity of neutrophils and monocytes in sepsis. Correlation to CD64 and CD14 antigen expression. *Clin Exp Immunol*. 154:87-97.

5. Cohen, J. 2009. Sepsis and septic shock: inching forwards. *Clin Med*. 9:256-257.

6. Antonucci, E., E. Fiaccadori, F.S. Taccone, and J.L. Vincent. 2014. Glucocorticoid administration in sepsis and septic shock: time for a paradigm change? *Minerva Anestesiol*. 80:1058-1062.

7. Delgado, M., C. Martinez, D. Pozo, J.R. Calvo, J. Leceta, D. Ganea, and R.P. Gomariz. 1999. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activation polypeptide (PACAP) protect mice from lethal endotoxemia through the inhibition of TNF-α and IL-6. *J. Immunol*. 162:1200-1205.

8. Du, B.H., J. Eng, J.D. Hulmes, M. Chang, Y.C. Pan, and R.S. Yalow. 1985. Guinea pig has a unique mammalian VIP. *Biochem Biophys Res Commun*. 128: 1093-1098.

9. Delgado, M., J.C. Marie, C. Martinez, C. Abad, and J. Leceta. 2000. Anti-inflammatory properties of the type 1 and type 2 vasoactive intestinal peptide receptors: role in lethal endotoxic shock. *Eur J. Immunol*. 30:3236-3246.

10. Storka, A., B. Burian, G. Führlinger, T. Clive R. Crevenna, A. Gsur, W. Mosgöller, and M. Wolzt. 2013. VPAC1 receptor expression in peripheral blood mononuclear cells in a human endotoxemia model. *J. Transl Med*. 11:117.

11. Askar, B., H. Ibrahim, P.A. Barrow, and N. Foster. 2015. Vasoactive intestinal peptide (VIP) differentially affects inflammatory immune responses in human monocytes infected with viable Salmonella or stimulated with LPS. *Peptides*. 71:188-195.

12. Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real time RT-PCR. *Nucleic acids Res*. 29:e45.

13. Ibrahim, H., B. Askar, P.A. Barrow, and N. Foster. 2018. Dysregulation of JAK/STAT genes by vasoactive intestinal peptide (VIP) in Salmonella-infected monocytes may inhibit its therapeutic potential in human sepsis. *Cytokine*. 105:49-56.

14. Foster, N., S.D. Hulme, and P.A. Barrow. 2006. Vasoactive intestinal peptide (VIP) prevents killing of virulent and phoP mutant Salmonella typhimurium by inhibiting IFN-gamma stimulated NADPH oxidative pathways in murine macrophages. *Cytokine.* 36:134-140.

15. Gogos, C.A., E. Drosou, H.P. Bassaris, and A. Skoutelis. 2000. Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J. Infect. Dis*. 16: 982-987.

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

17. Zhou, J., H. Chaudhry, Y. Zhong, M.M. Ali, L.A. Perkins, W.B. Owens, J.E. Morales, F.R. McGuire, E.E. Zumbrun, J. Zhang, P.S. Nagarkatti, and M. Nagarkatti. 2014. Dysregulation in microRNA expression in peripheral blood mononuclear cells of sepsis patients is associated with immunopathology. *Cytokine*. 71: 89-100.

18. Delgado, M., E.J. Munoz-Elias, R.P. Gomariz, and D. Ganea, 1999. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide enhance IL-10 production by murine macrophages: in vitro and in vivo studies. J. Immunol. 162: 1707-1716.

19. Fields, P.I., E.A. Groisman, and F.A. Heffron. 1989. *Salmonella* locus that controls resistance to microbicida proteins from phagocytic cells. *Science*. 243:1059–1062.

20. Miller, S.I., A.M. Kukral, and J.J. Mekalanos. 1989. A two componenet regulatory system (phoP-phoQ) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci USA*. 86:5054–5058.

21. Weighardt, H., C.D. Heidecke, K. Emmanuilidis, S. Maier, H. Bartels, J.R. Siewert, and B. Holzmann. 2000. Sepsis after major visceral surgery is associated with sustained and interferon-gamma-resistant defects of monocyte cytokine production. *Surgery*. 127: 309-315.

22. Wang, D., X. Zhong, D. Huang, R. Chen, G.Bai, Q. Li, B. Yu, Y. Fan, X. Sun. 2014. Functional polymorphisms of interferon-gamma affect pneumonia-induced sepsis. *PLoS One*. 9: e87049.

Figure Legends

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

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

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

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

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

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

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

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

Figure 5. VIP inhibits expression of TNFα and IFN-γ mRNA in *S*. Typhimurium infected tissue

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

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

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

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

Table 1. Primer pairs used in qPCR analysis

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

Fig 1. Askar et al

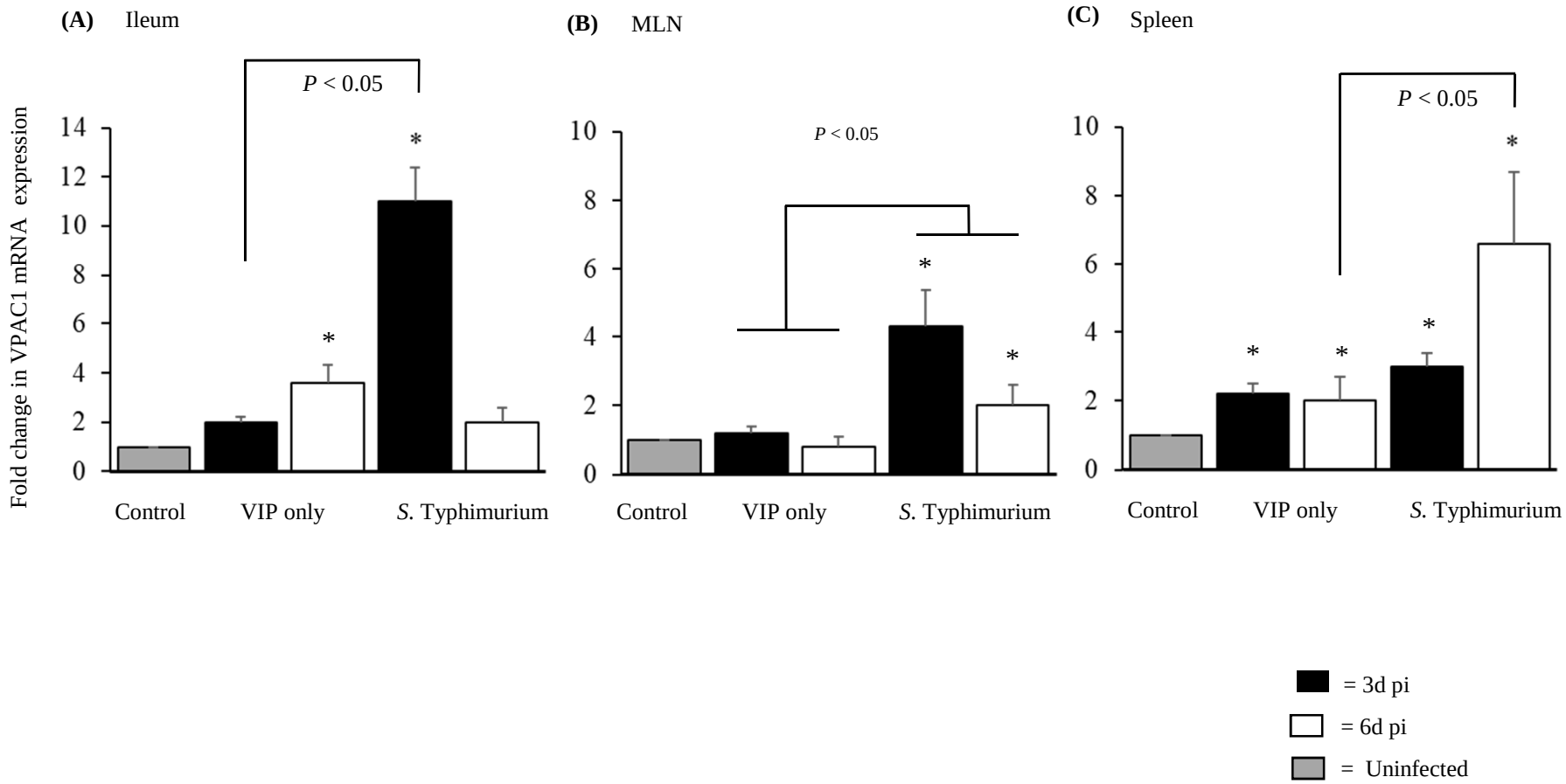

Fig 2. Askar et al

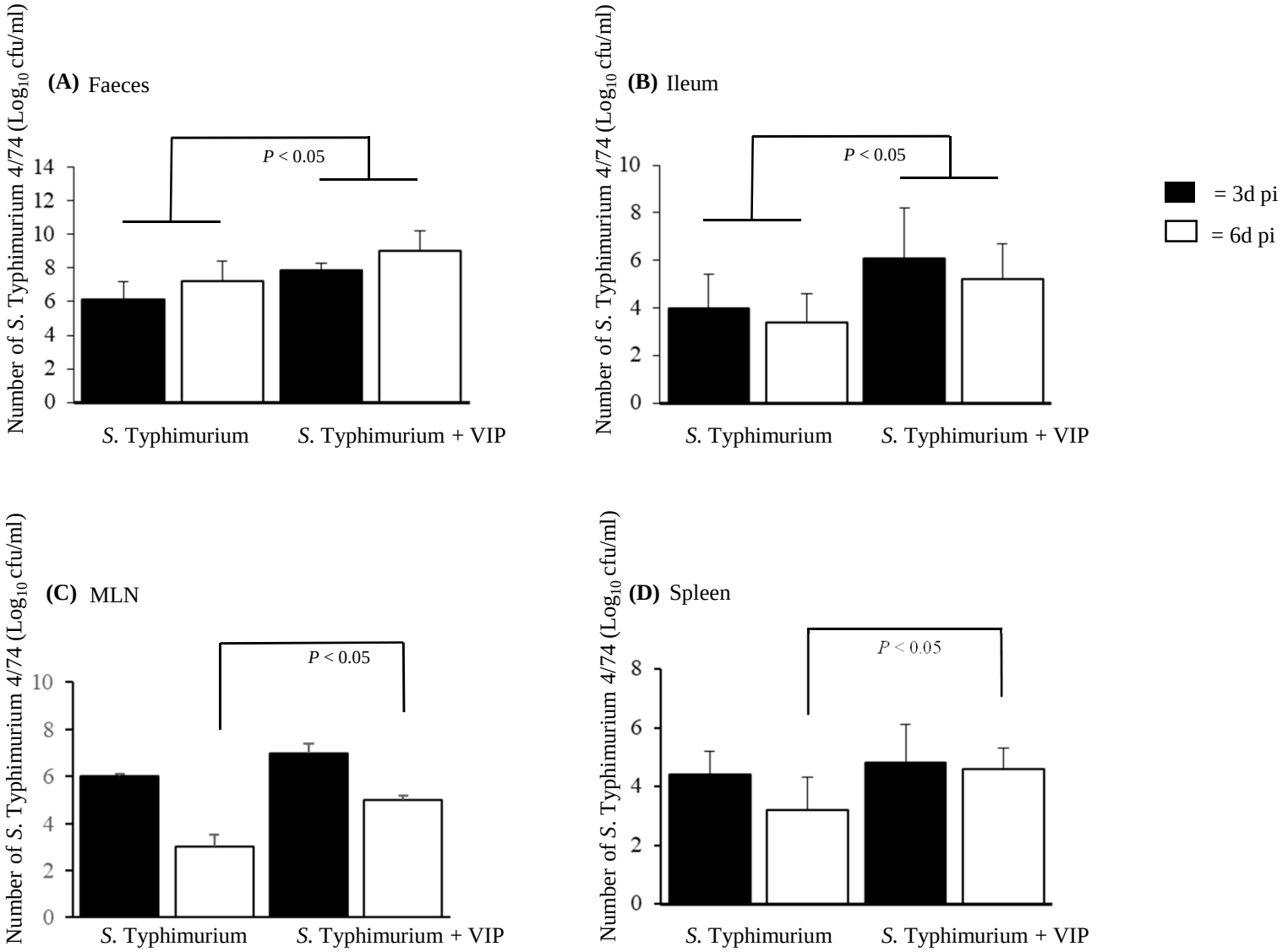

Fig 3. Askar et al

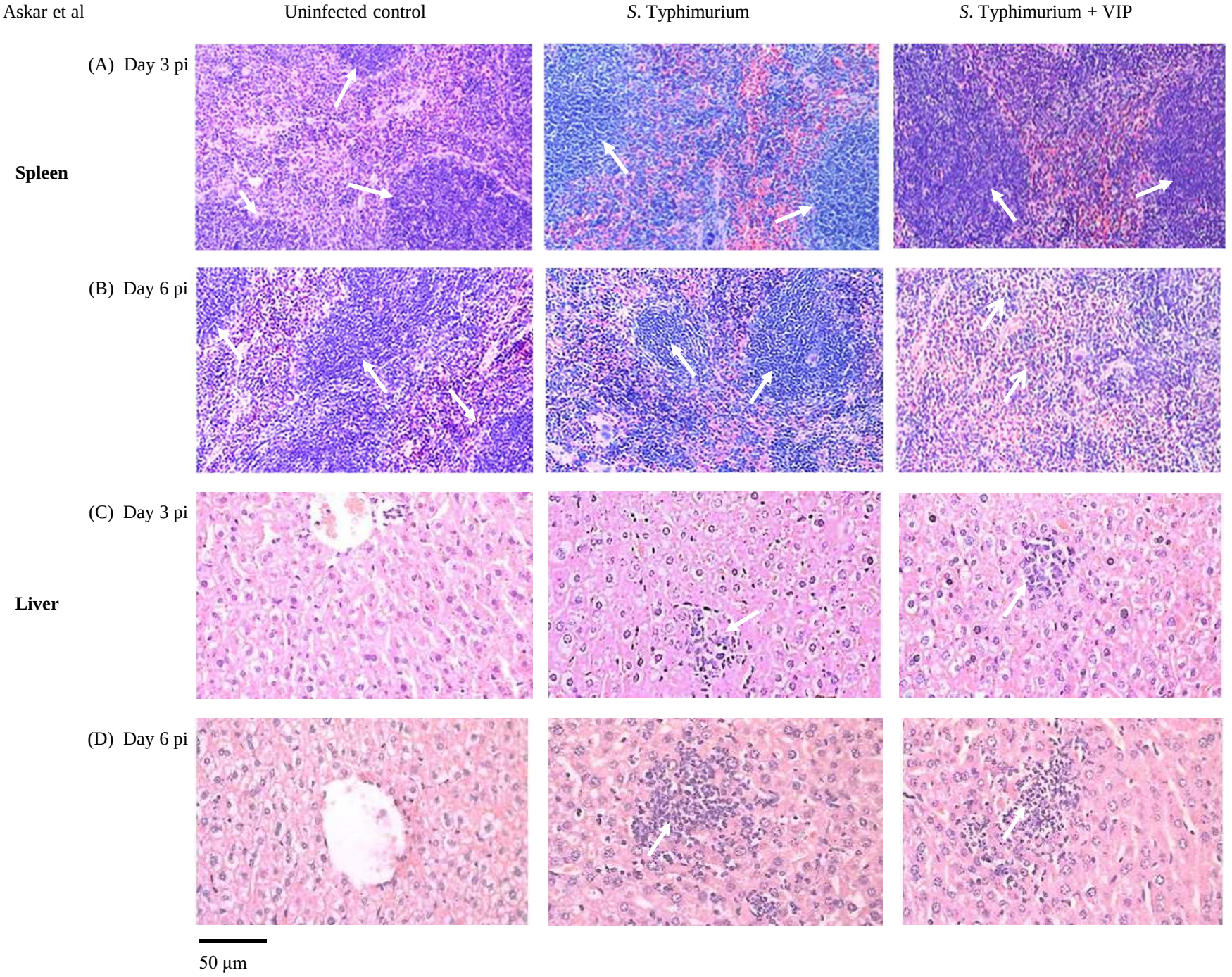

Fig 4. Askar et al

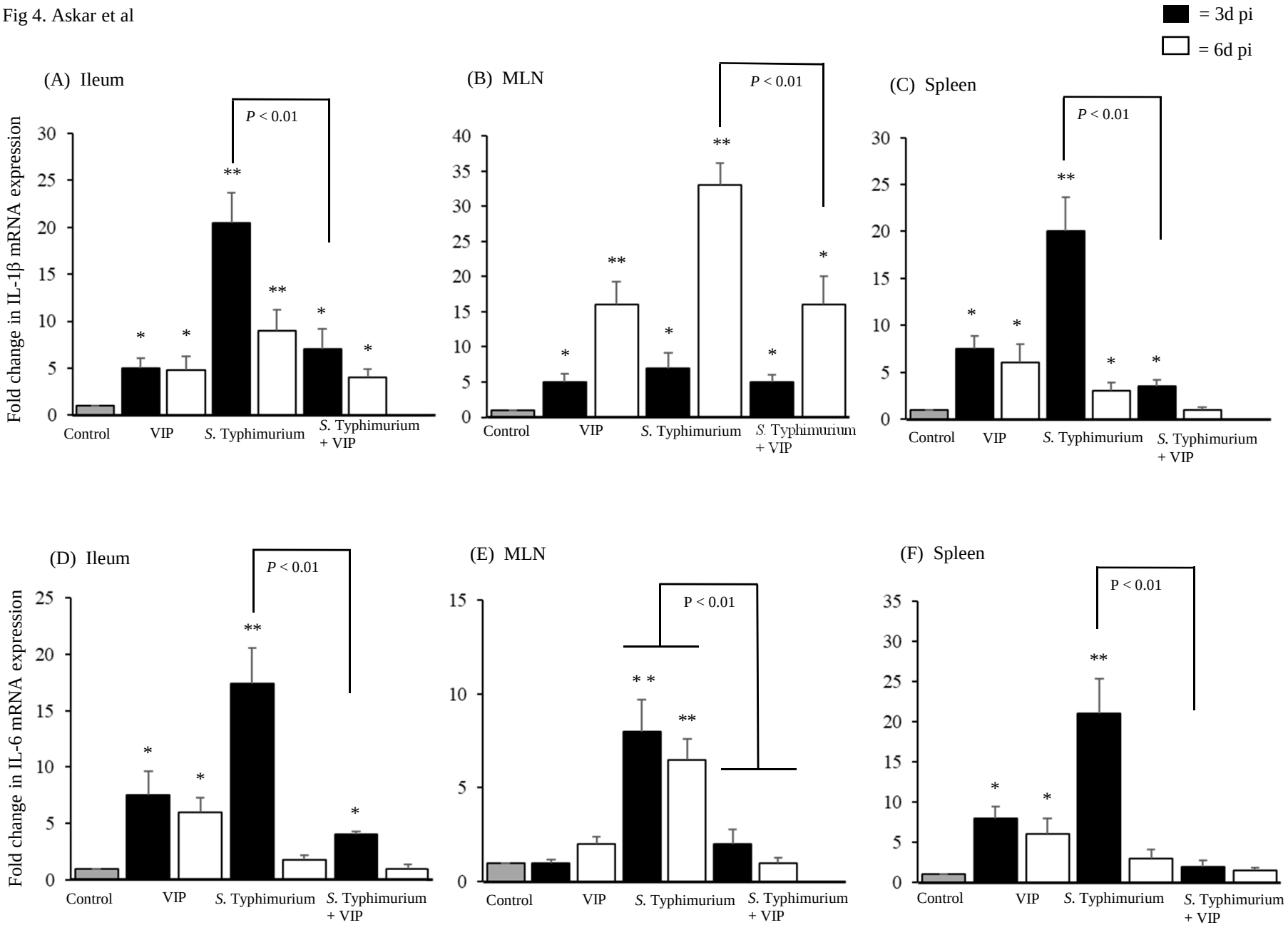

Fig 5. Askar et al

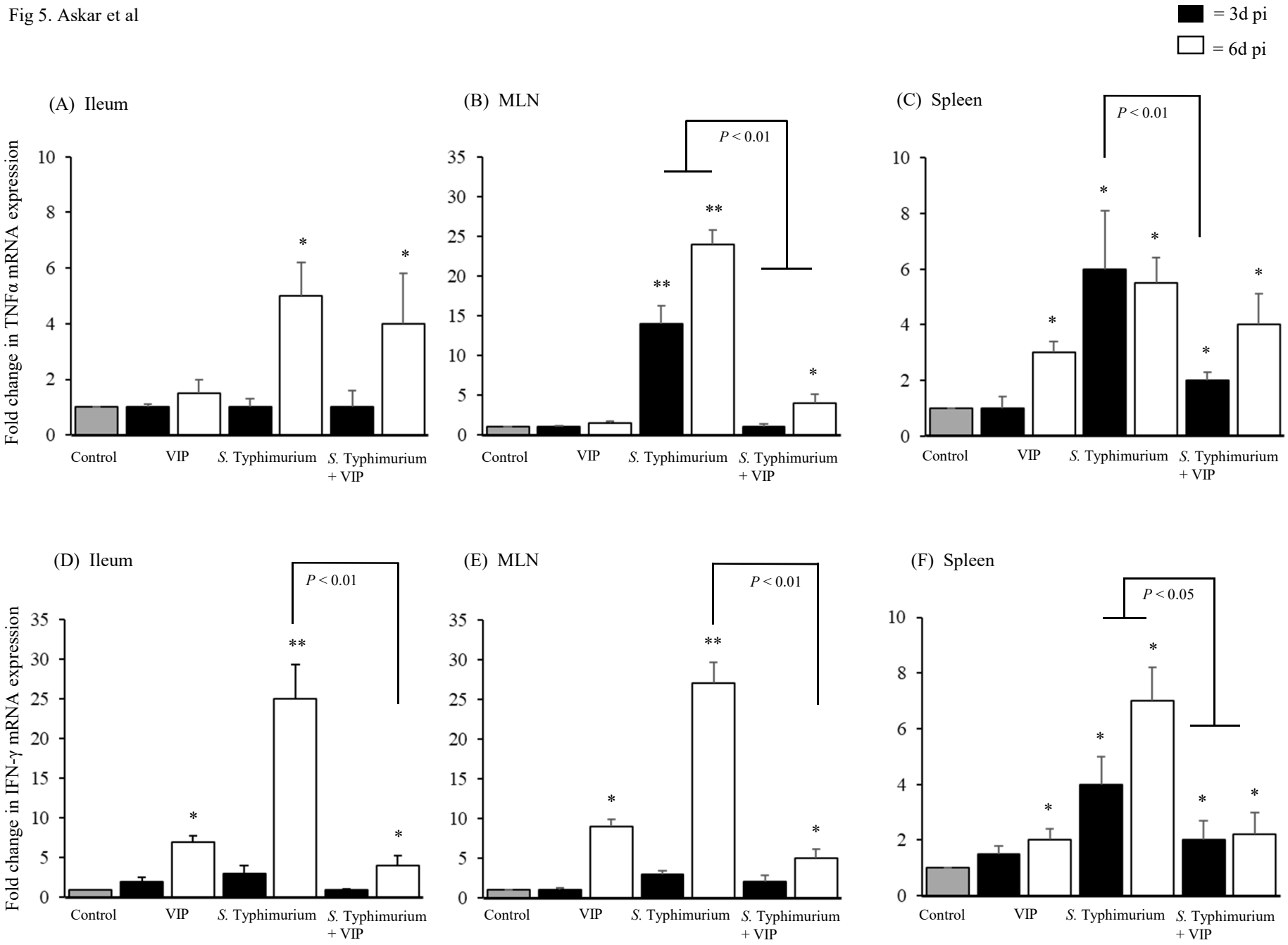

Fig 6. Askar et al

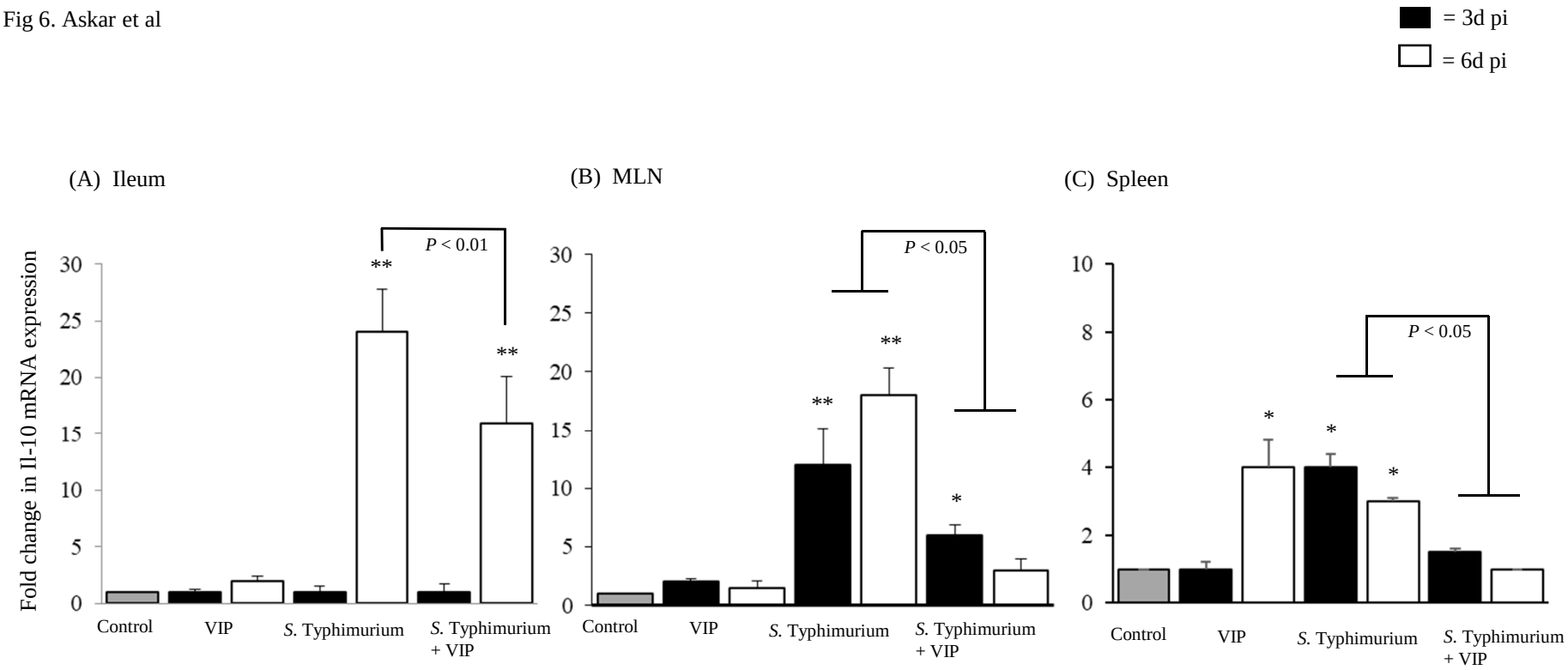

Table 1. Askar et al

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′