1 **Immune evasion by Salmonella: Exploiting the VPAC1/VIP axis in human monocytes**

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24   **Abstract**

25   Immune evasion is a critical survival mechanism for bacterial colonization of deeper tissues and

26   may lead to life-threatening conditions such as endotoxemia and sepsis. Understanding these

27   immune evasion pathways would be an important step for the development of novel anti-

28   microbial therapeutics.

29   Here, we report a hitherto unknown mechanisms by which *Salmonella* exploits an anti-

30   inflammatory pathway in human immune cells to obtain survival advantage. We show that

31   *Salmonella enterica* serovar Typhimurium strain 4/74 significantly (P <0.05) increased

32   expression of mRNA and surface protein of the type 1 receptor (VPAC1) for anti-inflammatory

33   vasoactive intestinal peptide (VIP) in human monocytes.

34   However, we also show that *S*. Typhimurium induced retrograde recycling of VPAC1 from early

35   endosomes to Rab11a-containing sorting endosomes, associated with the Golgi apparatus, and

36   anterograde trafficking via Rab3a and calmodulin 1. Expression of Rab3a and calmodulin 1 were

37   significantly increased by *S*. Typhimurium infection and W-7 (calmodulin antagonist) decreased

38   VPAC1 expression on the cell membrane while CALP-1 (calmodulin agonist) increased VPAC1

39   expression ($P < 0.05$). When infected monocytes were co-cultured with VIP, a significantly

40   higher number of *S*. Typhimurium were recovered from these monocytes, compared to S.

41   Typhimurium recovered from monocytes cultured only in cell media.

42   We conclude that *S*. Typhimurium infection exploits host VPAC1/VIP to gain survival

43   advantage in human monocytes.

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Introduction

Survival of some *Salmonella* serovars in innate immune cells is a critical step for immune evasion and colonization of deeper tissue, which may lead to life-threatening diseases such as Gram negative sepsis or typhoid. Understanding the pathways associated with immune evasion and survival may inform better therapeutic strategies for the treatment of such diseases.

The bi-phasic model for human sepsis proposes a phase of uncontrolled production of inflammatory mediators, which leads to systemic inflammatory response syndrome (SIRS) followed by a compensatory anti-inflammatory response syndrome (CARS) (1-3). 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).

Studies, to date, have indicated that vasoactive intestinal peptide (VIP) may have therapeutic potential in the treatment of sepsis. The amino acid structure of VIP is highly conserved throughout the vertebrates and is identical in all mammals apart from guinea pigs (7). In murine models of LPS-induced sepsis, intra-peritoneal administration of low concentrations of VIP (<5 nmol) prevented mortality and this was associated with inhibition of inflammatory cytokines (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). VPAC1 is constitutively expressed by some resting immune cells (9-10) and the immunosuppressive action of VIP on LPS-stimulated murine macrophages occurs via

71　this receptor (11). Increased VPAC1 expression was also reported in human peripheral blood

72　monocytes following intravenous administration of LPS and correlates with increased VIP

73　concentration in sera (12). However, using virulent *S*. Typhimurium (rather than LPS) we have

74　shown that VIP promotes survival of *S*. Typhimurium within human monocytes, which is

75　associated with inhibition of proinflammatory cytokines and increased survival of the infected

76　cells (13). This may indicate that *S*. Typhimurium increases receptivity of monocytes to VIP,

77　specifically to inhibit production of inflammatory mediators, thus gaining survival advantage. It

78　is known that upon activation, VPAC1 is internalized and may be recycled back to the cell

79　membrane (14-16) but nothing is known about the intracellular pathways utilized to achieve this

80　and nothing has been reported regarding the effect of *Salmonella* infection on VPAC1 or these

81　pathways.

82　The aim of this current study was to investigate whether *S*. Typhimurium induces VPAC1

83　expression in human monocytes, and, if so, to determine the intracellular pathways responsible

84　for this.

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86　**Material and methods**

87　**Reagents**

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

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

90　**Bioethics**

91　Human blood used in this study was obtained with patient consent. All studies were conducted

92　following approval by local ethics committees. Studies were performed on 5 separate occasions,

93　therefore blood from 5 individual humans.

Bacterial culture and strains.

S. Typhimurium 4/74 (17) 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

incubation with monocytes, bacteria were adjusted to a multiplicity of infection (MOI) of 10.

Isolation of peripheral blood monocytes (PBM)

Human blood was purchased from the blood transfusion service (Sheffield, UK). The blood was

diluted with sterile PBS then gently poured onto Histopaque-1077, prior to isolation of the buffy

coat, as standard procedure. After appropriate washing steps, buffy coat supernatants were

resuspended with appropriate amounts of cold MACS buffer and anti-CD14 antibody coated

micromagnetic beads (Miltenyi Biotech, Bisley, Surrey UK) according to the manufacturer's

instructions. The viability of isolated monocytes was assessed using Trypan blue (10% v/v) and

was found to be > 90 % prior to use.

Salmonella **invasion assays**

Monocytes were firstly washed with sterile PBS prior to culture with *S*. Typhimurium 4/74 at a

multiplicity of infection (MOI) of 10:1 at 37 ^{0}C and 5% CO_2 for 60 min with or without

VIP (10^{-7} M). The cells were then washed and cultured with RPMI media containing 100 μg/ml

gentamycin and incubated for a further 60 min. The monocytes were washed and the media was

substituted with RPMI containing 25 μg/ml of gentamycin for a further 2, 6 or 24 h post-

infection (pi) in total. The cells were then washed three times with PBS at room temperature and

then lysed using 1 % Triton X (Fisher Scientific LTD, Loughborough, UK) for 15 minutes at 37

^{0}C. Intracellular bacterial counts were determined by serial dilution at different time points of 2,

116 6 and 24h pi. Viable bacterial cell counts were measured as colony forming units per ml

117 (CFU/ml). All counts were performed in triplicate on 5 separate occasions.

PCR analysis

119 RNA was purified from treated or control monocytes by standard methods, using Rneasy plus

120 kits (Qiagen, Hilden, Germany). RNA quantity and quality were measured using a NanoDrop

121 8000 spectrophotometer (Thermo Scientific, Warrington, UK) and was converted into

122 complimentary DNA (cDNA) using a SuperScipt first strand DNA synthesis kit (Invitrogen,

123 Carlbad, CA, USA). For quantitative PCR analysis primers and probes were designed using a

124 universal probe library (Roche Diagnostics, Mannheim, Germany). A PCR reaction volume (20

125 μl) consisting of 10 μl Light Cycler 480 Probes Master (Roche, Germany), 1 μm of each forward

126 and reverse primer (Eurofins MWG, Operon, Germany) and 0.2 μl, labelled with Fluorescein and

127 dark quencher dye. The total volume was adjusted to 20 μl using PCR water. In negative control

128 wells, PCR water was added instead of cDNA. Standard curves of target and reference genes

129 were performed at dilution ranges between 1:10- 10^5. Thermal cycles consisted of denaturing at

130 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,

131 72 ^{0}C for 1 min and cooling at 40 ^{0}C for 30 secs, performed using a Roche applied sciences light

132 cycler 480 (Roche, Germany). All data was normalised to unstimulated monocytes, or expression

133 in uninfected mouse tissues for *in vivo* studies, and quantification was determined by comparison

134 to the reference gene using the Pfaffl method (18). All primers and probes used in PCR are

135 shown in Table 1.

Immunofluorescence studies

137 Freshly isolated monocytes (5 x 10^5) were cultured on glass cover slips (VWR International Ltd,

138 Leighton Buzzard, UK) placed in 24 well culture dishes, for 6h. The monocytes were then

139   infected with *S*. Typhimurium (6h pi) or cultured with VIP (10^{-7} M) in RPMI media for 6h, or

140   cultured for 6 h in RPMI media without bacteria or VIP (uninfected control). After 6h the

141   monocytes were washed 3 times in PBS and fixed in paraformaldehyde (4% v/v) for 10 min at

142   room temperature prior to permeabilization in Triton-X (Fisher Scientific, Loughborough, UK)

143   (0.2% v/v) for 10 min. After washing 3 times in PBS, the monocytes were incubated at room

144   temperature for 30 min in blocking buffer; bovine serum albumin (BSA) solution (3% w/v in

145   PBS). After washing 3 times in PBS the monocytes were incubated for 60 min with appropriate

146   primary antibody (300 μl), washed 3 times in PBS and incubated with secondary antibody (300

147   μl) in the dark for 45 min on an end-to-end shaker. After a final washing step, the coverslips

148   were placed onto microscope slides prior to adding Vectashield hard set mounting medium. All

149   slides were examined using a TCS SP2 confocal microscope (Leica Micosystems, Heidelburg,

150   Germany). Each panel shown in figures 3 and 4 are merged plane image captures 4 μm below the

151   cell surface. To prevent spectral overlap, Alexa 488 and Alexa 647 fluorophore conjugates were

152   chosen and bypass filtering was applied to prevent bleed through. Laser intensity and detector

153   gain were optimised and standardised throughout. Each experiment was repeated in triplicate on

154   5 separate occasions. All primary and secondary antibodies used during the confocal analysis,

155   together with suppliers are shown in Table 2.

156   **Flow cytometry**

157   *S*. Typhimurium infected monocytes (6h pi), monocytes cultured with VIP and monocytes

158   cultured only in RPMI media (unstimulated controls) for 6h were centrifuged at 300 x g for 10

159   min. The supernatant was discarded and the cell pellet was washed 3 times by resuspension in

160   PBS and centrifugation at 300 x g for 10 min, repeated a further 2 times. Monocytes were either

161   left non-permeabilised or permeabilised for 10 min in Triton-X (Fisher Scientific, UK) (0.2%

162 v/v) and washed a further 3 times prior to incubation at room temperature for 30 min in blocking

163 buffer (PBS and BSA 3% w/v). After washing 3 times in PBS the monocytes were incubated for

164 60 min with mouse anti-human VPAC1 IgG2a (300 μl), washed 3 times in PBS and incubated

165 with rat anti-mouse IgG2a-Alexa-488 (300 μl) (Table 2) in the dark for 45 min on an end-to-end

166 shaker. The monocytes were then washed 3 times prior to analysing antibody fluorescence using

167 a FACScan flow cytometer (Becton Dickinson, NJ, USA). Gating of the cell populations were

168 performed, according to journal guidelines, using CD14 expression (92% of the population) and

169 side scatter (SSC). In FACS plots, quadrants were set according to fluorescence expressed by

170 monocytes incubated only with secondary antibody (control) (lower left quadrant) and

171 fluorescence in test cell populations were compared to these. Data analysis was performed using

172 FACSDiva software (Becton Dickinson, USA). Each experiment was performed in triplicate on

173 5 separate occasions.

174 **Calmodulin 1 (CAM1) agonist and antagonist studies**

175 To investigate the effect of CAM1 on VPAC1 expression, freshly isolated monocytes (5 x 10^5)

176 were incubated for 2h with either the CAM1 agonist; calcium-like peptide 1 (CALP1) (20 μM)

177 (Tocris Bioscience, Abingdon, UK) or the CAM1 antagonist; W-7 (30 μM) (Sigma-Aldrich,

178 Poole, UK). The concentrations of agonist and antagonist chosen in these experiments were

179 determined via dose response and output curves (data not shown). PCR and flow cytometry

180 were then performed to assess VPAC1 expression in *S*. Typhimurium infected and uninfected

181 monocytes, as stated above at 6h pi. Mean data was obtained from experiments performed in

182 triplicate on 5 separate occasions.

183 **Statistical Analysis**

184 Two-tailed unpaired student's *t* test or one-way ANOVA were performed to determine

185 significant differences between different groups (control negative, *S*. Typhimurium infected,

186 with or without VIP) using Graph Pad Prism software. Bonferroni's multiple comparisons test

187 was applied to examine significant difference between the means of more than two groups,

188 following ANOVA. Significance values were determined at the 95% confidence limit ($P < 0.05$).

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190

191 **Results**

192 ***S*. Typhimurium increases VPAC1 expression**

193 High constitutive expression of VPAC1 was detected by RTPCR but following S. Typhimurium

194 infection, expression of VPAC1 increased further (Fig 1A). *S*. Typhimurium significantly

195 increased VPAC1 mRNA expression by around 5 fold above expression in resting monocytes (*P*

196 <0.05) but VPAC1 expression was not differentially expressed when monocytes were cultured

197 with VIP (Fig 1B). After 6 and 24h pi, significantly more *S*. Typhimurium were recovered from

198 infected monocytes which had been co-cultured with VIP, compared to infected monocytes

199 without VIP (*P* <0.05) (Fig 1C).

200 Following FACS analyses, we also detected a 50% increase in the immunoreactivity of VPAC1

201 protein in the cytosol of monocytes after 6h pi with *S*. Typhimurium, which was highly

202 significant (*P* <0.01) when compared to VPAC1 protein in the cytosol of monocytes incubated

203 with VIP or uninfected monocytes (Fig 2A and C). FACS analysis also showed thatVPAC1

204 protein was increased on the surface of monocytes infected with *S*. Typhimurium by around 30%

205 and this increase was also significant (P <0.05) when compared to VPAC1 protein in the cytosol

206 of monocytes incubated with VIP or uninfected monocytes (Fig 2B and D).

Retrograde recycling of VPAC1 occurs following *S*. Typhimurium infection of monocytes

Confocal microscopy showed that VPAC1 expression was similar in monocytes incubated with VIP to that in unstimulated monocytes (Fig 3A), this was low and was partially co-localized with the early endosome antigen 1 (EEA1) within the early/sorting endosome (SE). However, VPAC1 immunofluorescent intensity was greatly increased and was strongly co-localized with EEA1 following *S*. Typhimurium infection (Fig 3A). Following internalization of VPAC1 in the SE of *S*. typhimurium-infected monocytes, VPAC1 was trafficked from the SE to recycling endosomes (REs) associated with the TGN (retrograde trafficking). This was indicated by strong co-localization of VPAC1 with Rab11a (Fig 3B) and concomitant co-localisation of Rab11a with the trans-Golgi network (TGN), using TGN protein 46 (TGN46) as a marker Fig 3C.

***S*. Typhimurium induces anterograde recycling of VPAC1 via Rab3a/CAM1.**

Further trafficking of VPAC1 in monocyte exosomes, following *S*. Typhimurium Infection, was indicated by strong co-localisation of VPAC1 with the membrane docking protein Rab3a (Fig 4A) which was concomitantly co-localised with CAM1 (Fig 4B). Subsequent qPCR analysis showed that *S*. Typhimurium infection induced a >5 fold ($P < 0.05$) increase in both Rab3A and CAM1 above that measured in unstimulated monocytes or monocytes cultured with VIP (Fig 4C). Addition of a CAM1 agonist (CALP1) to cell cultures significantly increased VPAC1 mRNA expression in *S*. Typhimurium-infected monocytes ($P < 0.05$), whereas addition of a CAM1 antagonist (W-7) significantly decreased VPAC1 mRNA expression ($P < 0.05$) (Fig 4D). FACS analyses also indicated a causal association between CAM1 and VPAC1 protein expression. These showed that expression of VPAC1 protein on the surface of *S*. Typhimurium-infected monocytes was increased by CALP1 and decreased by W-7 (Fig 5G-I) compared to uninfected monocytes (Fig 5A-C) or monocytes cultured with VIP (Fig 5D-F) and in both cases

230   these changes were significant (*P* <0.05). P- values were calculated by ANOVA analysis of

231   mean expression detected for each experimental group (Shown in Fig 5K). A diagrammatic

232   model of intracellular cycling of VPAC1, induced by *S*. Typhimurium infection, is shown in Fig

233   5L.

234

235   **Discussion**

236   We show that *S*. Typhimurium increased expression of VPAC1 in human monocytes and this

237   was associated with increased intracellular survival of the bacteria, when infected monocytes

238   were co-cultured with VIP. This is in accordance with a study by Storka et al., (2013) (12) who

239   reported a >30% increase in VPAC1 expression in monocytes isolated from human volunteers

240   24h after infusion of lipopolysaccharide (LPS) from *Escherichia coli* and this was correlated

241   with an increased concentration of VIP in plasma. However, at least some of this effect is

242   probably due to increased monocyte survival (13, 19) but the effect is not merely due to LPS,

243   which we found to induce lower levels of VPAC1 by both qPCR and FACS analyses compared

244   to *Salmonella* infection (data not shown).

245   Murine studies have indicated that the immunosuppressive effect of VIP occurs uniquely via

246   VPAC1 (11) and that VIP/VPAC1 interaction regulates production of inflammatory mediators

247   associated with mortality in sepsis (8), which may have evolved to protect the host. However,

248   studies using viable *Salmonella,* rather than LPS have shown that VIP down-regulates

249   inflammatory mediators and increases *Salmonella* survival in human monocytes (13, 19) and

250   murine macrophages (20). We therefore hypothesised that *S*. Typhimurium actively utilises the

251   VPAC1/VIP axis for its own survival advantage.

252   We now show that *S*. Typhimurium not only increased VPAC1 mRNA and protein expression

253   but also induced VPAC1 recycling to the cell membrane. The initial step in this process was

254   internalization of VPAC1 into EEA-1-containing SE. EEA-1 is required for tethering, docking

255   and fusion of the SE to Soluble NSF Attachment Protein Receptor (SNARE), which

256   subsequently leads to endosomal shipment (21). A previous study has shown that internalisation

257   and localisation of VPAC1 in the SE occurred in VPAC1 transinfected CHO cells, following

258   culture with VIP (22). However, in this latter study, VPAC1 was not recycled back to the cell

259   membrane and in accordance with this, we show that VIP did not increase VPAC1 expression on

260   the cell membrane.

261   Internalised proteins may be trafficked via the SE to late endosomes and lysosomes (which

262   may prevent further recycling) or to the trans-Golgi network (TGN) (retrograde transport) which

263   may sort the proteins into recycling endosomes (REs) for subsequent transport back to the cell

264   membrane (anterograde transport) (23-24).   This requires interaction with Ras-associated

265   binding (Rab) proteins, which are small GTPases involved in intracellular trafficking of protein

266   cargo from the SE to downstream endosomes, including the RE, and the docking of transport

267   vesicles with membrane targets (25).   In our study, *S*. Typhimurium-induced retrograde

268   transport of VPAC1 was indicated by strong co-localisation of VPAC1 with Rab11a, one of the

269   best studied markers of REs (26) and TGN protein 46 (TGN46). The TGN is the critical region

270   on the Golgi apparatus, which collects and sorts newly synthesised proteins and proteins relayed

271   to it from REs (27-28). Some bacterial toxins, such as Shiga toxin, produced by *Shigella*

272   *dysenteriae* (29), HIV envelope protein (30) and the immunosuppressive HIV nef protein (31-32)

273   are trafficked from the cell surface via retrograde transport. However, our study is the first to

274   show that *S*. Typhimurium (or any other pathogen) induces retrograde transporting of VPAC1.

275 The results we obtained also suggested that *S*. Typhimurium induced anterograde transport of

276 VPAC1 via CAM1/Rab3a. The importance of Ca2+ in regulated exocytosis has been known for

277 a number of years (33) and a study by MacKenzie et al., (34) also reported a positive correlation

278 between Ca2+ concentration and VPAC1 density. Ca2+ binding by CAM1 forms Ca2+/CAM1

279 complexes that bind to Rab3a, which subsequently causes switching of Rab3a from a GDP-

280 bound (inactive form) to a GTP-bound (active) form (35). It is possible that the increase in

281 CAM1 and Rab3a mRNA we show may increase calcium binding and active Rab3a and thus

282 VPAC1 binding and transport to the cell membrane. Active Rab3a is a constituent protein in

283 secretory vesicles within PC-12 cells and, in newly formed secretory vesicles associated with the

284 TGN in pancreatic acinar cells (36) and is involved in the docking and exocytosis of secretory

285 vesicles (37). Very little has been reported regarding Rab3a in immune cells but a study by Abu-

286 Amer et al., (1999) (38) has shown that Rab3a expression is increased in murine bone marrow

287 derived macrophages stimulated with LPS, although this was not studied in the context of

288 VPAC1. We also show that *S*. Typhimurium (which contains LPS) significantly increased Rab3a

289 and CAM1 mRNA expression in monocytes. Moreover, our results show a causal link between

290 CAM1 and VPAC1 expression on the monocyte membrane. However, the effect of CAM1

291 agonist or antagonist was not absolute, thus suggesting that other factors (possibly Rab3a itself)

292 also directly affect VPAC1 expression on the monocyte surface.

293   Only a fraction of newly synthesised VPAC1 become inserted into cell membranes, due to

294 conformational misfolding (16) and although *S*. Typhimurium infection increased VPAC1

295 mRNA expression, it is possible that *S*. Typhimurium-induced recycling of VPAC1 via

296 retrograde/anterograde pathways overcomes low surface expression due to misfolding. This

297   would facilitate greater interaction with the increased concentration of VIP in serum, as shown

298   by Storka et al., (2013) (12).

299   In conclusion, *S*. Typhimurium exploits the VPAC1/VIP axis to increase survival in human

300   monocytes, this is achieved, at least in part, by retrograde and anterograde recycling of VPAC1

301   via CAM1.

306

307   **Conflict of interest**

308   The authors have none to declare.

309

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Figure Legends

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

Forward and reverse primers and probes are shown for each gene analysed by PCR.

Table 2. Primary and secondary antibodies used in confocal microscope analysis and flow cytometry.

The type and concentration of primary and secondary antibodies, together with secondary

antibody fluorophore conjugates, together with commercial suppliers, are shown.

Figure 1. Increased survival of *S*. Typhimurium 4/74 in human monocytes co-cultured with VIP is associated with increased expression of VPAC1.

Reverse transcription (rt) PCR showing constitutive and *S*. Typhimurium-induced expression of

VPAC1 in monocytes after 6h post-infection (pi.). Comparison between VPAC1 expression is

also shown with the reference gene β-actin, which is unchanged in both uninfected and infected

monocytes. (B) Quantitative PCR (qPCR) showing expression of VPAC1 after 6h pi in

monocytes infected with S. Typhimurium or cultured with VIP (10^{-7} M). Fold changes are

431 expressed in comparison with mRNA expression measured in unstimulated (control) monocytes,

432 assigned an arbitrary expression value of 1. (C) Recovery of *S*. Typhimurium from monocytes

433 cultured with or without VIP (10^{-7} M) at different times post-infection (pi). White bar = 2h pi,

434 Grey bar = 6h pi, Black bar = 24h pi. Histograms (B and C) show means calculated from 5

435 separate experiments performed in triplicate. Connecting bars show significant differences at P =

436 0.05, error bars show standard deviation from the mean.

437

438 **Figure 2. *S*. Typhimurium 4/74 increases VPAC1 protein in the cytosol and cell membrane**

439 **of human monocytes.**

440 VPAC1 expression is shown in representative FACS analyses of 5 independent experiments

441 performed in (A) the cytoplasm (permeabilised) and (B) cell membrane (non-permeablised) at 6h

442 pi. In all cases, lower left quadrants were set according to fluorescence expressed by monocytes

443 incubated with secondary antibody only (control). Histograms show mean population sizes

444 expressing VPAC1 after each treatment; permeabilised monocytes (C) and non-permeabilsed

445 monocytes (D) calculated from 5 independent experiments performed in triplicate. Connecting

446 bars show significant differences at P = 0.01-0.05, error bars show standard deviation from the

447 mean.

448

449 **Figure 3. *S*. Typhimurium infection stimulates retrograde transported of VPAC1 from**

450 **early/sorting endosome to Rab11a-containing recycling endosome localized with the trans-**

451 **Golgi network in human monocytes.**

452 Confocal Laser Scanning Microscopy showing co-localization of VPAC1 with EEA1 and

453 Rab11a in human monocytes, each panel shows a merged plane image 4 μm below the cell

454   surface. (A) Co-localization of VPAC1 immunofluorescence (green Alexa 488) with early

455   endosome antigen 1 (red Alexa 647) (white arrows) with increased levels of VPAC1/EEA1

456   association in *S*. Typhimurium infected monocytes at 6h pi.

457   (B) Co-localisation of VPAC1 (green Alexa 488) with Rab11a (red Alexa 647) (white arrows) is

458   shown only in *S*. Typhimurium infected cells and in (C) co-localisation of Rab11a (red Alexa

459   647) with TGN46 (green Alexa 488) (white arrows) is shown only in *S*. Typhimurium infected

460   cells. CLSM images shown are representative of 5 independent experiments performed in

461   triplicate. Scale bar (bottom left) = 20 μm.

462

463   **Figure 4. *S*. Typhimurium 4/74 induces packaging of VPAC1 within Rab3a/CAM1**

464   **containing secretory vesicles for anterograde transport to the cell membrane of human**

465   **monocytes.**

466   (A-B) Confocal Laser Scanning Microscopy showing co-localization of VPAC1 with Rab3a and

467   CAM1. Each panel shows a merged plane image 4 μm below the cell surface.

468   (A) Co-localisation of VPAC1 (green Alexa 488) with Rab3a (red Alexa 647) (white arrows) in

469   *S*. Typhimurium infected monocytes. (B) Co-localisation of CAM1 (green Alexa 488) with

470   Rab3a (red Alexa 647) (white arrows) in *S*. Typhimurium infected monocytes. Images shown are

471   representative of 5 independent experiments performed in triplicate. Scale bar (bottom left) = 20

472   μm. (C) qPCR showing increased expression of Rab3a and CAM1 mRNA in monocytes 6h pi

473   with *S*. Typhimurium. (D) qPCR showing fold changes in VPAC1 mRNA with or without CAM1

474   agonist (CALP1) or antagonist (W-7). Each bar is a mean of 5 independent experiments

475   performed in triplicate. White bar = Without agonist/antagonist, Black bar = + CALP1 (agonist),

476   Grey bar = + W-1 (antagonist). Connecting bars show significant differences at P = 0.05, error

477   bars show standard deviation from the mean. Fold changes are expressed as a comparison with

478   mRNA expression measured in unstimulated (control) monocytes after 6h, assigned an arbitrary

479   expression value of 1.

480

481   **Figure 5. CAM1 increases VPAC1 protein expression on the cell membrane of monocytes**

482   **infected with *S*. Typhimurium 4/74.**

483   (A-J) FACS analysis showing VPAC1 expression on the monocyte membrane 6h pi with *S*.

484   Typhimurium or monocytes cultured with VIP (10^{-7} M) for 6h, with or without CAM1 agonist

485   (CALP1; 20µg/ml) or CAM1 antagonist (W-7; 30 µg/ml). FACS analyses shown are

486   representative of experiments performed in triplicate on 5 separate occasions. Y-axes, SSC =

487   Side scatter, X-axis = VPAC1 expression. Quantitative analysis of experimental means are

488   shown in (K). Black bars = 2h post-treatment treatment/infection, White bars = 6h post-

489   treatment/infection and Grey bars = 24h post-treatment/infection. Connecting bars show

490   significant differences at P = 0.05, error bars show standard deviation from the mean.

491   (L) Diagrammatic representation of *S*. Typhimurium induced trafficking of VPAC1 receptors. (1)

492   *S*. Typhimurium invades monocyte. (2) VPAC1 is packages in the EEA1 positive sorting

493   endosome (SE). (3) Retrograde transport of VPAC1 occurs via Rab11a positive recycling

494   endosome (RE) which is associated with TGN46 on the Golgi apparatus. (4) Anterograde

495   transport of VPAC1 occurs via Rab3a/CAM1 positive secretory vesicle (SV).
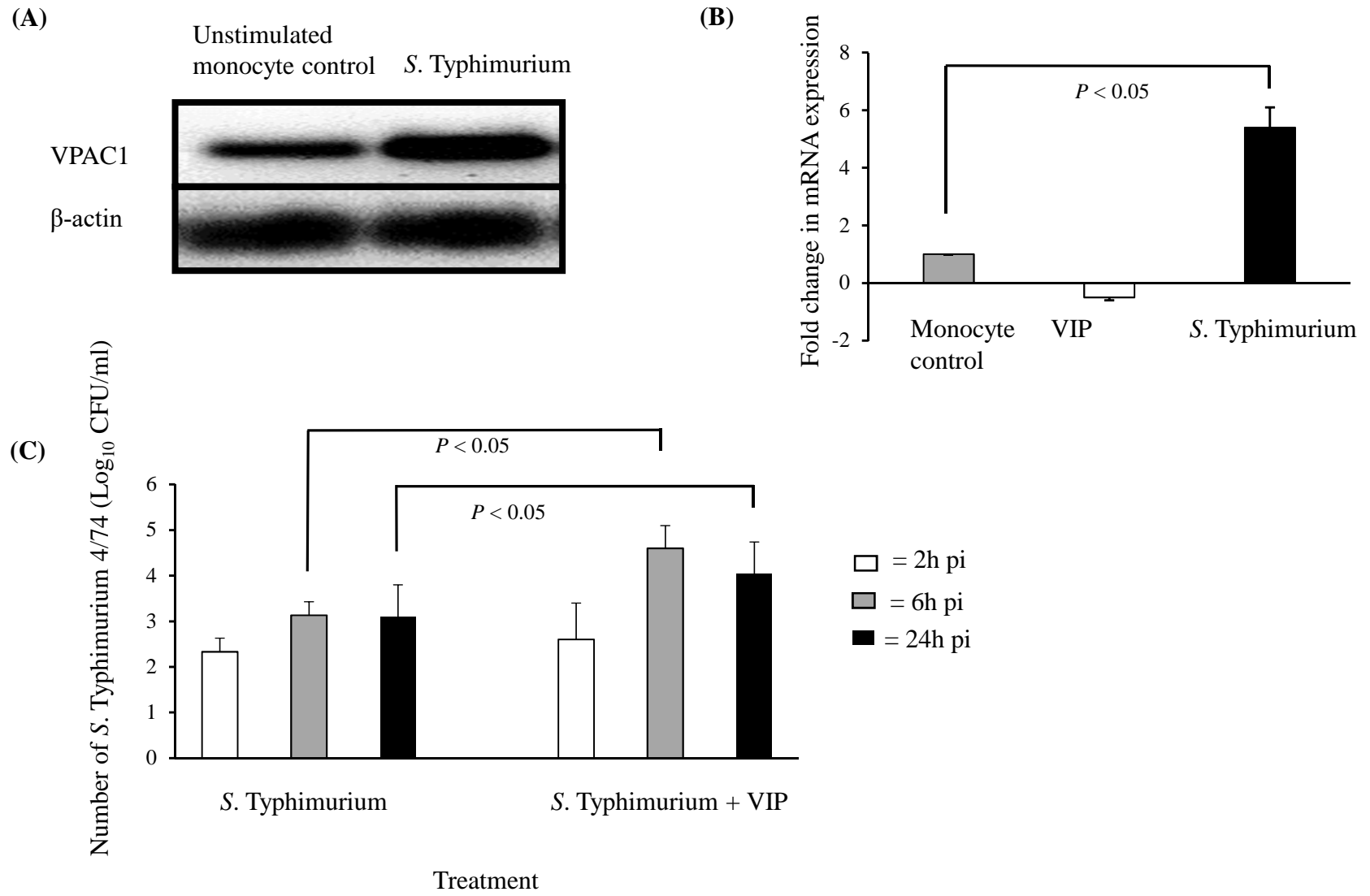
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Fig 1. Askar et al

(A)

Unstimulated monocyte control   *S*. Typhimurium

VPAC1

β-actin

(B)

Fold change in mRNA expression

$P < 0.05$

Monocyte control   VIP   *S*. Typhimurium

(C)

Number of *S*. Typhimurium 4/74 (Log$_{10}$ CFU/ml)

$P < 0.05$

$P < 0.05$

S. Typhimurium   *S*. Typhimurium + VIP

Treatment

□ = 2h pi
▨ = 6h pi
■ = 24h pi

Fig 2. Askar et al

Permeabilised monocytes

(A) Secondary antibody — 98.5% | 1.5%

(B) Monocyte control — 79.8% | 20.2%

(C) VIP — 84% | 16%

(D) *S.* Typhimurium — 26.8% | 73.2%

(E) Mean number of permeabilised monocytes expressing VPAC1

P <0.05   *P* <0.01

Secondary antibody   Monocyte control   *S.* Typhimurium

SSC

Non-permeabilised monocytes

(F) Secondary antibody — 99% | 1%

(G) Monocyte control — 90.6% | 9.4%

(H) VIP — 93.3% | 6.7%

(I) *S.* Typhimurium — 60.9% | 39.1%

VPAC1

(J) Mean number of non-permeabilised monocytes expressing VPAC1

P <0.05   *P* <0.05

Secondary antibody   Monocyte control   *S.* Typhimurium

Fig 3. Askar et al

(A) VPAC1   EEA1   Overlay

Monocyte
control

VIP

S. Typhimurium

Secondary antibody
only

20 µm

(B) VPAC1   Rab11a   Overlay

(C) Rab11a   TGN46   Overlay

Fig 4. Askar et al

(A) VPAC1　　Rab3a　　Overlay

(B) CAM1　　Rab3a　　Overlay

20 μm

(C)

(D)

Fig 5. Askar et al

(A) Monocyte

83.2%   16.6%

(B) Monocyte + CaM1 agonist

76.8%   23.2%

(C) Monocyte + CaM1 antagonist

92.0%   8.0%

(D) Monocyte + VIP

85.9%   14.1%

(E) VIP + CaM1 agonist

83.4%   16.6%

(F) VIP + CaM1 antagonist

90.6%   9.4%

(G) Monocyte + *S*. Typhimurium

64.0%   36.0%

(H) *S*. Typhimurium + CaM1 agonist

51.9%   48.1%

(I) *S*. Typhimurium + CaM1 antagonist

72.6%   27.4%

(J) Secondary antibody

97.5%   2.5%

SSC

VPAC1

(K)

$P < 0.05$

$P < 0.05$

Mean number of monocytes expressing membrane VPAC1

Secondary antibody   Unstimulated monocytes   Monocytes + VIP   Monocytes + *S*. Typhimurium

Fig 5. Askar et al

Table 1. Askar et al

Gene	Forward primer (5'-3')	Reverse primer (3'-5')	Probe
VPAC1	TCCGCCAGC CACTCTATC	GCTCGAGCC TGCACAATC	#19
Rab3a	AACGAGGAA TCCTTCAATGCA	TGGGCATTGT CCCATGAGTA	TGCAGGACTGGT CCACCCAGATCA
CAM1	TGCATTCAGGGC TGATTTATAGAG	AACAAGCTACAA AATGCCAGAAAGA	CCCTTGGCTTCTC CTTCTCCTACTCCCT
β-actin	CCAACCGCG AGAAGATGA	CCAGAGGAGT ACAGGGATAG	#64

Table 2. Askar et al

Receptor	Primary antibody	Conc. (µg/ml)	Secondary antibody	Conc. (µg/ml)
VPAC1	Mouse anti-human IgG2a (Abcam, Cambs, UK)	1	Rat anti-mouse IgG2a-Alexa 488 (Abcam, Camb, UK)	0.1
EEA1	Rabbit anti-human IgG (Abcam, Camb, UK)	1	Donkey anti-rabbit IgG-Alexa 647 (Abcam, Camb, UK)	2
Rab3a	Rabbit anti-human IgG (Abcam, Camb, UK)	1	Donkey anti-rabbit IgG-Alexa 647 647 (Abcam, Camb, UK)	2
CAM1	Mouse anti-human IgG1 (Abcam, Camb, UK)	2	Rat anti-mouse IgG1-Alexa 488 (Invitrogen, Frederick, USA)	1
Rab11a	Rabbit anti-human IgG (Abcam, Camb, UK)	1	Donkey anti-rabbit IgG Alexa 647 647 (Abcam, Camb, UK)	2
TGN46	Mouse anti-human IgG1 (Abcam, Camb, UK)	5	Rat anti-mouse IgG1-Alexa 488 (Invitrogen, Frederick, USA)	1