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Abstract

Under normal physiological conditions, the intestinal immunity remains largely hyporesponsive to the commensal microbiota, yet also retains the inherent ability to rapidly respond to pathogenic antigens. However, immunomodulatory activities of extracellular products from commensal bacteria have been little studied, with previous investigations generally utilising the live bacterium to study microbiota-epithelial interactions. In this study, we demonstrate that extracellular products of a commensal bacterium, *Escherichia coli* C25, elicit a moderate release of proinflammatory IL-8 and stimulate transcriptional up-regulation of Toll-like receptors (TLRs) in intestinal epithelial cell lines, HT29-19A and Caco-2. Additionally, we show that removal of outer membrane vesicles (OMVs) reduces the proinflammatory effect of secreted products from *E. coli* C25. Furthermore, we show that isolated OMVs have a dose-dependent proinflammatory effect on IECs. Interestingly, a relatively high concentration (40 µg/ml protein) of OMVs had no significant regulatory effects on TLR mRNA expression in both cell lines. Finally, we also demonstrate a that pre-incubation with *E. coli* C25-derived OMVs subsequently inhibited the internalisation of the bacterium itself in both cell lines. Taken together, our results suggest that commensal-derived extracellular products, in particular OMVs, could significantly contribute to intestinal homeostasis. We also demonstrate a unique interaction between commensal-derived OMVs and host cells.

Introduction

The intestinal commensal microbiota, consisting of ~10^{14} bacteria [1], is considered one of the densest and most diverse microbial communities on the planet [2]; consequently, our knowledge of the highly dynamic role the microbiota plays in host immunity is still very basic. Nevertheless, advances in technology have allowed some compositional characterisation of the commensal microbiota via metagenomic analyses [1,3]. For example, in early neonatal life**,** *E. coli* are among the first bacteria to colonise the human intestine [4] and these early commensal pioneers offer a preliminary defence against enteropathogens, due to physical and nutritional competition [5]. An early example of a commensal bacterium is *E. coli* C25 which was originally isolated from the faeces of a healthy individual in the mid-1950s [6] and was subsequently demonstrated to have antagonistic activities against the enteric pathogen, *Shigella flexneri* [7,8]. Also, C25 lacks the traditional virulence genes found in pathogenic strains of *E. coli*, such as extraintestinal pathogenic (ExPEC), enterohaemorrhagic (EHEC) and enteropathogenic (EPEC) [9,10]; moreover, it is a poor recipient of plasmid transfer [11], so is unlikely to acquire such genes from other bacteria. Nevertheless, studies utilising live C25 have demonstrated its ability to translocate through the intestinal epithelial barrier [10,12,13] and to initiate a proinflammatory response in intestinal epithelial cell lines [9,10,12,14]. Yet, the immunoregulatory ability of the extracellular products from C25 have only been briefly considered previously [15].

Gram negative bacteria, and *E. coli* in particular, are well characterised in their production of outer membrane vesicles (OMVs) [16-20], which are small (50-250 nm diameter), spherical, bilayered membranous structures naturally secreted into the bacterium's immediate surroundings [21]. OMVs have been isolated from a diverse range of environments, from liquid and solid lab cultures to river beds and waste water pipes [22], and even from the human body [23,24]. The composition, conformation and surface chemistry of

OMVs is representative of the intact outer membrane (OM) of Gram-negative bacteria, with lipopolysaccharides (LPSs), outer membrane proteins (OMPs), phospholipids and periplasmic proteins all present [17,21]. Therefore, it is unsurprising that OMVs from pathogens, such as *Pseudomonas aeruginosa*, *Helicobacter pylori* and *Vibrio cholera* have been suggested to contribute to the pathology of chronic inflammatory diseases, as they exhibit the ability to elicit IL-8 from gastric [25], bronchial [26] and intestinal epithelial cells [27,28], respectively. However, more recent studies have focussed on OMVs derived from probiotic bacteria, such as *E. coli* Nissle 1917 [18,29] and commensal bacteria, such as *Bacteroides fragilis* [30], *Bacteroides thetaiotaomicron* [31] and *E. coli* strain ECOR12 [29]. Nevertheless, with the recent exception of Fábrega *et al*., who showed that the two strains of *E. coli* studied were able to stimulate cytokine release from explanted colonic tissue [29], the direct interactions of OMVs from non-pathogenic bacteria with the host intestinal epithelium have been little studied [32]. Therefore, the current study aimed to investigate the direct inflammatory potential of OMVs derived from *E. coli* C25 on the intestinal epithelial cell lines, HT29-19A and Caco-2.

Materials and Methods

Cell culture

HT29-19A and Caco-2 cell lines were kindly donated by Prof. G. Warhurst (Royal NHS Foundation Trust and University of Salford, UK). Both cell lines were cultured in a standard media of high glucose (4500mg/l) Dulbecco's Modified Eagles Media (DMEM), 10 % foetal bovine serum (FBS), 4 mM glutamine and a mixture of 50 IU/ml penicillin and 50 µg/ml streptomycin (PenStrep). Additionally, HT29-19A cells were supplemented with 20 mM HEPES and Caco-2 cells had 0.1 mM MEM NEAA (non-essential amino acids) added. Both cell lines were seeded at a density of 0.5 x 10^5 cells/cm^2 and cultured to confluence (~7 days) in 35 mm x 10 mm cell culture dishes. After culturing the cells to confluence on tissue culture plastic over 7 days, both cell lines became semi-polarised; indeed, Caco-2 cells were observed to undergo dome formation, which is indicative of unidirectional water transport and polarisation.

Bacterial products

E. coli C25 was a kind gift from Prof. G. Warhurst and was cultured on tryptone soy agar (TSA) at 37 °C. DMEM, supplemented with 4 mM glutamine was inoculated with *E. coli* C25 and incubated overnight (~15 h), until the culture reached the stationary phase of growth (Supplementary Fig 1; ~1 x 10^9 CFU/ml). Subsequent to incubation, the culture was centrifuged at 6000 x *g* for 10 min to pellet out the bacteria. The supernatant was removed, had its pH adjusted to 7.4 and was subsequently filtered using 0.45 µm syringe-driven filters (Millex®, Millipore UK Ltd.). The cell-free supernatant was diluted 1:10 in cell culture medium and used in cell challenge experiments.

10 ml aliquots of overnight *E. coli* C25 cultures in DMEM with 4 mM glutamine (containing ~1 x 10^9 CFU/ml) were sonicated, using a Vibracell VCX 130 (Sonics and

Materials Inc.) at 85 % amplitude for a 5 x 6 s pulse program. Cultures were sonicated on ice and with a 24 s cooling step between pulses, in order to minimise denaturation of bacterial products. Resultant solutions were filtered through a 0.45 µm syringe-driven filter, diluted 1:10 in cell culture medium and subsequently used in cell challenge experiments.

Flagellin isolated from *Salmonella typhimurium* strain 14028 was purchased from Enzo Life Sciences Ltd.

Cytokine stimulation and analysis

Cells were challenged with the bacterial stimuli for 24 h, at 37 °C, 5 % CO_2 and constant humidity. Supernatants were collected and frozen at -80 °C until assayed for IL-8 by enzyme-linked immunosorbant assay (ELISA) analysis (IL-8 Human Antibody Pairs, Invitrogen). ELISA analysis was carried out according to the manufacturer's instructions.

qPCR

Epithelial cells were challenged with the bacterial stimuli for 24 h. The cells were subsequently lysed and the total RNA was extracted using the RNeasy® Mini Kit and RNase-free DNase Set (Qiagen). RNA was quantified spectrophotometrically using the absorbance at 260 nm (A_{260}) x 44 µg/ml x dilution factor and the purity was measured using A_{260}/A_{280}. cDNA was synthesised from 2 µg of total RNA by the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Ltd.). cDNA synthesis was carried out to the manufacturer's instructions.

PCR primers (Table 1) were purchased from Eurofins MWG Operon. Universal ProbeLibrary probes and Lightcycler® Taqman® Master Mix were purchased from Roche Diagnostics Ltd. Amplification was carried out in 20 µl reaction volume containing 1.5 µl cDNA, 0.5 µl F-primer and R-primer (0.4 µM), 0.5 µl Universal probe, 4 µl 5x Mastermix and 13 µl DNase/RNase-free water. The following program was used: 95 °C for 10 min

followed by 45 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 s. Target gene expression was normalised to the housekeeping gene GAPDH, and the fold difference of expression from the control was calculating using the $2^{-\Delta\Delta Ct}$ method [33].

OMV isolation

Outer membrane vesicle (OMV) isolation was achieved by a well-established method first described by Kadurugamuwa and Beveridge [34] and more recently modified by Vanaja *et al*. [35] and Fabrega *et al*. [29]. Briefly, 250 ml overnight (15 h) cultures of *E. coli* C25 in tryptone soy broth (TSB) were centrifuged at 6000 x *g* for 10 min to pellet out the bacteria. The supernatant was sequentially filtered through 0.80 and 0.45 μm pore size vacuum-driven bottle top filters. A sample of the filtrate was transferred to TSA plates and incubated at 37 °C to ensure there was no contaminating bacteria were present. The filtrates were ultracentrifuged at 150,000 x *g* for 1.5 h, at 5 °C, to pellet out the OMVs. The supernatant was removed and the pellet was resuspended in 50 mM HEPES buffer (pH 6.8) and ultracentrifuged again for 30 min at 120,000 x *g*, 5 °C. The supernatant was again removed and the pellet was resuspended in 50 mM HEPES buffer (pH 6.8), filtered through a 0.45 μm syringe filter and stored at 4 °C. Approximately 400 μg OMVs by protein concentration were isolated from 250 ml culture supernatant, measured by the modified Lowry assay as per the manufacturer's instructions (DC™ Protein Assay; Bio-Rad). Subsequently, OMVs were serially diluted (to give 4-100 μg/ml protein) in cell culture medium and utilised in cell challenge experiments.

Outer membrane isolation

E. coli C25 outer membrane (OM) was isolated using a slightly modified protocol from that previously described by Zhou *et al*. [36]. Briefly, 250 ml overnight (15 h) cultures of *E. coli*

C25 grown in TSB were centrifuged at 10,000 x *g* for 10 min and the resultant pellet was washed twice in PBS. The bacterial pellet was then resuspended in 10ml PBS with 0.01 M EDTA, incubated at room temperature for 30 min and sonicated for 10 s at 85% amplitude. The mixture was then centrifuged again at 10,000 x *g* for 10 min at 4°C and the supernatant was collected, with the pellet being discarded. The supernatant was subsequently centrifuged at 80,000 x *g* for 2 h at 4°C. The translucent yellow pellet was resuspended in sterile water and was centrifuged again at 80,000 x *g* for 2 h at 4°C. The final pellet was resuspended in sterile water and frozen at -80°C [36].

Transmission electron microscopy

OMVs were isolated and resuspended at approximately 1 mg/ml protein content in 50 mM HEPES buffer (pH 6.8). Vesicles were placed on Carbon Films on 400 Copper Mesh Grids (Agar Scientific) for 1 min. Grids were then negatively stained with 1% aqueous uranyl acetate for 1 min and visualized on a LoJeol 1200EX TEM.

SDS-PAGE

Protein concentrations of isolated OMVs and OMs were measured using the modified Lowry assay as per the manufacturer's instructions (DC™ Protein Assay; Bio-Rad), with BSA used as a protein standard (Sigma-Aldrich). 30 µg of samples were heated at 70°C for 10 minutes and subsequently resolved on a 4-12% NuPAGE Novex Bis-Tris precast protein gel (Invitrogen) in 1x MES buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.0). The gel was then stained using the Pierce™ Silver Stain Kit (Thermo Scientific) to the manufacturer's instructions and viewed using the InGenius gel viewing cabinet (Syngene) and GeneSnap software (Syngene).

Bacterial internalisation assay

The method for this assay was developed from the technique described by Macutkiewicz *et al.* [10]. Epithelial cells were cultured to confluence (~7 days) and treated with fresh medium 24 h in advance of the assay. 10 ml sterile tryptone soy broth (TSB) was inoculated with *E. coli* C25 and incubated at 37 °C overnight (15 h), giving a culture entering the stationary phase of growth (Supplementary Fig 1) and at a density of ~1 x 10^9 CFU/ml (serial dilutions and plating out on to tryptone soy agar (TSA) gave exact numbers retrospectively). The culture was centrifuged at 10,000 x *g* for 10 min and the bacterial pellet was resuspended in 10 ml HBSS supplemented with 10 mM sodium bicarbonate and 180 mg/dl glucose (Trans-HBSS) in order to reduce bacterial growth during the assay [11]. Epithelial cells were washed twice in sterile Trans-HBSS, had ~1 x 10^9 CFU C25 in 2 ml Trans-HBSS added and were incubated at 37 °C for 4 h. After incubation epithelial cells were washed twice with Trans-HBSS, thus removing any non-adherent/non-internalised bacteria. 1 ml Trans-HBSS/50 µg/ml gentamicin was added to the cells and incubated at room temperature for 15 min, killing all but the internalised bacteria. The supernatant was removed and plated out neat, in TSA, to confirm that the antibiotic had killed the extracellular bacteria. The epithelial cells were lysed by osmotic pressure with the addition of 1 ml sterile deionised water, and subsequently by sheer force with repeated pipetting, thus releasing the bacteria contained within the cells. A serial dilution of the resultant lysates was performed to give 10^{-3} and 10^{-4} dilutions, which were subsequently plated out on TSA, using the agar pour plate method. All resultant plates were incubated overnight at 37°C and bacterial colonies were manually counted.

Statistical Analysis

Results are expressed as median ± interquartile range (IQR) for the stated number of experimental repeats (*n*). Statistical significance was calculated using Mann Whitney *U*-test or Kruskal-Wallis test with Dunn's *post hoc* analysis and a p value ≤ 0.05 was considered significant. All statistical analyses were undertaken using Prism® 6 (GraphPad Software Inc.).

Results

Extracellular products derived from *E. coli* C25 elicit a moderate proinflammatory response from IECs

Earlier studies have shown that live *E. coli* C25 can induce secretion of proinflammatory cytokines from intestinal epithelial cells [9,14]; however, the extracellular products from this bacterium have only briefly been considered before [15]. To investigate the inflammatory potential of *E. coli* C25-derived extracellular products, HT29-19A and Caco-2 intestinal epithelial cells lines were challenged with the cell-free supernatant from cultures of *E. coli* C25 and sonicated samples of *E. coli* C25 cultures for 24 h. Sonicated samples were used to give maximal levels of antigenic material and mimic cells lysed by antimicrobial peptides (AMPs) *in vivo*. Additionally, the responses to both cell-free supernatant and sonicates were compared to the pathogenic antigen, flagellin, which is known to be a major contributing antigen in the pathology of inflammatory bowel disease [37] and therefore represents a relevant positive control. Moreover, significant IL-8 release in response to flagellin has previously been described in both cell lines utilised in this study [38,39].

E. coli C25 cell-free supernatant was shown to elicit a significant (~5-fold; $p \leq 0.005$) increase in IL-8 release in HT29-19A cells (Fig. 1a). The 1.5-fold increase in IL-8 release in Caco-2 cells was much more modest (Fig. 1b), but still statistically significant ($p \leq 0.05$). The increased IL-8 release in both cell lines was also reflected at the transcriptional level, with up-regulation of IL-8 mRNA in both HT29-19A (~9-fold increase; Fig 1c) and Caco-2 (~5-fold increase; Fig. 1d) cells, but neither was statistically significant. Additionally, both HT29-19A and Caco-2 cells exhibited an increased release (~7-fold; $p \leq 0.01$ and ~2-fold; $p \leq 0.05$, respectively) of IL-8 when challenged with the sonicated samples of *E. coli* C25, with levels comparable to those seen for the cell-free supernatant challenges (Figs. 1a and 1b). Furthermore, as with cell-free supernatants, challenging with sonicates increased IL-8 mRNA

expression in both cell lines, with HT29-19A cells exhibiting ~20-fold increase (Fig. 1c) and Caco-2 cells demonstrating a ~4-fold increase (Fig 1 d), although neither of these trends were calculated to be statistically significant.

Although the release and transcriptional up-regulation of IL-8 was potentiated in response to the extracellular products of *E. coli* C25 in both cell lines, when compared to that observed in response to the pathogenic positive control, 100 ng/ml final concentration flagellin, the increase is relatively modest (Fig 1); on addition of flagellin, IL-8 release was ~20-fold ($p \leq 0.005$) higher in HT29-19A (Fig 1a) and ~40-fold ($p \leq 0.005$) in Caco-2 cells (Fig. 1b), compared to control. mRNA expression was up-regulated ~250-fold ($p \leq 0.01$) and ~35-fold ($p \leq 0.01$) in HT29-19A (Fig. 1(c)) and Caco-2 cells (Fig. 1d), respectively. Despite this, upon direct comparison of cells treated with C25 extracellular products and those treated with flagellin, none of the trends were calculated to be statistically significant.

Removal of OMVs from *E. coli* C25 extracellular products reduces their proinflammatory effect on IECs

Gram negative bacteria are well characterised in their production of outer membrane vesicles (OMVs) [21,40], which, when derived from either pathogenic or commensal bacteria, have previously shown immunomodulatory activity on different intestinal cell types [27,29,30]. Therefore, to elucidate the contribution of OMVs to the proinflammatory profile of *E. coli* C25-derived extracellular products, we removed them from the C25 cell-free supernatant via ultracentrifugation. HT29-19A and Caco-2 cells were subsequently challenged with OMV-free cell-free supernatant (1:10 dilution in cell culture media) for 24 h and the resultant release of IL-8 was quantified.

In both cell lines, the release of IL-8 in response to the OMV-free cell-free supernatant was significantly ($p \leq 0.001$) increased from the control (Fig. 1a, 1b and 2). Once

the OMVs had been removed from the supernatant, the level of IL-8 expressed from both cell lines in response to OMV-free cell-free supernatant appeared to be reduced in comparison to untampered cell-free supernatant (Fig. 2); nevertheless, it was only statistically significant ($p \leq 0.01$) in Caco-2 cells (Fig. 2b) and not HT29-19A (Fig. 2a). Despite appearing to be increased compared to the control cells, the IL-8 production in cells challenged with the OMV-free cell-free supernatant was not calculated to be significantly different from these controls (Fig. 2).

E. coli C25-derived OMVs elicit a dose-dependent proinflammatory response from IECs, but have no regulatory effects on TLR mRNA expression.

To investigate the immunomodulatory potential of isolated *E. coli* C25 outer membrane vesicles (OMVs), HT29-19A and Caco-2 cells were challenged with a 4-100 µg/ml protein concentration range of OMVs for 24 h. Release and expression of proinflammatory IL-8 was investigated by ELISA and qPCR analysis.

In the HT29-19A cell line, an increased level of IL-8 was observed at a 4 µg/ml concentration of OMVs and increased in a dose-dependent manner; nevertheless, the increase was only calculated to be statistically significant ($p \leq 0.05$) from 20 µg/ml OMVs (Fig. 3a). Similarly, in Caco-2 cells, 20 µg/ml OMVs was the minimum concentration required to elicit a statistically significant ($p \leq 0.05$) increase in IL-8 secretion (Fig. 3b). A ~6-fold increase in IL-8 mRNA was produced by 40 µg/ml OMVs in HT29-19A cells ($p \leq 0.001$), in contrast, no change was observed in Caco-2 cells (Fig. 3c). This distinct difference in the responsiveness to OMVs was again indicative of the phenotypic variance between the two cell lines. OMVs, which were shown to measure 50-100 nm (Fig 3d), had their protein content compared to that of the outer membrane (OM) via SDS-PAGE (Fig 3e). The two had very similar protein compositions, with only subtle differences in band intensity evident; therefore, we can

speculate that the surface protein composition of the OMVs is representative of antigens present on the whole parent bacterium.

Previous studies have reported that agonist binding results in the up-regulation of their cognate TLR receptors [41-43]; we confirmed this phenomenon in the current study by measuring the regulation of TLR-5 mRNA expression in both cell lines, in response to a 24 h challenge with 100 ng/ml flagellin. Significant ($p \leq 0.001$) up-regulation of TLR-5 mRNA was observed in HT29-19A cells (~110-fold increase; Supplementary Fig. 2) and Caco-2 cells (~26-fold increase; Supplementary Fig. 2). To investigate whether this was also true in reaction to commensal-derived antigenic material, we monitored transcriptional expression of the TLRs most relevant to bacterial antigens (TLRs-1, -2, -4, -5 and -9) in response to *E. coli* C25 cell-free supernatant. In HT29-19A cells, we observed a significant ($p \leq 0.05$) increase in all the TLRs tested in response to challenge with cell-free supernatant (Fig 4a). Similarly, the Caco-2 cell line showed up-regulation in mRNA expression of TLR-1, -4, -5 and -9, although only the data for TLRs-1 and -9 was considered statistically significant ($p \leq 0.05$; Fig. 4b). Interestingly, cell-free supernatant-challenged Caco-2 cells did not exhibit any regulation in TLR-2 mRNA expression, as the levels remained comparable to the control. This was in complete contrast to HT29-19A cells, which showed the largest increase in TLR-2 mRNA expression (~17-fold increase (Fig. 4a)). This contradiction in reaction is likely to arise from the distinct phenotypic differences between the two cell lines, as Caco-2 cells exhibit a significant ($p \leq 0.001$; ~540-fold) increased constitutive expression in TLR-2 mRNA, when compared to HT29-19A cells (data not shown).

Surprisingly, given the fact that the OMVs possess all the surface antigens of the parent bacterium (Fig. 3e) when HT29-19A cells were challenged with a 40 μg/ml OMVs no significant differences were observed in TLR mRNA expression (Fig. 4a), despite TLRs-2, -4 and -5 appearing to be slightly up-regulated and TLR-9 was completely undetectable in the

presence of cell-free supernatant, when compared to the control. Indeed, a number of TLRs appeared to be down-regulated in Caco-2 cells; however, none of these were statistically significant (Fig. 4b).

Pre-incubation with *E. coli* C25-derived OMVs inhibits the internalisation of the parent bacterium

As mentioned previously, past studies have utilised *E. coli* C25 as a model strain for bacterial translocation across the intestinal epithelium [10,12]; therefore, we sought to investigate the regulatory ability of OMVs on this process. To explore this, we performed a bacterial internalisation assay in both HT29-19A and Caco-2 cells. Interestingly, we observed a reduction in the number of bacteria internalised in both cell lines which had been pre-treated with 40 µg/ml OMVs (Fig. 5); however, only the decrease seen in Caco-2 cells (~3.5-fold) was statistically significant ($p \leq 0.05$).

Discussion

We have previously described the potential of specific extracellular products derived from commensal enteric bacteria to modulate the low-level inflammation which exists in intestinal homeostasis [44,45]; however, there is still a paucity of research in this field. In the present study, we aimed to explore the inflammatory profile of extracellular products secreted by the commensal enteric bacterium *E. coli* C25 on two immortalised intestinal epithelial cell lines, HT29-19A and Caco-2. Here, we principally demonstrate that the extracellular products of *E. coli* C25 (both naturally secreted and after the artificial enhancement of their production via bacterial sonication) elicit a moderate proinflammatory response, via secretion of the potent neutrophil chemoattractant, IL-8, from the intestinal cell lines, HT29-19A and Caco-2. In this study, the cells were cultured on tissue culture plastic, which allowed them to become semi-polarised; however, in future studies it may be more physiologically accurate to culture the cell lines to complete polarisation on Transwell inserts and examine the effects of *E. coli* C25-derived factors on both the apical and basolateral surfaces.

In corroboration with previous studies on these cell lines [46], it is evident that the two possess a marked difference in constitutive secretion of IL-8 and that their responsiveness to antigenic material is relatively dissimilar. It has previously been speculated that HT29 and Caco-2 cell lines were isolated from different cell type populations within the epithelial layer. HT29 cell lines are thought to originate from hyper-responsive intestinal epithelial crypt cells [47,48], whereas Caco-2 cells were derived from the more immunotolerant villus enterocytes [48-50]. Nevertheless, despite the distinct phenotypic differences between HT29-19A and Caco-2 cells, we were able to confirm a mild proinflammatory response in both cells lines during challenges with extracellular products derived from *E. coli* C25. Also, we show that the naturally secreted products present in cell-free supernatant from cultures of *E. coli* C25 can induce a modest up-regulation of the major

TLRs associated with recognition of bacterial antigens. As mentioned previously, agonist binding of TLRs results in the up-regulation of their cognate receptor [41-43] and we confirmed this phenomenon occurs in IECs in response to flagellin; therefore, from the mRNA up-regulation of multiple TLRs observed in this study, we can speculate that C25 cell-free supernatant contains multiple TLR ligands. One such secretory product which we hypothesised to contribute to this was outer membrane vesicles (OMVs).

OMVs isolated from Gram-negative bacteria are receiving increasing interest in microbiological research [40,51]; yet, despite the vast number of Gram-negative bacteria present within the intestinal microbiota, there is a lack of studies considering the immnuoregulatory activity of OMVs derived from this population [32]. Also, the limited studies performed to date are divided in their opinion of the pontential role of OMVs in the intestinal niche. It has recently been suggested that macrophage-induced immune responses to OMVs from the commensal bacterium *B. thetaiotaomicron* could drive colitis in genetically susceptible hosts [31]; however, this is contradicted by an elegant study previously undertaken by Shen *et al*., which suggests a more beneficial role for commensal-derived OMVs. In their study, they demonstrated that capsular polysaccharide (PSA)-containing OMVs, isolated from *B. fragilis*, can protect against inflammation in the 2,4,6-trinitrobenzenesulfonic acid (TNBS) experimental model of colitis in mice via the production of anti-inflammatory cytokines by DCs, which subsequently enhanced the protective regulatory T cell response [30]. Additionally, Fábrega *et al*. have recently shown that OMVs from both probiotic and commensal strains of *E. coli* stimulate a more anti-inflammatory cytokine profile from explanted colonic tissue, despite a moderate increase in proinflammatory cytokines, such as IL-6 and IL-8 [29]. In the current study, we corroborate the findings of Fábrega *et al*., as we show that the naturally secreted OMVs of an enteric commensal bacterium have a direct proinflammatory effect on the intestinal epithelial cell

lines, HT29-19A and Caco-2. However, we suggest that, should this proinflammatory effect also be observable *in vivo*, then it is moderate enough to be beneficial to the host by contributing to the homeostatic low-level inflammatory environment which is characteristic of the normal intestine.

Previously, it has been shown that OMVs are able to directly interact with host cells via TLRs [52-55]; nevertheless, we have demonstrated that a relatively high concentration (40 µg/ml) of C25-derived OMVs does not elicit an up-regulation of TLR mRNA expression, as was observed in response to the cell-free supernatant from cultures of the parent bacterium. It is well established that activation of TLRs by their agonists significantly enhances the internalisation of bacteria in both professional immune cells, such as macrophages [56,57], and non-professional immune cells, such as intestinal epithelial cells [58]. In addition to this, *E. coli* C25 has been used as a model strain for bacterial translocation through the intestinal epithelial barrier [10,12,13]; therefore, we decided to explore the regulatory effects of OMVs on this process. Consequently, we demonstrate that pre-treatment with C25 OMVs was able to reduce the subsequent internalisation of the C25 bacterium in intestinal epithelial cells.

Therefore, we propose that, through limiting the up-regulation of TLRs by other secretory products, OMVs can reduce the number of their parent bacterium which translocate the intestinal epithelial layer. It has been suggested that indigenous bacteria constitutively translocate transcellularly from the intestinal lumen of healthy, immunocompetent individuals, but are subsequently killed en route or *in situ* by professional immune cells once they reach the lymphoid organs [59]. Furthermore, Lichtman *et al.* suggested that bacterial translocation is required to generate immunocompetent cells within the gut-associated lymphoid tissue (GALT) [60]; however, prolonged and excessive immune reaction to the microflora leads to the chronic inflammation of the intestinal mucosa classically associated

with inflammatory bowel disease (IBD) [61]. Consequently, in order to maintain the fine balance of intestinal homeostasis, it is necessary to allow low numbers of commensal bacteria to translocate the intestinal epithelium; however, it is evident that this process must be stringently regulated. Here, we propose that, through the production of OMVs, the commensal microbiota themselves are able to directly contribute to the regulation of their own translocation, thus maintaining the mutually beneficial symbiosis with a healthy host and avoiding the pathogenesis of IBD.

In summary, these data demonstrate that, *in vitro*, extracellular products derived from a commensal bacterium have a mild proinflammatory effect on host intestinal epithelial cells and stimulate a moderate up-regulation of TLRs. We hypothesise these effects could be beneficial *in vivo* by priming the intestine and subsequently allowing a rapid, but more controlled, response to pathogenic bacteria and their associated antigens. Also, we show that OMVs are key contributors to the proinflammatory effect of the *E. coli* C25-derived extracellular products. Furthermore, we demonstrate a novel interaction between the commensal microbiota and host cells; through the inhibition of TLR up-regulation, membrane vesicles derived from a commensal bacterium are able limit the internalisation of the parent bacterium into intestinal epithelial cells. Finally, we hypothesise that, were the results presented here to be representative of the *in vivo* environment, then the products secreted into the intestinal milieu by the commensal microbiota, and OMVs in particular, could play a key role in the induction of the homeostatic low-level inflammatory response that is highly characteristic of the healthy intestine.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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620 **Table 1 – qPCR primers and probes**

Gene	Primer Sequence		Universal Probe No.
GAPDH	F – gctctctgctcctcctgttc	R – acgaccaaatccgttgactc	#60
IL-8	F – agacagcagagcacacaagc	R – aggaaggctgccaagagag	#72
TLR-1	F – aaacaacattgaaacaacttggaa	R – cacgtttgaaattgagaaatacca	# 65
TLR-2	F – ctctcggtgtcggaatgtc	R – aggatcagcaggaacagagc	#56
TLR-4	F – gaaggttcccagaaaagaatgtt	R – cctgattgtccttttcttgaatg	# 75
TLR-5	F – ctccacagtcaccaaaccag	R – cctgtgtattgatgggcaaa	# 72
TLR-9	F – tgtgaagcatccttccctgta	R – gagagacagcgggtgcag	#56

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Figure Legends

Figure 1 – *E. coli* C25-derived extracellular products elicit IL-8 release expression in IECs. HT29-19A and Caco-2 cells were challenged with *E. coli* C25 cell-free supernatant (1:10 dilution) and sonicated bacteria (1:10 dilution) for 24 h; 100 ng/ml of flagellin was used a positive pathogenic control. (**a**) IL-8 release from HT29-19A cells (n = 3-12). (**b**) IL-8 release from Caco-2 cells (n = 3-12). (**c**) mRNA expression of IL-8 in HT29-19A cells (n = 3-6). (**d**) mRNA expression of IL-8 in HT29-19A cells (n = 3-6). Results are median ± IQR. *, ** and *** indicate significance from the control, where $p \leq 0.05$, 0.01 and 0.005, respectively.

Figure 2 – Removal of OMVs decreases the proinflammatory activity of *E. coli* C25-derived extracellular products. *E. coli* C25 cell-free supernatant was ultracentrifuged to remove OMVs. The resultant OMV-free cell-free supernatant was diluted 1:10 in cell culture media and (**a**) HT29-19A and (**b**) Caco-2 cells were challenged for 24 h and IL-8 release was measured via ELISA (n = 6-12). Results are median ± IQR. ** and **** indicate statistical significance, where $p \leq 0.01$ and 0.001, respectively.

Figure 3 – OMVs isolated from *E. coli* C25 mediate a dose dependent release of IL-8 from IECs. (**a**) HT29-19A and (**b**) Caco-2 cells were challenged with serial dilutions (4-100 µg/ml) of *E. coli* C25 OMVs for 24 h and IL-8 expression was measured (n = 6). (**c**) mRNA expression of IL-8 in cells challenged with 40 µg/ml OMVs for 24 h (n = 3). Results are median ± IQR. *, ** and *** indicate significance from the control, where $p \leq 0.05$, 0.01 and 0.005, respectively. (**d**)TEM micrograph of C25 OMVs. (**e**) Comparison of protein content of *E. coli* C25 outer membrane vesicles (OMV) and outer membrane (OM) preparations by SDS-PAGE.

Figure 4 – *E. coli* C25-derived OMVs have no regulatory effects on TLR mRNA expression. HT29-19A (a) and Caco-2 (b) cells were challenged with 1:10 dilutions of *E. coli*

C25 cell-free supernatant or 40 µg/ml OMVs culture concentration of *E. coli* C25 outer membrane vesicles (C25 OMVs) for 24 h and qPCR was utilised to study TLR mRNA expression. Results are median ± IQR, $n = 3$. * and ** indicate significance from the control, where $p \leq 0.05$ and 0.01, respectively.

Figure 5 – Commensal- derived OMVs block internalisation of their parent bacterium. HT29-19A (**a**) and Caco-2 (**b**) cells were challenged with 40 µg/ml *E. coli* C25 OMVs for 24 h. Subsequently, the supernatants were removed and cell layers were co-cultured with ~1 x 10^9 CFU of *E. coli* C25 for 4 h. Non-internalised bacteria were killed and epithelial cells were lysed, releasing internalised bacteria. Lysates were serially diluted, plated out and incubated for 24h. Resultant colonies were counted and expressed as a % of the original inoculum. Results are median ± IQR, $n = 4$-6. * indicates significance from the control, where $p \leq 0.05$.

660 **Figure 1**

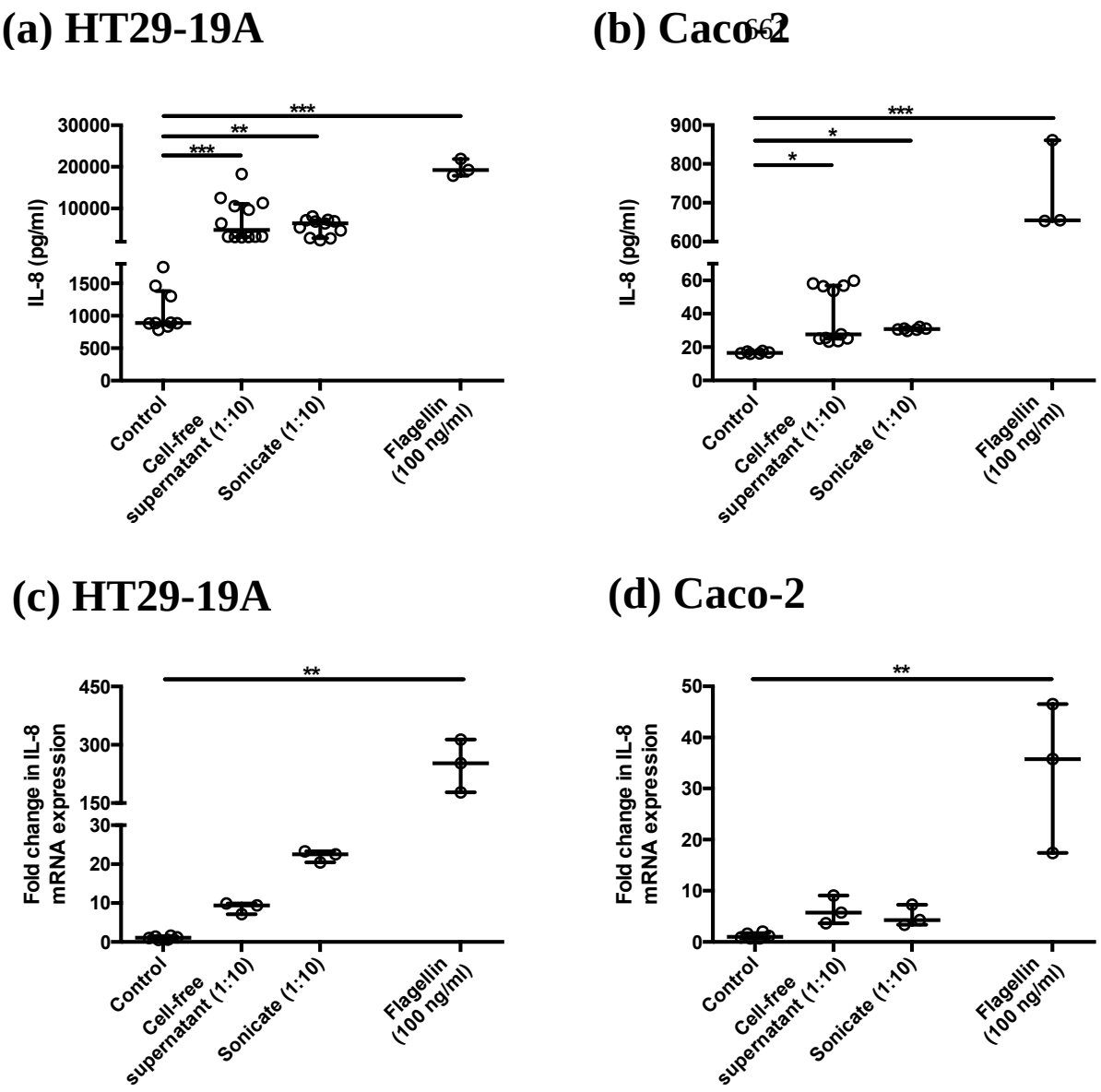

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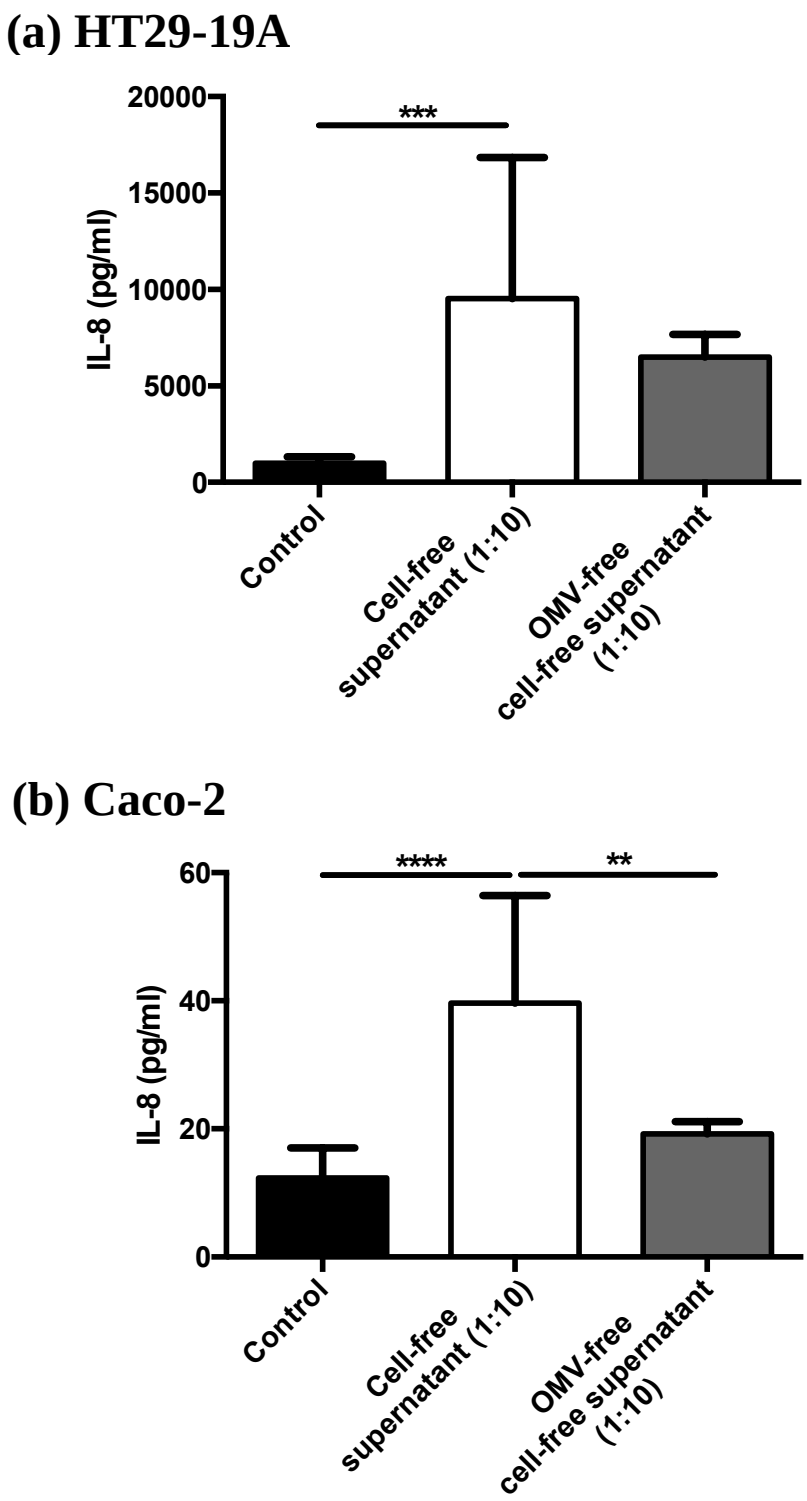
Figure 2


(a) HT29-19A
20000
15000
10000
5000
0
IL-8 (pg/ml)
***
Control
Cell-free supernatant (1:10)
OMV-free cell-free supernatant (1:10)
(b) Caco-2
60
40
20
0
IL-8 (pg/ml)
****
**
Control
Cell-free supernatant (1:10)
OMV-free cell-free supernatant (1:10)

677 **Figure 3**

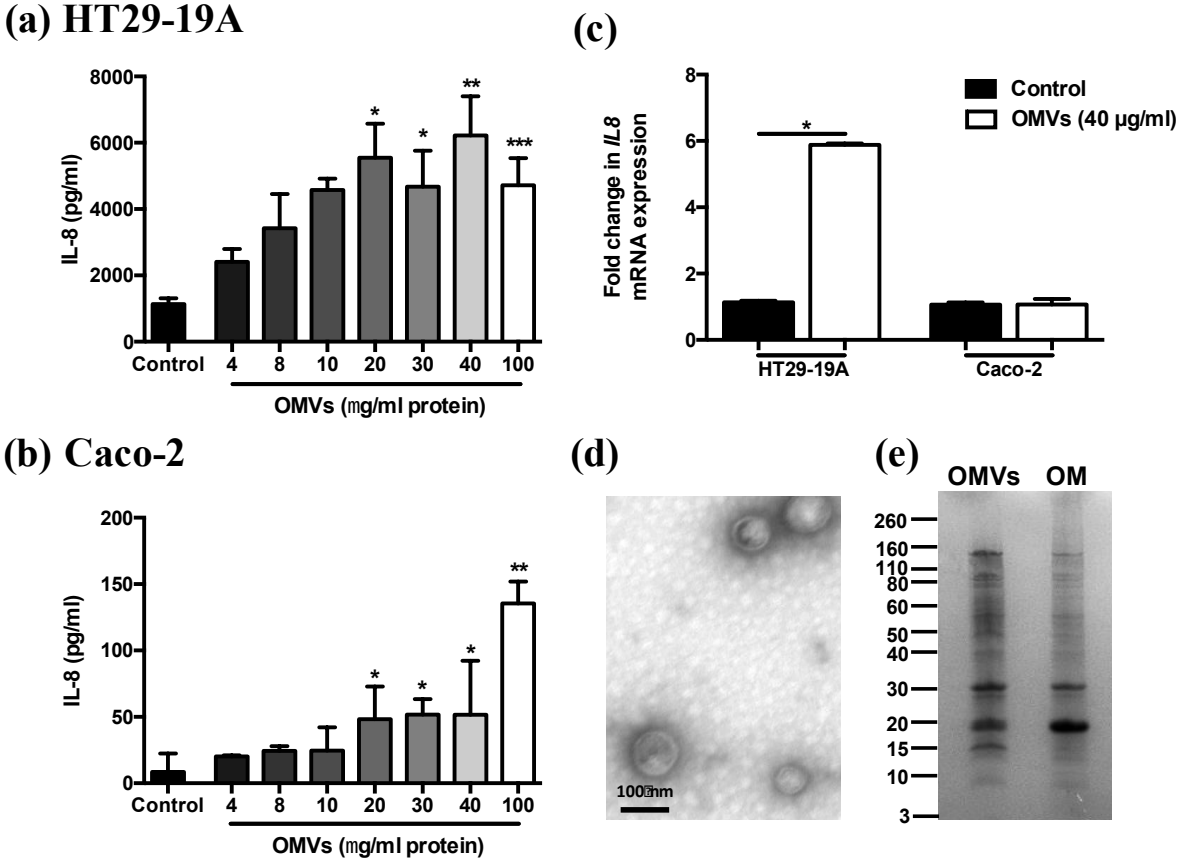


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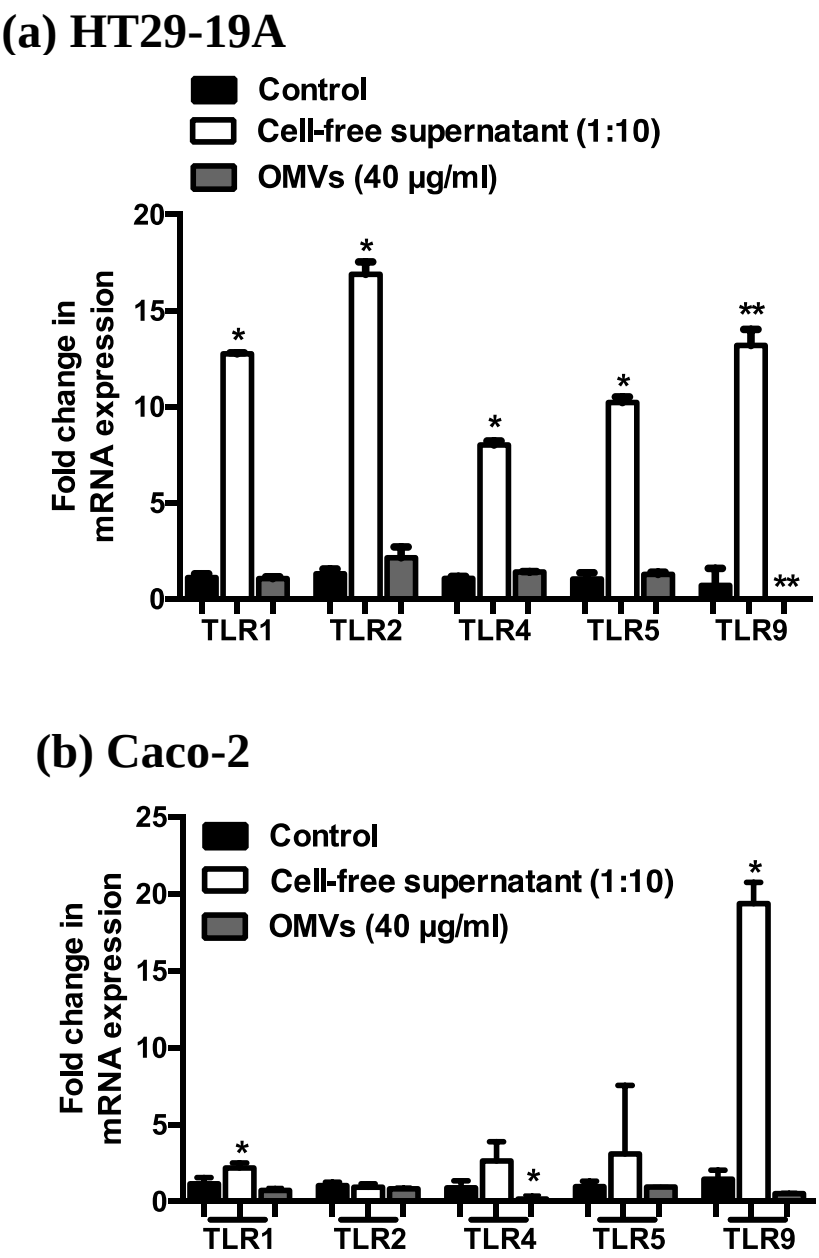
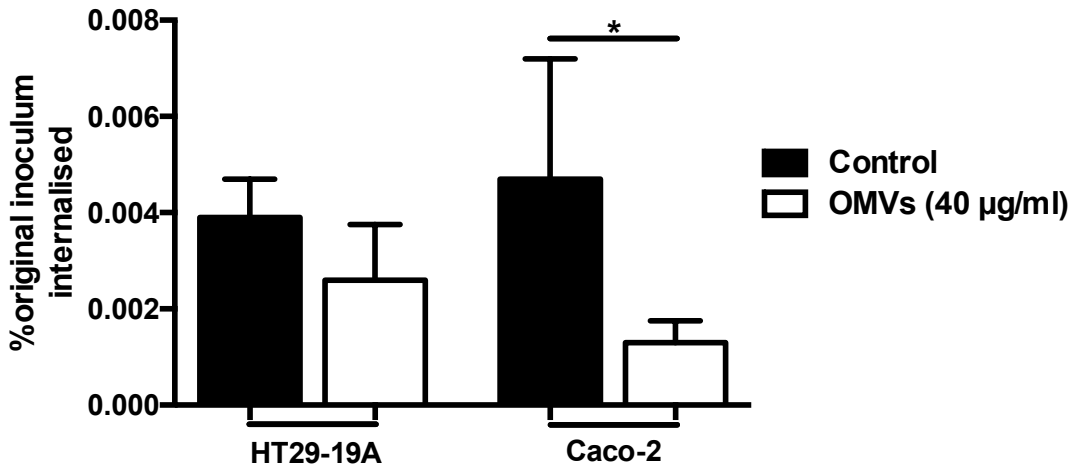
Figure 4


(a) HT29-19A
Control
Cell-free supernatant (1:10)
OMVs (40 µg/ml)
Fold change in mRNA expression
20
15
10
5
0
*
*
*
*
**
**
TLR1
TLR2
TLR4
TLR5
TLR9
(b) Caco-2
Control
Cell-free supernatant (1:10)
OMVs (40 µg/ml)
Fold change in mRNA expression
25
20
15
10
5
0
*
*
*
TLR1
TLR2
TLR4
TLR5
TLR9

690 **Figure 5**



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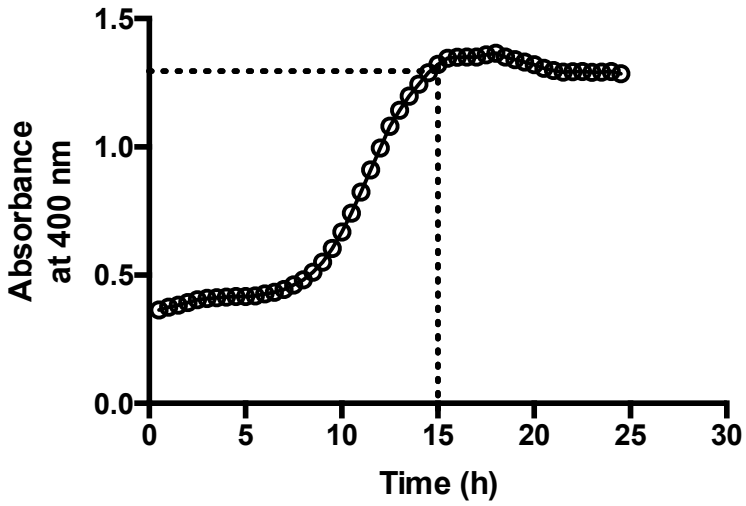
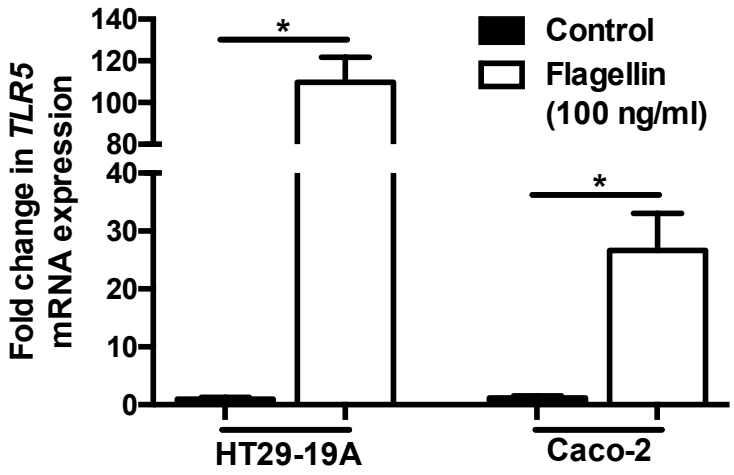
Supplementary Figure 1



Supplementary Figure 1 – *E. coli* C25 growth curve. *E. coli* C25 was cultured in TSB broth for 24 h, with the absorbance at 400 nm measured every 30 min. The 15 h culture time utilised in the experiments in this study is indicated by the dotted line.

Supplementary Figure 2



Supplementary Figure 2 – Flagellin up-regulates TLR5 mRNA expression in IECs. **H**T29-19A and Caco-2 cells were challenged with 100 ng/ml final concentration of flagellin for 24 h and TLR5 mRNA expression was measured. Results are **median ± IQR**, *n* = 4-6. *** indicates significance from the control, where $p \leq 0.05$.**