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

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Summary

Immune evasion is a critical survival mechanism for bacterial colonization of deeper tissues and may lead to life-threatening conditions such as endotoxaemia and sepsis. Understanding these immune evasion pathways would be an important step for the development of novel anti-microbial therapeutics. Here, we report a hitherto unknown mechanism by which *Salmonella* exploits an anti-inflammatory pathway in human immune cells to obtain survival advantage. We show that *Salmonella enterica* serovar Typhimurium strain 4/74 significantly ($P < 0{\cdot}05$) increased expression of mRNA and surface protein of the type 1 receptor (VPAC1) for anti-inflammatory vasoactive intestinal peptide (VIP) in human monocytes. However, we also show that *S.* Typhimurium induced retrograde recycling of VPAC1 from early endosomes to Rab11a-containing sorting endosomes, associated with the Golgi apparatus, and anterograde trafficking via Rab3a and calmodulin 1. Expression of Rab3a and calmodulin 1 were significantly increased by *S.* Typhimurium infection and W-7 (calmodulin antagonist) decreased VPAC1 expression on the cell membrane while CALP-1 (calmodulin agonist) increased VPAC1 expression ($P < 0{\cdot}05$). When infected monocytes were co-cultured with VIP, a significantly higher number of *S.* Typhimurium were recovered from these monocytes, compared with *S.* Typhimurium recovered from monocytes cultured only in cell media. We conclude that *S.* Typhimurium infection exploits host VPAC1/VIP to gain survival advantage in human monocytes.

Keywords: calmodulin; immune evasion; Rab; *Salmonella*; vasoactive intestinal peptide.

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.[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 tumour necrosis factor-α, interleukin-1 and interleukin-6.[4] Intervention with anti-inflammatories has been proposed as a rational therapeutic avenue during SIRS but specific inhibition of interleukin-1β or tumour necrosis factor-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 debatable, 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 lipopolysaccharide (LPS) -induced sepsis, intraperitoneal 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 that is also activated via the pituitary adenylate-cyclase activating polypeptide. VPAC1 is constitutively expressed by some resting immune cells[9,10] and the immunosuppressive action of VIP on LPS-stimulated murine macrophages occurs via this receptor.[11] Increased VPAC1 expression was also reported in human peripheral blood monocytes following intravenous administration of LPS and correlates with increased VIP concentration in sera.[12] However, using virulent *Salmonella enterica* serovar 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.[13] This may indicate that *S.* Typhimurium increases receptivity of monocytes to VIP, specifically to inhibit the production of inflammatory mediators, so gaining survival advantage. It is known that upon activation, VPAC1 is internalized and may be recycled back to the cell membrane[14–16] but nothing is known about the intracellular pathways used to achieve this, and nothing has been reported regarding the effect of *Salmonella* infection on VPAC1 or these pathways.

The aim of this current study was to investigate whether *S.* Typhimurium induces VPAC1 expression in human monocytes, and, if so, to determine the intracellular pathways responsible for this.

Material and methods

Reagents

Unless otherwise stated all reagents were purchased from Sigma-Aldrich (Poole, UK). Polymerase chain reaction and microarray reagents were purchased from Qiagen (Manchester, UK).

Bioethics

Human blood used in this study was obtained with patient consent. All studies were conducted following approval by local ethics committees. Studies were performed on five separate occasions, so used blood from five individuals.

Bacterial culture and strains

Salmonella Typhimurium 4/74[17] were grown in Luria–Bertani broth (Life Technologies Ltd, Paisley, UK) for 18 hr at 37° under agitation. The bacteria were then subcultured in fresh Luria–Bertani broth for 4 hr to late log phase (established by conventional counts of colony-forming units). Before incubation with monocytes, bacteria were adjusted to a multiplicity of infection of 10.

Isolation of peripheral blood monocytes

Human blood was purchased from the blood transfusion service (Sheffield, UK). The blood was diluted with sterile phosphate-buffered saline (PBS) then gently poured onto Histopaque-1077, before 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, UK) according to the manufacturer's instructions. The viability of isolated monocytes was assessed using Trypan blue [10% volume/volume (v/v)] and was found to be >90% before use.

Salmonella invasion assays

Monocytes were firstly washed with sterile PBS before culture with *S.* Typhimurium 4/74 at a multiplicity of infection of 10 : 1 at 37° in 5% CO_2 for 60 min with or without VIP (10^{-7} M). The cells were then washed and cultured with RPMI-1640 medium containing 100 μg/ml gentamycin and incubated for a further 60 min. The monocytes were washed and the medium was substituted with RPMI-1640 containing 25 μg/ml of gentamycin for a further 2, 6 or 24 hr post-infection (p.i.) 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 min at 37°.

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

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

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

Receptor	Primary antibody	Conc. (μg/ml)	Secondary antibody	Conc. (μg/ml)
VPAC1	Mouse anti-human IgG2a (Abcam, Cambridge, UK)	1	Rat anti-mouse IgG2a-Alexa 488 (Abcam, Cambridge, UK)	0·1
EEA1	Rabbit anti-human IgG (Abcam, Cambridge, UK)	1	Donkey anti-rabbit IgG-Alexa 647 (Abcam, Cambridge, UK)	2
Rab3a	Rabbit anti-human IgG (Abcam, Cambridge, UK)	1	Donkey anti-rabbit IgG-Alexa 647 647 (Abcam, Cambridge, UK)	2
CAM1	Mouse anti-human IgG1 (Abcam, Cambridge, UK)	2	Rat anti-mouse IgG1-Alexa 488 (Invitrogen, Frederick, MD)	1
Rab11a	Rabbit anti-human IgG (Abcam, Cambridge, UK)	1	Donkey anti-rabbit IgG Alexa 647 647 (Abcam, Cambridge, UK)	2
TGN46	Mouse anti-human IgG1 (Abcam, Cambridge, UK)	5	Rat anti-mouse IgG1-Alexa 488 (Invitrogen, Frederick, MD)	1

The type and concentration of primary and secondary antibodies, together with secondary antibody fluorophore conjugates, together with commercial suppliers, are shown.

Intracellular bacterial counts were determined by serial dilution at different time-points of 2, 6 and 24 hr p.i. Viable bacterial cell counts were measured as colony-forming units per ml. All counts were performed in triplicate on five separate occasions.

PCR analysis

RNA was purified from treated or control monocytes by standard methods, 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 cDNA using a SuperScipt first-strand DNA synthesis kit (Invitrogen, Carlbad, CA). For quantitative PCR analysis primers and probes were designed using a universal probe library (Roche Diagnostics, Mannheim, Germany). A PCR volume (20 μl) consisting of 10 μl Light Cycler 480 Probes Master (Roche Diagnostics), 1 μm of each forward and reverse primer (Eurofins MWG, Operon,

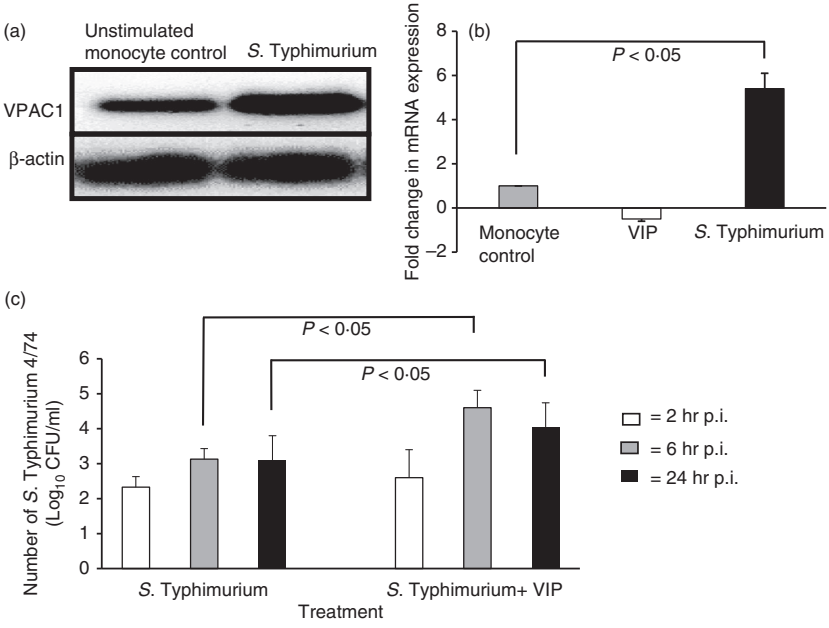

Figure 1. Increased survival of *Salmonella enterica* serovar Typhimurium 4/74 in human monocytes co-cultured with vasoactive intestinal peptide (VIP) is associated with increased expression of VIP receptor 1 (VPAC1). (a) Reverse transcription PCR showing constitutive and *S.* Typhimurium-induced expression of VPAC1 in monocytes after 6 hr post-infection (p.i.). 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 showing expression of VPAC1 after 6 hr p.i. in monocytes infected with *S.* Typhimurium or cultured with VIP (10^{-7} M). Fold changes are expressed in comparison with mRNA expression measured in unstimulated (control) monocytes, assigned an arbitrary expression value of 1. (c) Recovery of *S.* Typhimurium from monocytes cultured with or without VIP (10^{-7} M) at different times p.i.: white bar = 2 hr p.i., grey bar = 6 hr p.i., black bar = 24 hr p.i. Histograms (b and c) show means calculated from five separate experiments performed in triplicate. Connecting bars show significant differences at $P = 0{\cdot}05$, error bars show standard deviation from the mean.

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 produced at dilution ranges between 1 : 10 and 10^5. Thermal cycles consisted of denaturing at 95° for 10 min and 40 cycles of sample amplification at 95° for 10 seconds, 60° for 30 seconds, 72° for 1 min and cooling at 40° for 30 seconds, performed using a Roche Applied Sciences Light Cycler 480 (Roche Diagnostics). All data were normalized to unstimulated monocytes, and quantification was determined by comparison to the reference gene using the Pfaffl method.[18] All primers and probes used in PCR are shown in Table 1.

Immunofluorescence studies

Freshly isolated monocytes (5×10^5) were cultured on glass cover slips (VWR International Ltd, Leighton Buzzard, UK) placed in 24-well culture dishes, for 6 hr. The monocytes were then infected with *S.* Typhimurium (6 hr p.i.) or cultured with VIP (10^{-7} M) in RPMI-1640 medium for 6 hr, or cultured for 6 hr in RPMI-1640 medium without bacteria or VIP (uninfected control). After 6 hr the monocytes were washed three times in PBS and fixed in paraformaldehyde (4% v/v) for 10 min at room temperature before permeabilization in Triton-X (Fisher Scientific) (0·2% v/v) for 10 min. After washing three times in PBS, the monocytes were incubated at room temperature for 30 min in blocking buffer; bovine serum albumin solution [3% weight (w)/v in PBS]. After washing three times in PBS the monocytes were incubated for 60 min with appropriate primary antibody (300 μl), washed three times in PBS and incubated with secondary antibody (300 μl) in the dark for 45 min on an end-to-end shaker. After a final washing step, the coverslips were placed onto microscope slides before adding Vectashield hard set mounting medium. All slides were examined using a TCS SP2 confocal microscope (Leica Microsystems, Heidelburg, Germany). Each panel shown in Figs 3 and 4 comprises merged plane image captures 4 μm below the cell surface. To prevent spectral overlap, Alexa 488 and Alexa 647 fluorophore conjugates were chosen and bypass filtering was applied to prevent bleed through. Laser intensity and detector gain were optimized and standardized throughout. Each experiment was repeated in triplicate on five separate occasions. All primary and secondary antibodies used during the

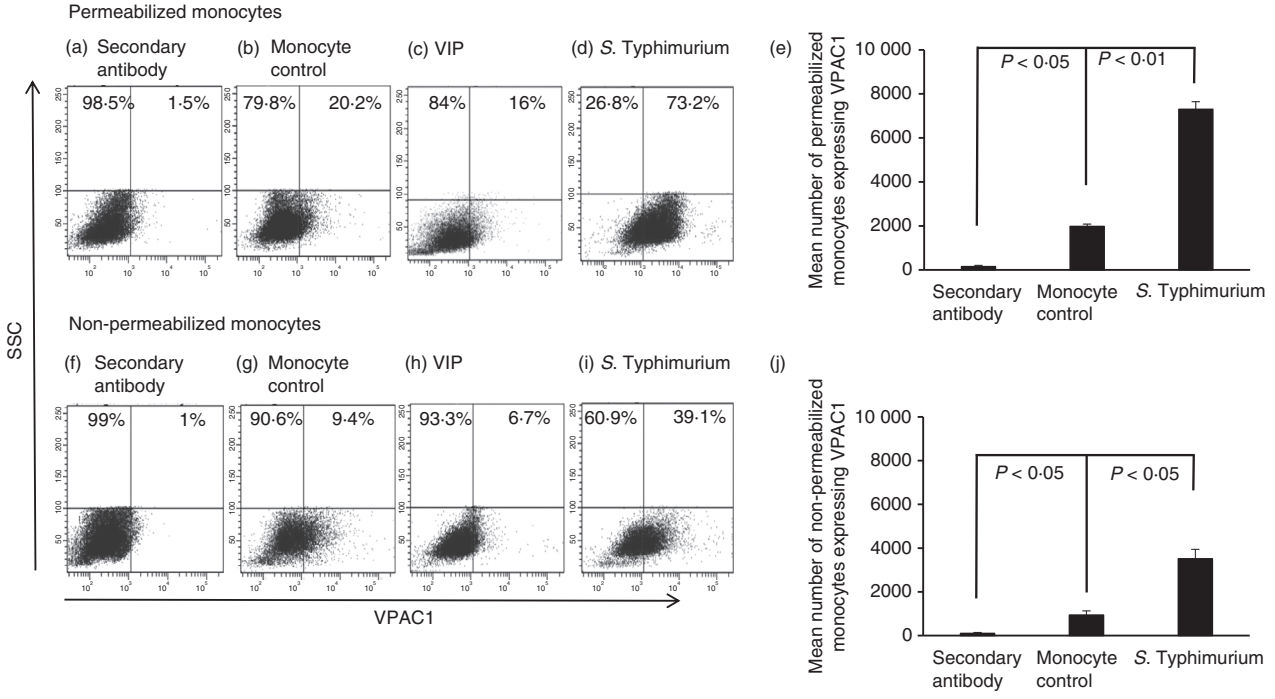

Figure 2. *Salmonella enterica* serovar Typhimurium 4/74 increases vasoactive intestinal peptide receptor 1 (VPAC1) protein in the cytosol and cell membrane of human monocytes. VPAC1 expression is shown in representative FACS analyses of five independent experiments performed in (a–e) the cytoplasm (permeabilized) and (f–j) cell membrane (non-permeabilized) at 6 hr post-infection (p.i.). In all cases, lower left quadrants were set according to fluorescence expressed by monocytes incubated with secondary antibody only (control). Histograms show mean population sizes expressing VPAC1 after each treatment; permeabilized monocytes (e) and non-permeabilized monocytes (j) calculated from five independent experiments performed in triplicate. Connecting bars show significant differences at $P = 0{\cdot}01$ to $P = 0{\cdot}05$, error bars show standard deviation from the mean.

confocal analysis, together with suppliers are shown in Table 2.

Flow cytometry

S. Typhimurium-infected monocytes (6 hr p.i.), monocytes cultured with VIP and monocytes cultured only in RPMI medium (unstimulated controls) for 6 hr were centrifuged at 300 *g* for 10 min. The supernatant was discarded and the cell pellet was washed three times by resuspension in PBS and centrifugation at 300 *g* for 10 min, repeated twice more. Monocytes were either left non-permeabilized or permeabilized for 10 min in Triton-X (Fisher Scientific) (0·2% v/v) and washed a further three times before incubation at room temperature for 30 min in blocking buffer (PBS and bovine serum albumin 3% w/v). After washing three times in PBS the monocytes were incubated for 60 min with mouse anti-human VPAC1 IgG2a (300 µl), washed three times in PBS and incubated with rat anti-mouse IgG2a-Alexa-488 (300 µl) (Table 2) in the dark for 45 min on an end-to-end shaker. The monocytes were then washed three times before analysing antibody fluorescence using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Gating of the cell populations was performed, according to journal guidelines, using CD14 expression (92% of the population) and side scatter. In FACS plots, quadrants were set according to fluorescence expressed by monocytes incubated only with secondary antibody (control) (lower left quadrant) and fluorescence in test cell populations were compared to these. Data analysis was performed using FACSDIVA software (Becton Dickinson). Each experiment was performed in triplicate on five separate occasions.

Calmodulin 1 agonist and antagonist studies

To investigate the effect of calmodulin 1 (CAM1) on VPAC1 expression, freshly isolated monocytes (5×10^5) were incubated for 2 hr with either the CAM1 agonist,

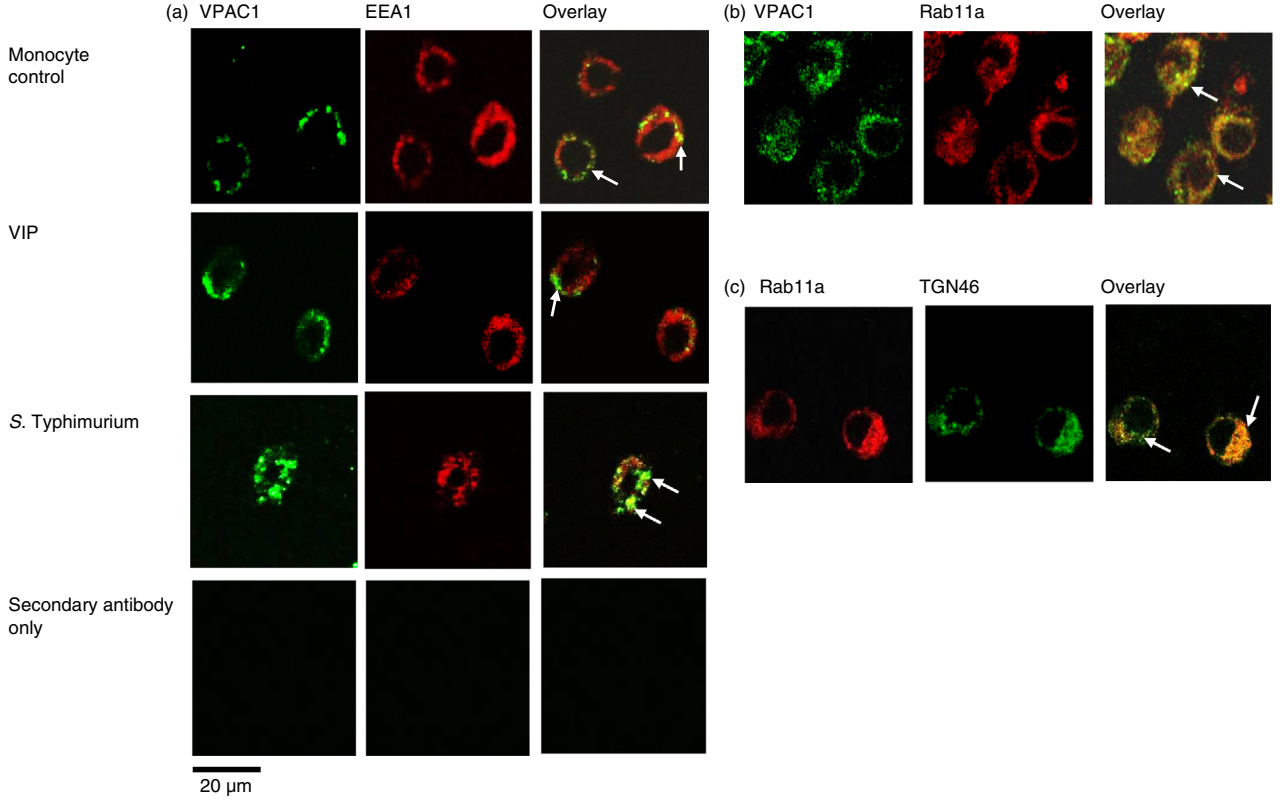

Figure 3. *Salmonella enterica* serovar Typhimurium infection stimulates retrograde transport of vasoactive intestinal peptide receptor 1 (VPAC1) from early/sorting endosome to Rab11a-containing recycling endosome localized with the trans-Golgi network in human monocytes. Confocal laser scanning microscopy showing co-localization of VPAC1 with endosome antigen 1 (EEA1) and Rab11a in human monocytes, each panel shows a merged plane image 4 µm below the cell surface. (a) Co-localization of VPAC1 immunofluorescence (green Alexa 488) with early endosome antigen 1 (red Alexa 647) (white arrows) with increased levels of VPAC1/EEA1 association in *S.* Typhimurium-infected monocytes at 6 hr postinfection. (b) Co-localization of VPAC1 (green Alexa 488) with Rab11a (red Alexa 647) (white arrows) is shown only in *S.* Typhimurium-infected cells, and in (c) co-localization of Rab11a (red Alexa 647) with TGN46 (green Alexa 488) (white arrows) is shown only in *S.* Typhimurium-infected cells. Confocal laser scanning microscopy images shown are representative of five independent experiments performed in triplicate. Scale bar (bottom left) = 20 µm.

calcium-like peptide 1 (CALP1) (20 μM) (Tocris Bioscience, Abingdon, UK) or the CAM1 antagonist, W-7 (30 μM) (Sigma-Aldrich). The concentrations of agonist and antagonist chosen in these experiments were determined using dose–response and output curves (data not shown). The PCR and flow cytometry were then performed to assess VPAC1 expression in *S.* Typhimurium-infected and uninfected monocytes, as stated above at 6 hr p.i. Mean data were obtained from experiments performed in triplicate on five separate occasions.

Statistical analysis

Two-tailed unpaired Student's *t*-test or one-way analysis of variance were performed to determine significant differences between different groups (control negative, *S.* Typhimurium-infected, with or without VIP) using GraphPad Prism software (GraphPad, San Diego, CA). Bonferroni's multiple comparisons test was applied to examine significant difference between the means of more than two groups, following analysis of variance. Significance values were determined at the 95% confidence limit ($P < 0{\cdot}05$).

Results

S. Typhimurium increases VPAC1 expression

High constitutive expression of VPAC1 was detected by RT-PCR but following *S.* Typhimurium infection, expression of VPAC1 increased further (Fig. 1a). *S.* Typhimurium significantly increased VPAC1 mRNA

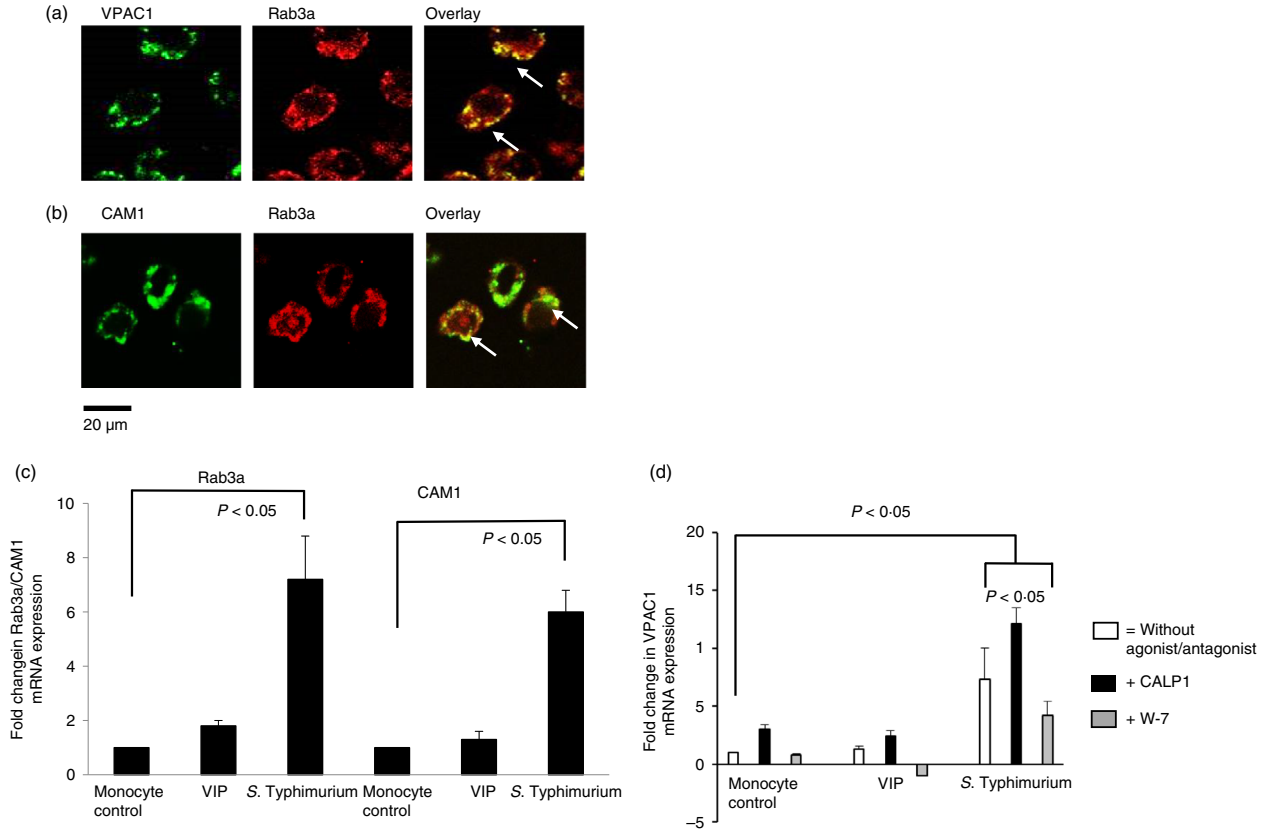

Figure 4. *Salmonella enterica* serovar Typhimurium 4/74 induces packaging of vasoactive intestinal peptide receptor 1 (VPAC1) within Rab3a/CAM1 containing secretory vesicles for anterograde transport to the cell membrane of human monocytes. (a, b) Confocal laser scanning microscopy showing co-localization of VPAC1 with Rab3a and CAM1. Each panel shows a merged plane image 4 μm below the cell surface. (a) Co-localization of VPAC1 (green Alexa 488) with Rab3a (red Alexa 647) (white arrows) in *S.* Typhimurium-infected monocytes. (b) Co-localization of CAM1 (green Alexa 488) with Rab3a (red Alexa 647) (white arrows) in *S.* Typhimurium-infected monocytes. Images shown are representative of five independent experiments performed in triplicate. Scale bar (bottom left) = 20 μm. (c) Quantitative PCR showing increased expression of Rab3a and CAM1 mRNA in monocytes 6 hr postinfection with *S.* Typhimurium. (d) Quantitative PCR showing fold changes in VPAC1 mRNA with or without CAM1 agonist (CALP1) or antagonist (W-7). Each bar is a mean of five independent experiments performed in triplicate. White bar = without agonist/antagonist, black bar = + CALP1 (agonist), grey bar = + W-1 (antagonist). Connecting bars show significant differences at $P = 0{\cdot}05$, error bars show standard deviation from the mean. Fold changes are expressed as a comparison with mRNA expression measured in unstimulated (control) monocytes after 6 hr, assigned an arbitrary expression value of 1.

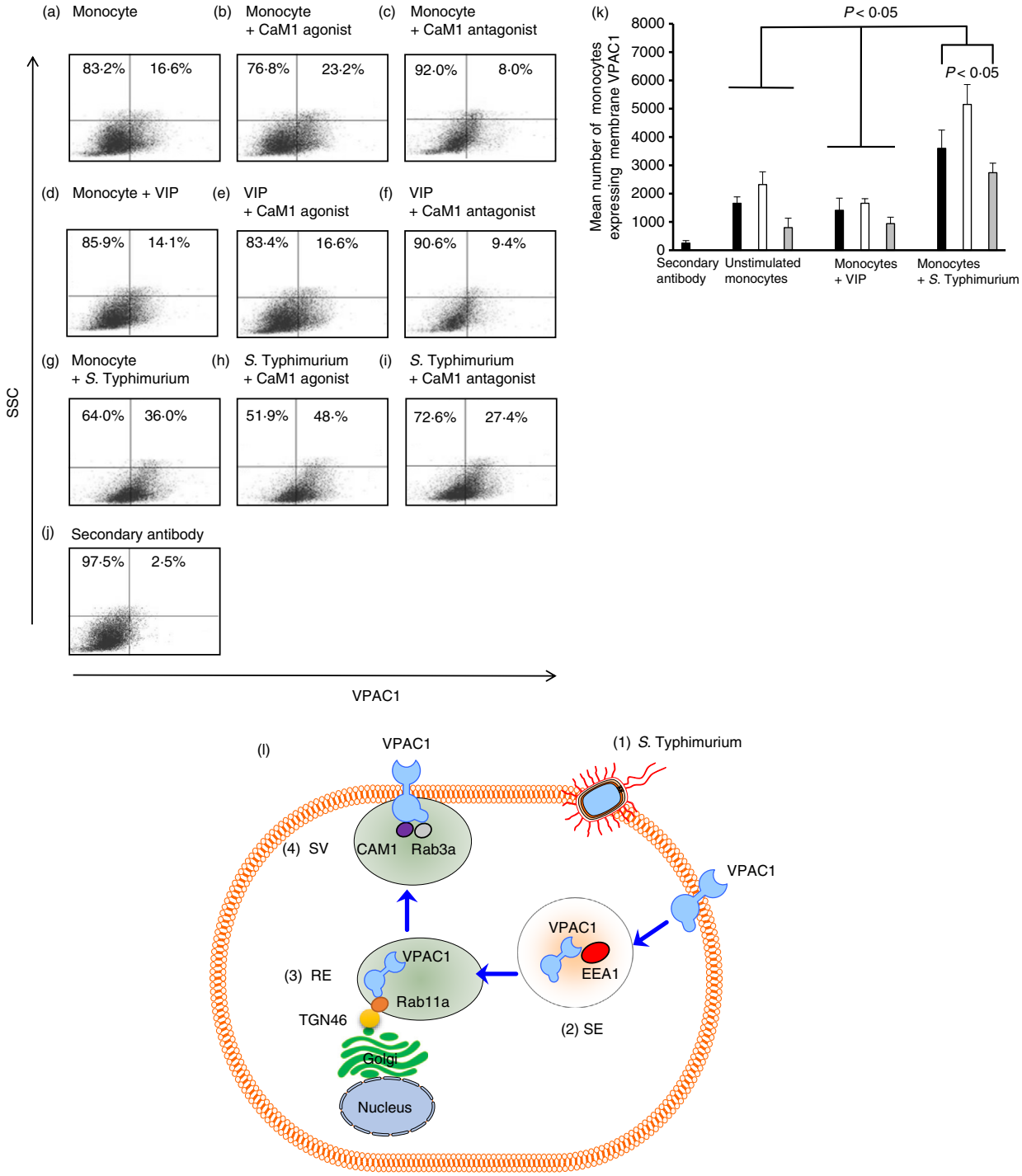

Figure 5. CAM1 increases vasoactive intestinal peptide receptor 1 (VPAC1) protein expression on the cell membrane of monocytes infected with *Salmonella enterica* serovar Typhimurium 4/74. (a–j) FACS analysis showing VPAC1 expression on the monocyte membrane 6 hr postinfection with *S.* Typhimurium or monocytes cultured with vasoactive intestinal peptide (VIP; 10^{-7} M) for 6 hr, with or without CAM1 agonist (CALP1; 20 μg/ml) or CAM1 antagonist (W-7; 30 μg/ml). FACS analyses shown are representative of experiments performed in triplicate on five separate occasions. *y*-axes, SSC = Side scatter; *x*-axis, VPAC1 expression. Quantitative analysis of experimental means are shown in (k). Black bars = 2 hr post-treatment/infection, white bars = 6 hr post-treatment/infection and grey bars = 24 hr post-treatment/infection. Connecting bars show significant differences at $P = 0{\cdot}05$, error bars show standard deviation from the mean. (l) Diagrammatic representation of *S.* Typhimurium-induced trafficking of VPAC1 receptors.(1) *S.* Typhimurium invades monocyte. (2) VPAC1 is packaged in the EEA1-positive sorting endosome (SE). (3) Retrograde transport of VPAC1 occurs via Rab11a-positive recycling endosome (RE), which is associated with TGN46 on the Golgi apparatus. (4) Anterograde transport of VPAC1 occurs via Rab3a/CAM1 positive secretory vesicle (SV).

expression by around fivefold above expression in resting monocytes ($P < 0{\cdot}05$) but VPAC1 expression was not differentially expressed when monocytes were cultured with VIP (Fig. 1b). After 6 and 24 hr p.i., significantly more *S.* Typhimurium were recovered from infected monocytes that had been co-cultured with VIP, compared with infected monocytes without VIP ($P < 0{\cdot}05$) (Fig. 1c).

Following FACS analyses, we also detected a 50% increase in the immunoreactivity of VPAC1 protein in the cytosol of monocytes after 6 hr p.i. with *S.* Typhimurium, which was highly significant ($P < 0{\cdot}01$) when compared with VPAC1 protein in the cytosol of monocytes incubated with VIP or uninfected monocytes (Fig. 2b–c and d). FACS analysis also showed that VPAC1 protein was increased on the surface of monocytes infected with *S.* Typhimurium by around 30% and this increase was also significant ($P < 0{\cdot}05$) when compared with VPAC1 protein in the cytosol of monocytes incubated with VIP or uninfected monocytes (Fig. 2g–i and j).

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 immunofluorescence 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 trans-Golgi network (TGN; retrograde trafficking). This was indicated by strong co-localization of VPAC1 with Rab11a (Fig. 3b) and concomitant co-localization of Rab11a with the 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-localization of VPAC1 with the membrane docking protein Rab3a (Fig. 4a), which was concomitantly co-localized with CAM1 (Fig. 4b). Subsequent quantitative PCR analysis showed that *S.* Typhimurium infection induced a more than fivefold ($P < 0{\cdot}05$) 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 ($P < 0{\cdot}05$), whereas addition of a CAM1 antagonist (W-7) significantly decreased VPAC1 mRNA expression ($P < 0{\cdot}05$) (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 with uninfected monocytes (Fig. 5a–c) or monocytes cultured with VIP (Fig. 5d–f) and in both cases these changes were significant ($P < 0{\cdot}05$). *P*-values were calculated by analysis of variance of mean expression detected for each experimental group (shown in Fig. 5k). A diagrammatic model of intracellular cycling of VPAC1, induced by *S.* Typhimurium infection, is shown in Fig. 5(l).

Discussion

We show that *S.* Typhimurium increased expression of VPAC1 in human monocytes and this was associated with increased intracellular survival of the bacteria, when infected monocytes were co-cultured with VIP. This is in accordance with a study by Storka *et al.*,[12] who reported a >30% increase in VPAC1 expression in monocytes isolated from human volunteers 24 hr after infusion of LPS from *Escherichia coli*, and this was correlated with an increased concentration of VIP in plasma. However, at least some of this effect is probably due to increased monocyte survival[13,19] but the effect is not merely due to LPS, which we found to induce lower levels of VPAC1 by both quantitative PCR and FACS analyses compared with *Salmonella* infection (data not shown).

Murine studies have indicated that the immunosuppressive effect of VIP occurs uniquely via VPAC1,[11] and that VIP/VPAC1 interaction regulates the production of inflammatory mediators associated with mortality in sepsis,[8] which may have evolved to protect the host. However, studies using viable *Salmonella*, rather than LPS, have shown that VIP down-regulates inflammatory mediators and increases *Salmonella* survival in human monocytes[13,19] and murine macrophages.[20] We therefore hypothesized that *S.* Typhimurium actively utilizes the VPAC1/VIP axis for its own survival advantage.

We now show that *S.* Typhimurium not only increased VPAC1 mRNA and protein expression but also induced VPAC1 recycling to the cell membrane. The initial step in this process was internalization of VPAC1 into EEA1-containing SE. The EEA1 is required for tethering, docking and fusion of the SE to soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor, which subsequently leads to endosomal shipment.[21] A previous study has shown that internalization and localization of VPAC1 in the SE occurred in VPAC1 transinfected Chinese

hamster ovary cells, following culture with VIP.[22] However, in this latter study, VPAC1 was not recycled back to the cell membrane and in accordance with this, we show that VIP did not increase VPAC1 expression on the cell membrane.

Internalized proteins may be trafficked via the SE to late endosomes and lysosomes (which may prevent further recycling) or to the TGN (retrograde transport), which may sort the proteins into REs for subsequent transport back to the cell membrane (anterograde transport).[23,24] This requires interaction with Ras-associated binding (Rab) proteins, which are small GTPases involved in intracellular trafficking of protein cargo from the SE to downstream endosomes, including the RE, and the docking of transport vesicles with membrane targets.[25] In our study, *S.* Typhimurium-induced retrograde transport of VPAC1 was indicated by strong co-localization of VPAC1 with Rab11a, one of the best studied markers of REs[26] and TGN protein 46 (TGN46). The TGN is the critical region on the Golgi apparatus that collects and sorts newly synthesized proteins and proteins relayed to it from REs.[27,28] Some bacterial toxins, such as Shiga toxin, produced by *Shigella dysenteriae*,[29] HIV envelope protein[30] and the immunosuppressive HIV nef protein,[31,32] are trafficked from the cell surface via retrograde transport. However, our study is the first to show that *S.* Typhimurium (or any other pathogen) induces retrograde transporting of VPAC1.

The results we obtained also suggested that *S.* Typhimurium induced anterograde transport of VPAC1 via CAM1/Rab3a. The importance of Ca^{2+} in regulated exocytosis has been known for a number of years[33] and a study by MacKenzie *et al.*[34] also reported a positive correlation between Ca^{2+} concentration and VPAC1 density. Calcium binding by CAM1 forms Ca^{2+}/CAM1 complexes that bind to Rab3a, which subsequently causes switching of Rab3a from a GDP-bound (inactive) form to a GTP-bound (active) form.[35] It is possible that the increase in CAM1 and Rab3a mRNA that we show may increase calcium binding and active Rab3a and hence may increase VPAC1 binding and transport to the cell membrane. Active Rab3a is a constituent protein in secretory vesicles within PC-12 cells and in newly formed secretory vesicles associated with the TGN in pancreatic acinar cells,[36] and is involved in the docking and exocytosis of secretory vesicles.[37] Little has been reported regarding Rab3a in immune cells but a study by Abu-Amer *et al.*[38] has shown that Rab3a expression is increased in murine bone-marrow-derived macrophages stimulated with LPS, although this was not studied in the context of VPAC1. We also show that *S.* Typhimurium (which contains LPS) significantly increased Rab3a and CAM1 mRNA expression in monocytes. Moreover, our results show a causal link between CAM1 and VPAC1 expression on the monocyte membrane. However, the effect of CAM1 agonist or antagonist was not absolute, suggesting that other factors (possibly Rab3a itself) also directly affect VPAC1 expression on the monocyte surface.

Only a fraction of newly synthesized VPAC1 becomes inserted into cell membranes, as a result of conformational misfolding,[16] and although *S.* Typhimurium infection increased VPAC1 mRNA expression, it is possible that *S.* Typhimurium-induced recycling of VPAC1 through retrograde/anterograde pathways overcomes low surface expression due to misfolding. This would facilitate greater interaction with the increased concentration of VIP in serum, as shown by Storka *et al.*[12]

In conclusion, *S.* Typhimurium exploits the VPAC1/VIP axis to increase survival in human monocytes, this is achieved, at least in part, by retrograde and anterograde recycling of VPAC1 via CAM1.

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Disclosure

The authors have no conflicts of interest to declare.

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