The WxxxE effector EspT triggers expression of immune mediators in an Erk/JNK and NF-κB-dependent manner

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Summary

Enteropathogenic *Escherichia coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) and *Citrobacter rodentium* colonize their respective hosts while forming attaching and effacing lesions. Their infection strategy relies on translocation of a battery of type III secretion system effectors, including Map, EspM and EspT, which belong to the WxxxE/SopE family of guanine nucleotide exchange factors. Using the *C. rodentium* mouse model we found that EspT triggers expression of *KC* and *TNFα in vivo*. Indeed, a growing body of evidence suggests that, in addition to subversion of actin dynamics, the SopE and the WxxxE effectors activate signalling pathways involved in immune responses. In this study we found that EspT induces expression of the pro-inflammatory mediators *cyclooxygenase-2* (COX-2) an enzyme involved in production of prostaglandin E(2) (PGE2), interleukin (*Il*)-*8* and *Il-1β* in U937 human macrophages by activating the nuclear factor kappa-B (NF-κB), the extracellular signal-regulated kinases 1 and 2 (Erk1/2) and c-Jun N-terminal kinase (JNK) pathways. Since EspT modulates the activation of Cdc42 and Rac1, which mediates bacterial invasion into epithelial cells, we investigated the involvement of these Rho GTPases and bacterial invasion on pro-inflammatory responses and found that (i) Rac1, but not Cdc42, is involved in EspT-induced Il-8 and Il-1β secretion and (ii) cytochalasin D inhibits EspT-induced EPEC invasion into U937 but not Il-8 or Il-1β secretion. These results suggest that while EPEC translocates a number of effectors (i.e. NleC, NleD, NleE, NleH) that inhibit inflammation, a subset of strains, which encode EspT, employ an infection strategy that also involves upregulation of immune mediators.

Introduction

The human pathogens enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) and the mouse pathogen *Citrobacter rodentium* colonize the gut epithelium via attaching and effacing (A/E) lesions, which are characterized by localized effacement of the intestinal brush border microvilli, the rearrangement of host cytoskeletal proteins beneath the intimately attached bacteria and alteration of tight junctions (Spitz *et al.*, 1995), which together contribute to the diarrhoeal disease. Similarly to other Gram-negative pathogens, EPEC, EHEC and *C. rodentium* encode a type III secretion system (T3SS), which is central to their infection strategy (Frankel et al., 1998). The T3SS translocates effector proteins

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directly from the bacteria to the eukaryotic cell cytoplasm, which target various cell-signalling pathways and subvert host cell responses (Wong et al., 2011).

The mucosal inflammation represents one of the main host defences against invading pathogens. Mucosal inflammation in the intestine is characterized by a distinct intestinal epithelial cell (IEC) response following the recognition of invading pathogens and subsequent infiltration of the mucosa with lymphocytes, in particular professional phagocytic cells including macrophages and neutrophils. Collectively, inflammation causes the upregulation of inflammatory genes, due to the activation of a number of transcription factors in both IECs and infiltrating leucocytes. Recently EPEC have been shown to use multiple strategies to downregulate IEC's inflammation. T3SS effectors NleB, NleE, NleH, NleC and NleD attenuate pro-inflammatory neutrophilic chemokine CXCL-8 [also known as interleukin-8 (Il-8)] expression by blocking NF-κB or c-Jun N-terminal kinase (JNK) pathways (Gao et al., 2009; Nadler et al., 2010; Newton et al., 2010; Vossenkamper et al., 2010; Yen et al., 2010; Baruch et al., 2011; Pearson et al., 2011; Wan et al., 2011). Despite the immunosuppressive activity of multiple bacterial effectors, it is established that *C. rodentium* induces inflammatory responses *in vivo* characterized by an inflammatory cell infiltrate in the colon lamina propria and hyperplasia of the colonic crypts (Eckmann, 2006). This suggests that the immunosuppressive effectors may only partially block innate responses *in vivo*, and that host mechanisms (i.e. Toll-like receptors – TLRs – or Nod-like receptors – NLRs – that recognize pathogen-associated molecular patterns – PAMPs) offset their effect or that other translocated effectors can moderate and counterbalance the effect of the anti-inflammatory effectors.

Based on a conserved Trp–xxx–Glu motif, the T3SS effector proteins IpgB1 and IpgB2 of *Shigella*, SifA and SifB of *Salmonella*, and Map, EspM and EspT of EPEC and EHEC were grouped together into the WxxxE effectors family (Alto et al., 2006). Most of the WxxxE effectors, similarly to the *Salmonella* effector SopE, subvert actin dynamic by acting as guanine nucleotide exchange factors (GEF) of Rho GTPases (Bulgin et al., 2010). SopE, IpgB1 and EspT trigger membrane ruffles (Hardt et al., 1998; Ohya et al., 2005; Bulgin et al., 2009b), IpgB2 and EspM trigger stress fibres (Arbeloa et al., 2008) and Map trigger filopodia (Kenny et al., 2002) via activation of Rac1, RhoA and Cdc42 respectively. Despite the fact that WxxxE effectors and SopE are mainly involved in subversion of actin dynamics, a growing body of evidence suggests that these effectors are also implicated in activation of pathways involved in immune response. Indeed, by activating mitogen-activated protein kinases (MAPKs) and/or NF-κB pathway, SopE (Hobbie et al., 1997; Bruno et al., 2009; Muller et al., 2009), IpgB1 and IpgB2 (Fukazawa et al., 2008) induce innate immune responses.

To date, the involvement of EPEC effectors in the upregulation of the innate immune response has not been studied. To investigate the role of the WxxxE effectors Map, EspM2 and EspT on the inflammatory responses we used a human macrophage model of infection (U937), as this cell type is one of the first to respond to bacterial pathogens during infection. Here we report that EspT, which is implicated in EPEC cell invasion, also plays an important role in production of the immune mediators (Il-1β, Il-8 and PGE2) through mechanisms involving extracellular signal-regulated kinases (Erk), JNK and NF-κB.

Results

EspT induces expression of COX-2, Il-8 and Il-1β in U937 human macrophages

As the *Shigella* WxxxE effector IpgB1 triggers expression of Il-8 (Fukazawa et al., 2008) we investigated the impact of the IpgB1 EPEC homologue EspT and the other EPEC WxxxE effectors, Map and EspM2, on innate immune responses in macrophages. U937 macrophages were infected with either wild-type EPEC strain E2348/69 or E2348/69 expressing Map, EspM2 or EspT for 3 h then washed and incubated for a further 16 h in fresh media containing gentamicin to kill adherent bacteria. Compared with wild-type E2348/69, E2348/69 expressing EspT increased the level of *cyclooxygenase-2* (*COX-2*) (Fig. 1A), *Il-8* (Fig. 1B) and *Il-1β* (Fig. 1C) mRNA expression and in parallel the level of secreted prostaglandin E (2) (PGE2) (Fig. 1D), Il-8 (Fig. 1E) and Il-1β (Fig. 1F), while the transcript level of GAPDH control did not change (Fig. S1). In contrast expression of EspM2 or Map had no effect on gene expression or protein secretion of the proinflammatory mediators.

EspT induces Il-8 and Il-1β expressions through activation of NF-κB and Erk/JNK pathways

As NF-κB is a key transcription factor of inflammatory gene expression including *COX-2* and *Il-1β* (Hoffmann et al., 2002), we examined its involvement in EspT-induced inflammatory responses in macrophages. U937 cells, infected either with E2348/69 expressing EspT or with wild-type E2348/69 as a control, were stained using antibodies directed against the NF-κB p65 subunit and Dapi (to stain nucleic acids). This has shown that in contrast to infection with wild-type E2348/69, E2348/69 expressing EspT induced targeting of p65 to the nucleus (Fig. 2A). Quantification of U937-infected cells revealed that $5 \pm 4.7\%$ and $29.3 \pm 11.6\%$ of adherent E2348/69 and E2348/69 expressing EspT respectively were associated with translocated p65 into the nucleus (Fig. 2B). Consistently, pre-incubation of cells with MG132, a proteasome inhibitor known to inhibit NF-κB activation by blocking the degradation of IκB, significantly reduced EspT-induced Il-1β (Fig. 2C) and Il-8 (Fig. 2D) secretion.

Since the MAPK cascade modulates innate immune responses (Hoffmann et al., 2002) we analysed the effect of EspT on MAPK activation. Using the p38, JNK and Erk inhibitors SB202190, SP600125 and PD98059, respectively, revealed that EspT induced Il-8 (Fig. 3A) and Il-1β (Fig. 3B) protein secretion via the Erk and JNK pathways but independently of p38 since only SP600125 and PD98059 significantly reduced the level of EspT-mediated Il-8 secretion. Western blotting has shown that EspT induced phosphorylation/activation of Erk (Fig. 3C) and JNK (Fig. 3D), but not of p38 (Fig. S2).

As the PI3K/AKT pathway is also involved in immune response (Arbibe et al., 2000), we analysed the effect of PI3K inhibitors LY294002 and Wortmannin on EspT-mediated Il-8 secretion. This revealed that infection of U937 cells with E2348/69 expressing EspT in the presence or absence of either LY294002 or Wortmannin resulted in secretion of Il-8 at comparable levels (Fig. 3E).

EspT induces inflammatory response independently of bacterial uptake

While various EPEC effectors are able to block phagocytosis of non-opsonized bacteria by macrophages (e.g. EspF and EspH) (Marches *et al.*, 2008; Dong *et al.*, 2010), we have recently reported that EspT can mediate invasion of EPEC into non-phagocytic cell (Bulgin *et al.*, 2009a). Thus, we analysed whether EPEC expressing EspT can invade macrophages and whether bacterial uptake is required for induction of the inflammatory mediators. We observed by immunostaining that E2348/69 expressing EspT invaded U937 better (29.6 ± 6.7%) than E2348/69 alone (11.8 ± 4.4%) (Fig. 4A and B), a process that was associated with actin remodelling and cell spreading as observed by immunostaining and scanning electron microscopy (Fig. 4C). As expected, we found that cytochalasin D, a well-characterized inhibitor of actin polymerization and hence bacterial invasion/phagocytosis, blocked EPEC internalization and macrophage cell spreading (data not shown). However cytochalasin D did not prevent EspT-induced Il-8 (Fig. 4D) and Il-1β (Fig. 4E) secretions, indicating that EspT translocation, rather than microfilament reorganization or bacterial internalization, is involved in the observed inflammatory response.

EspT-mediated Il-8 production is regulated via RAC1

Subversion of actin dynamics by Map, IpgB1, IpgB2, EspM2, EspM3 and EspT is dependent on their invariant structural WxxxE motif (Alto *et al.*, 2006). More recently, the active site of the WxxxE effectors has been described (Arbeloa *et al.*, 2010). Using sequence alignment we identified the putative active site of EspT (Fig. 5A). In order to determine whether the WxxxE motif or the putative active site are required for EspT-induced pro-inflammatory mediator release, residue W63 from the WxxxE site and the Q117 from the putative active site were substituted by A and Y respectively (Fig. 5A), mutations that were already shown to drastically reduce the activity of WxxxE effectors (Huang *et al.*, 2009; Arbeloa *et al.*, 2010). U937 cells were then infected with E2348/69 expressing EspT$_{WT}$, EspT$_{W63A}$ or EspT$_{Q117Y}$; infection with wild-type E2348/69 was used as a control. We found that EPEC expressing EspT$_{W63A}$ and EspT$_{Q117Y}$ triggered significantly lower level of Il-8 secretion compared with E2348/69 expressing EspT$_{WT}$ (Fig. 5B). Since EspT was shown to specifically activate Rac1 and Cdc42, we investigated their involvement in EspT-induced secretion of pro-inflammatory mediators. We found that EspT-mediated Il-8 production in U937 was significantly reduced following depletion of Rac1 (Fig. 5D), but not Cdc42, by siRNA (Fig. 5D). We used a pharmacological approach to confirm that Rac1 is required for EspT-mediated Il-8 production. Towards this end U937 cells were treated with NSC23766, a Rac1-specific inhibitor interfering with its interaction with Rac-specific GEFs. This has shown that NSC23766 prevented EspT-induced Il-8 secretion (Fig. 5E). Thus, the observed inflammatory activation in macrophages by bacteria expressing EspT is independent of cellular internalization but dependent on activation of Rac1.

EspT induces expression of KC and TNFα *in vivo*

As *C. rodentium* induces mucosal inflammation in mice (Hirata *et al.*, 2010), we determined the relevance of the *in vitro* EspT function on induction of immune mediator *in vivo*. Towards this end we infected C57/BL-6 mice with wild-type *C. rodentium* and an isogenic *espT* mutant. Wild-type and the mutant *C. rodentium* strains colonized the mouse intestine at

comparable levels (Fig. 6A). Importantly, while we did not detect expression of the proinflammatory cytokine Il-1β following infection with either strain, the *espT* mutant triggered significantly reduced levels of *KC*, the murine equivalent of Il-8 (Fig. 6B) and *TNFα* (Fig. 6C) mRNA compared with infection with the parental, wild-type strain.

Discussion

Enteropathogenic *E. coli*, EHEC and *C. rodentium* utilize their T3SS to inject effector proteins into the mammalian cell in order to manipulate cell-signalling pathways and subvert host cell responses. The WxxxE effector EspT has recently been shown to trigger bacterial invasion into epithelial cell (Bulgin *et al.*, 2009a). Here we demonstrated for the first time that EspT mediates internalization of EPEC into macrophages, suggesting that it overrides the anti-phagocytic activity of EspF (Marches *et al.*, 2008) and EspH (Dong et al., 2010).

Toll-like receptors and NLRs are the key host receptors involved in the recognition of microbial PAMPs (e.g. TLR4 and TLR5 which recognize LPS and flagelin respectively) leading to production of inflammatory mediators through various adaptors (e.g. MyD88, IRAK, TRAF6 or RIP2) (Medzhitov, 2001). However, a number of studies have recently shown that bacterial effectors involved in cell invasion are also able to induce inflammatory response. For example, Fukazawa *et al.* (2008) showed that induction of NF-κB activation and initiation of innate immune responses by the *Shigella* WxxxE effector IpgB2 is dependent on activation of RhoA, RIP2 and ROCK pathways (Fukazawa *et al.*, 2008). Invasion of epithelial cells by *Salmonella* stimulates innate immune responses by mechanisms that do not involve TLRs or NLRs but rather the bacterial effector proteins SopE, SopE2 and SopB (Hobbie *et al.*, 1997; Bruno *et al.*, 2009). *Yersinia* invasion into epithelial cells activates the NF-κB pathway and Il-8 expression in a mechanism involving invasin-mediated activation of Rac1 (Grassl *et al.*, 2003). In this study we identified EspT as an effector of A/E pathogens that can trigger inflammatory response *in vivo*. Epithelial cells, macrophages and dendritic cells represent the first sentinel cells implicated in the recognition of invading pathogens leading to the induction of inflammatory response. Since we did not record any significant EspT-induced cytokine production using epithelial cells (data not shown), we investigated its impact on macrophages. We found that EspT, but not the EPEC WxxxE effectors Map and EspM2, induces production of Il-8, Il-1β and PGE2 by U937 macrophages. Il-8 and Il-1β are important mediators of inflammatory responses. PGE2, which is one of the most abundant metabolites of arachidonic acid generated through an enzymatic cascade controlled by COX-1 and COX-2, also plays a role in inflammatory response by increasing vascular permeability, fever generation and hyperalgesia (Trebino *et al.*, 2003) *Salmonella* has already been shown to induce *COX-2* expression in macrophages (Uchiya *et al.*, 2004; Cristina Cerquetti *et al.*, 2008). Importantly, in our study, induction of the proinflammatory responses by EspT occurred independently of bacterial invasion. Mutagenesis of the putative EspT active loop residue Q117 (Huang *et al.*, 2009) and the structural residue W63 (Alto *et al.*, 2006; Arbeloa *et al.*, 2010) severely attenuated the ability of EspT to trigger production of Il-8 suggesting that activation of Rho GTPase by EspT is involved in Il-8 production. Moreover, we found using siRNA and specific inhibitors that Rac1, but not Cdc42, is involved in EspT-induced Il-8 production. Rac1 regulates cell signalling by a variety of downstream effectors including p21-activated kinases (PAKs)

(Naumann *et al.*, 1998) and Phosphatidylinositol 3-kinases (PI3K) (Arbibe *et al.*, 2000), which are required for MAPKs and NF-κB activation. By using the PAK inhibitor IPA3 and the PI3K inhibitors LY294002 and Wortmannin we found that these pathways are not involved in EspT-induced Il-8 production in the U937 cells. In contrast, we found that EspT-induced Il-8 and Il-1β secretion by U937 cells is dependent on NF-κB signalling and involves MAPK, Erk and JNK (Fig. 7). The JNK and Erk inhibition did not affect NF-κB nuclear translocation (data not shown). In addition, the global caspase inhibitor z-VAD-FMK, known to block caspases including caspase-1 which process pro-Il-1β into the mature secreted form Il-1β, did not affect EspT-induced secretion of Il-1β (data not shown).

Most of the recent studies of EPEC effectors interfering with inflammatory response have been performed using epithelial cells. When testing the function of EspT in HeLa, Caco-2 and Beas-2B cell we did not observe any significant EspT-induced Il-8 production. However, we found that EspT also triggered Il-8 production in human THP-1 macrophages. These results suggest that while the function of EspT in the context of epithelial cells is more likely limited to cell invasion, it triggers internalization, cell spreading and expression of pro-inflammatory responses in macrophages.

Many EPEC effectors have been shown to inhibit immune responses. Ruchaud-Sparagano *et al.* (2007) have shown that wild-type EPEC suppresses the ability of Caco-2 cells to activate cell signalling leading to secretion of Il-8 in a T3SS-dependent manner (Ruchaud-Sparagano *et al.*, 2007). More recently, NleB, NleE, NleH, NleC and NleD have been shown to inhibit Il-8 production in epithelial cells by targeting different stages of the inflammatory response (Gao *et al.*, 2009; Nadler *et al.*, 2010; Newton *et al.*, 2010; Vossenkamper *et al.*, 2010; Yen *et al.*, 2010; Baruch *et al.*, 2011; Pearson *et al.*, 2011; Wan *et al.*, 2011). Our finding that EspT activates immune responses is therefore intriguing, although not surprising considering that translocation of effectors of antagonistic functions is becoming a common theme in bacterial pathogenesis. Indeed, *Salmonella* (Collier-Hyams *et al.*, 2002; Le Negrate *et al.*, 2008) and *Shigella* (Arbibe *et al.*, 2007; Ashida *et al.*, 2010) secrete multiple T3SS effectors that interfere with inflammatory signalling and WxxxE/SopE effectors that induce pro-inflammatory responses (Fukazawa *et al.*, 2008; Bruno *et al.*, 2009). Our data show that overexpression of EspT overrides both the antiphagocytic activity exhibited by EspF and EspH and the immunosuppressive action of NleB, NleE, NleH, NleC and NleD.

Although we have shown that EspT is involved in induction of inflammatory responses during *C. rodentium* infection, *espT* is not found in EHEC and is only rarely found in human EPEC strains (Arbeloa *et al.*, 2009). Importantly, the *espT* positive EPEC strain E110019 was responsible for a severe diarrhoeal outbreak in Finland in 1987, which uniquely affected children and adults alike (Viljanen *et al.*, 1990). As EspT can affect the balance between pro and anti-inflammatory responses we suspect this may contribute, at least in part, to virulence. Indeed, our *in vivo* study has shown that although *C. rodentium* expresses all the known immunosuppressive effectors it induces a mild inflammatory response *in vivo* in a mechanism involving EspT. These results add to the growing recognition that bacterial pathogens use T3SS effectors to optimize their colonization niche by affecting the resident microbiota (Stecher *et al.*, 2007; Sekirov *et al.*, 2010) via mechanisms involving redundant, synergistic or antagonistic activities.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli strains and plasmids used in this study are listed in Table S1 (Levine *et al.*, 1978; Schlosser-Silverman *et al.*, 2000; Wiles *et al.*, 2004; Simpson *et al.*, 2006). Bacteria were cultured in Luria–Bertani (LB) medium or in Dulbecco's modified Eagle's medium (DMEM) supplemented with ampicillin (100 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) and nalidixic acid (50 μg ml^{-1}) as required.

Cell culture and infection

U937 human promyeloid cells were obtained from American Type Culture Collection (Rockville, MD). The cells were grown in RPMI 1640 medium (Invitrogen, Green Island, NY) supplemented with 10% heat-inactivated FBS (Invitrogen), 2 mM L-glutamine at 37°C in a 5% CO_2 humidified atmosphere as described. U937 cells were differentiated 24-well plates into macrophage-like cells by incubation with 15 nM PMA (Calbiochem, San Diego, CA) for 48 h. One hour before infection, the cells were washed in phosphate buffer (PBS), and incubated with 500 μl of fresh DMEM without FBS and supplemented or not with pharmacological inhibitors as required. After 1 h 500 μl of the primed and IPTG (1 μM final)-induced bacteria were added to each well and the infection assays were carried out at 37°C in 5% CO_2. After 1–3 h, the cells were then washed three times in PBS and processed or incubated for 16 h with fresh DMEM without FBS supplement with 200 μg ml^{-1} gentamicin.

Site-directed mutagenesis

Site-directed mutagenesis was carried out using a Quickchange II kit (Stratagene) according to the manufacturer's instructions. Primers listed in Table S1 were designed using the Quickchange mutagenic primer design program (Stratagene). Plasmids pSA10 containing *espT* was used as templates for the mutagenic reactions. Colonies were screened by sequencing and alignment to wild-type sequences to confirm mutagenesis.

Scanning electron microscopy

Glass coverslips were seeded with cells and infected for 2 h with the appropriate strains. The cells were washed three times in PBS pH 7.2 and then fixed with 2.5% gluteraldehyde (Sigma) in phosphate buffer pH 7.2 for 15 min. The coverslips were then washed in phosphate buffer pH 7.2 three times before being post-fixed in 1% Osmium Tetroxide for 1 h. The cells were then washed three times in phosphate buffer before being washed for 15 min in graded ethanol solutions from 50% to 100% to dehydrate the samples. The cells were then transferred to an Emitech K850 Critical Point drier and processed according to the manufacturer's instructions. The coverslips were coated in gold/palladium mix using an Emitech Sc7620 minisputter to a thickness of approximately 370A°. Samples for scanning electron microscopy (SEM) were then examined blindly at an accelerating voltage of 25 kV using a Jeol JSM-6390.

Immunofluorescence staining and microscopy

Glass coverslips were seeded with cells and infected for 2 h with the appropriate strains. The cells were washed three times in PBS and then fixed with 3% paraformaldehyde (PFA) for 15 min before washing three more times in PBS. After quenching for 10 min with 50 mM NH4Cl and permeabilized for 4 min in PBS 0.2% Triton X-100, cells were then washed three times in PBS and blocked for 1 h with PBS 5% BSA before incubation with primary and secondary antibodies. The primary antibody rabbit anti-NF-κB p65 subunit (SantaCruz Biotechnology) was used at a dilution of 1:100, while rabbit anti-O127 was used at a dilution of 1:500. Coverslips were incubated with the primary antibody for 1 h, washed three times in PBS and incubated with the secondary antibodies. Donkey anti-rabbit IgG conjugated to a Cy2 or Cy3 fluorophore (Jackson laboratories) were used at a 1:200. Actin was stained using AlexaFluor 647 phalloidin or Oregon Green phalloidin (Invitrogen) at a 1:100 dilution. All dilutions were in PBS 5% BSA. Coverslips were mounted on slides using Pro-LongH Gold antifade reagent (Invitrogen) and visualized by Zeiss Axioimager immunofluorescence microscope using the following excitation wavelengths: Cy3 – 550 nm, Cy5 – 650 nm and Oregon Green – 488 nm. All images were analysed using the Axiovision Rel 4.5 software.

RNA extraction and real-time reverse-transcription PCR analysis

After 3 h infection, the cells were then washed three times in PBS and incubated 16 h with fresh DMEM without FBS supplement with 200 μg ml^{-1} gentamicin. Regarding the *in vivo* assay, at day 12 post inoculum, the mice were culled and the colonic tissues were collected and preserved in RNAlater RNA Stabilization Reagent (Qiagen). Then, total RNA was extracted using an RNeasy kit from Qiagen. DNase treatment was performed using 2 μg of extracted RNA, 1 μl of DNase I (Amersham Biosciences) and 0.5 μl of RNasin (Promega) in a total volume of 20 μl in the manufacturer's buffer. cDNA were obtained by incubating RNA with 1 mM dNTP (Promega), 1.5 μl of hexamers as primers, 20 units of RNasin (Promega), and 300 units of Moloney murine leukaemia virus reverse transcriptase RNase H minus (Promega) in a total volume of 50 μl of the manufacturer's buffer for 1 h at 42°C and 10 min at 70°C. Real-time PCR was performed using the SYBR Green kit (Stratagene Brilliant II) and analysed using the MxPro software (Stratagene). The primers listed in Table S1 were used.

Cytokines and PGE2 enzyme immunoassays

After 3 h infection, the cells were then washed three times in PBS and incubated 16 h with fresh DMEM without FBS supplement with 200 μg ml^{-1} gentamicin. Then the supernatants were collected and PGE2 concentration was measured using Enzyme Immunoassay (EIA) kit (Cayman chemical). Il-8, Il-1β concentrations were measured using DuoSet ELISA kits from R&D systems.

Protein extraction and Western blot analyses

After 1 h infection, proteins were extracted from U937 cells using RIPA supplement with anti-proteases (Roche) and anti-phosphatases (Roche). Five micrograms of total proteins were subjected to SDS-PAGE. Proteins were transferred to membrane from Millipore, which

were blocked with 5% BSA and probed overnight at 4°C with the indicated antibodies (Erk, phospho Erk, JNK, phospho JNK – cell signalling – and tubulin – Sigma). After washing with TBS tween, the immunoreactive bands were visualized with a specific peroxidase-conjugated anti-rabbit or anti-mousse IgG antibody (Jackson laboratories) and using an ECL Plus Western Blotting Detecting System (Amersham, Biosciences).

Mice

Pathogen-free 18–20 g of C57Bl/6 mice were purchased from Charles River. All animals were housed in individually HEPA-filtered cages with sterile bedding and free access to sterilized food and water. All animal experiments were performed in accordance with the Animals Scientific Procedures (Act 1986) and were approved by the local Ethical Review Committee. Independent single infection experiments were performed three times using three to six mice per group. Mice inoculated with wild-type strain and uninfected mice were included in parallel with mutant strains by oral gavage with 200 μl of overnight LB-grown *C. rodentium* suspension in PBS ($1 \cdot 10^{11}$cfu). The number of viable bacteria used as inoculum was determined by retrospective plating onto LB agar containing antibiotics. Stool samples were recovered aseptically at various time points after inoculation and the number of viable bacteria per gram of stool was determined by plating onto LB agar (Wiles et al., 2004). At day 12 post inoculum, the mice were culled and segments of the terminal colon of each mouse were collected for mRNA studies as described above.

Supporting information

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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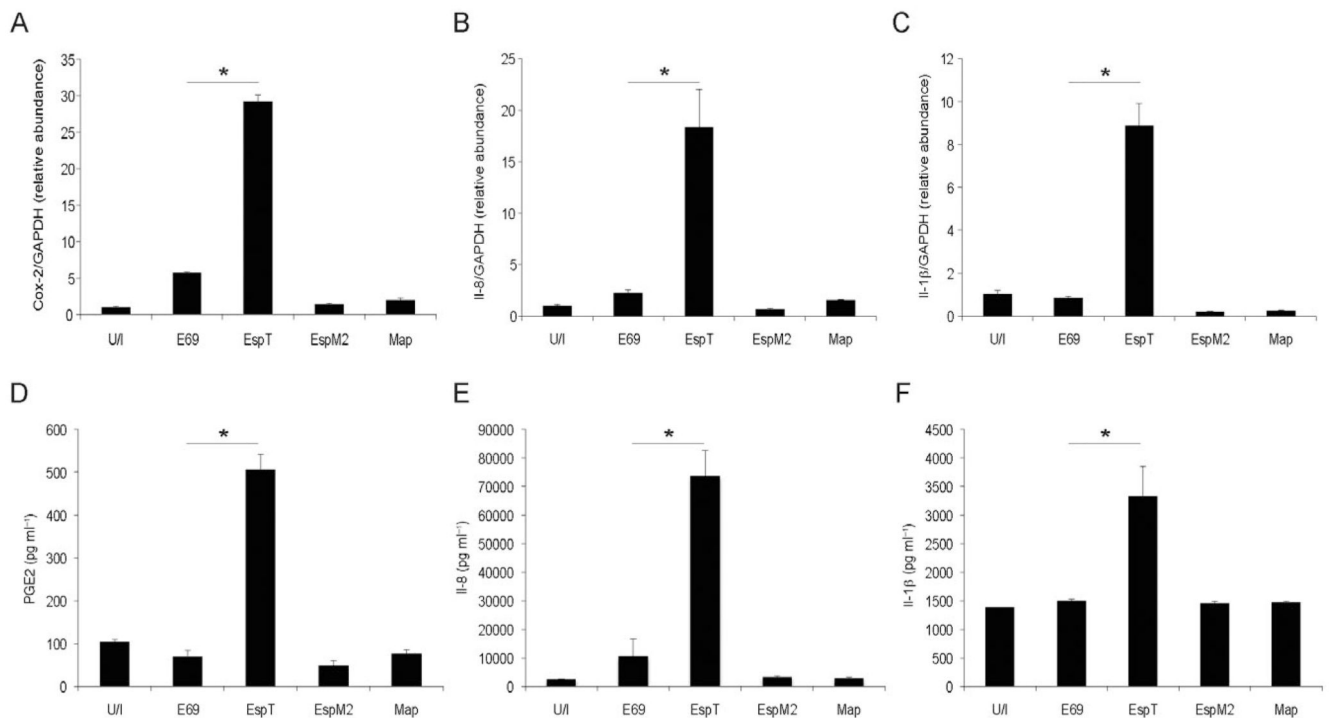
Fig. 1.

EspT upregulates production of pro-inflammatory mediators by macrophages. U937 cells were either left uninfected (U/I) or infected with EPEC E2348/69 (E69) or E2348/69 expressing EspT (EspT), EspM2 (EspM2) or Map (Map) for 3 h. Sixteen hours post infection, *COX-2* (A), *Il-8* (B) and *Il-1β* (C) mRNA expression and PGE2 (D), Il-8 (E) and Il-1β (F) secretion were determined. EPEC expressing EspT triggers expression and secretion of the analysed inflammatory mediators compare to wild-type EPEC, or EPEC expressing EspM2 or Map. Data are means + SD and are representative of at least three independent experiments; *P* value determined by Student's *T*-test (*P < 0.05) comparing E69 with all other strains.
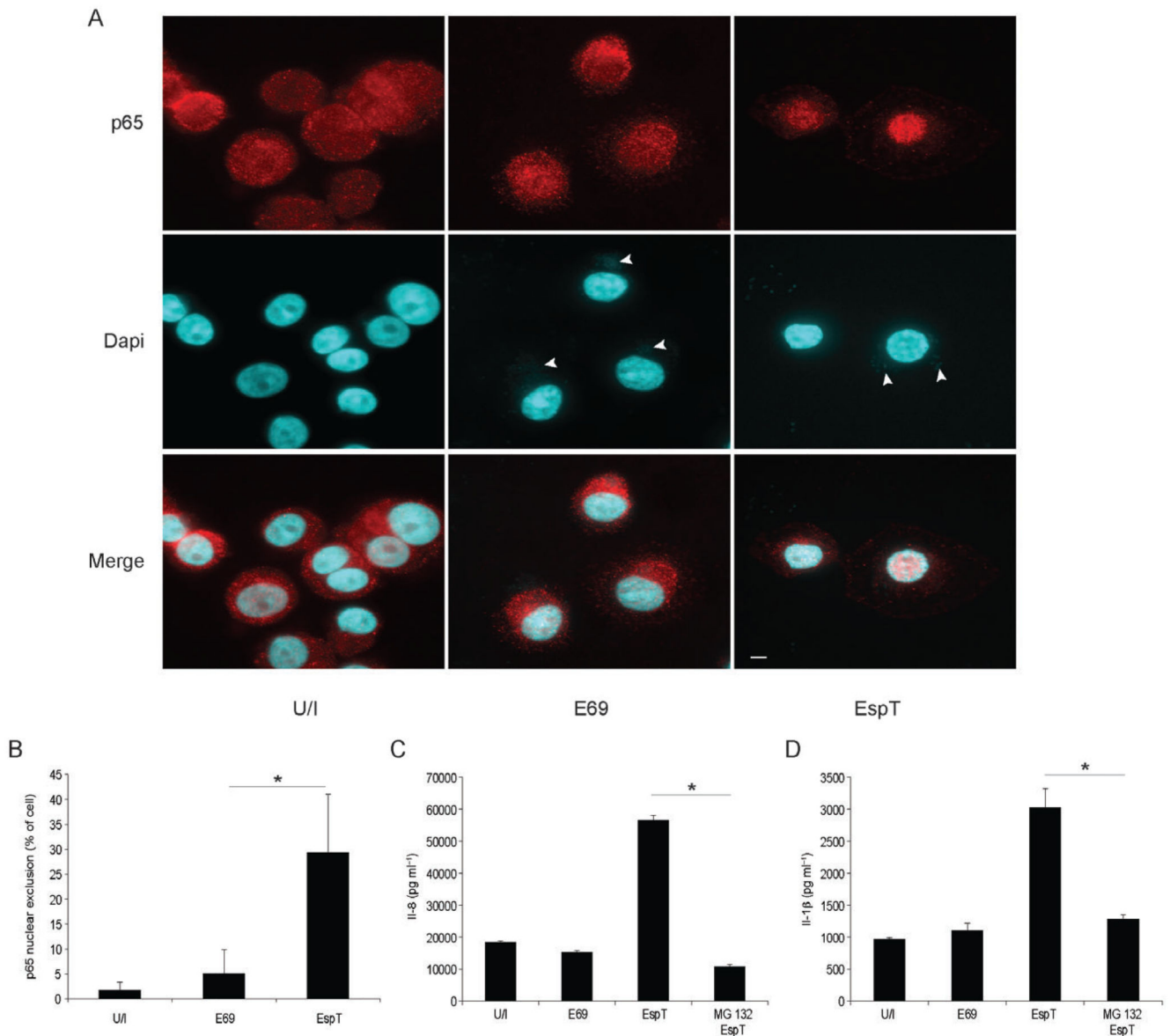
Fig. 2.

EspT induces Il-8 and Il-1β secretion via NF-κB activation. U937 cells were either left uninfected (U/I), or infected with E2348/69 (E69) or with E2348/69 expressing EspT (EspT). Two hours post infection p65 (red) was colocalized with Dapi (blue) in the nucleus specifically following infection with E2348/69 expressing EspT (A). Scale bar 5 μM. Quantification of infected cells with nuclear p65 staining (B). Pre-treatment of U937 cells with MG132 (MG132) 1 h before infection significantly reduces the level of EspT-mediated Il-8 (C) and Il-1β (D) secretion. Data are means + SD and are representative of at least two independent experiments; *P* value determined by Student's *T*-test (*$P < 0.05$) comparing EspT strain with MG132/EspT condition.
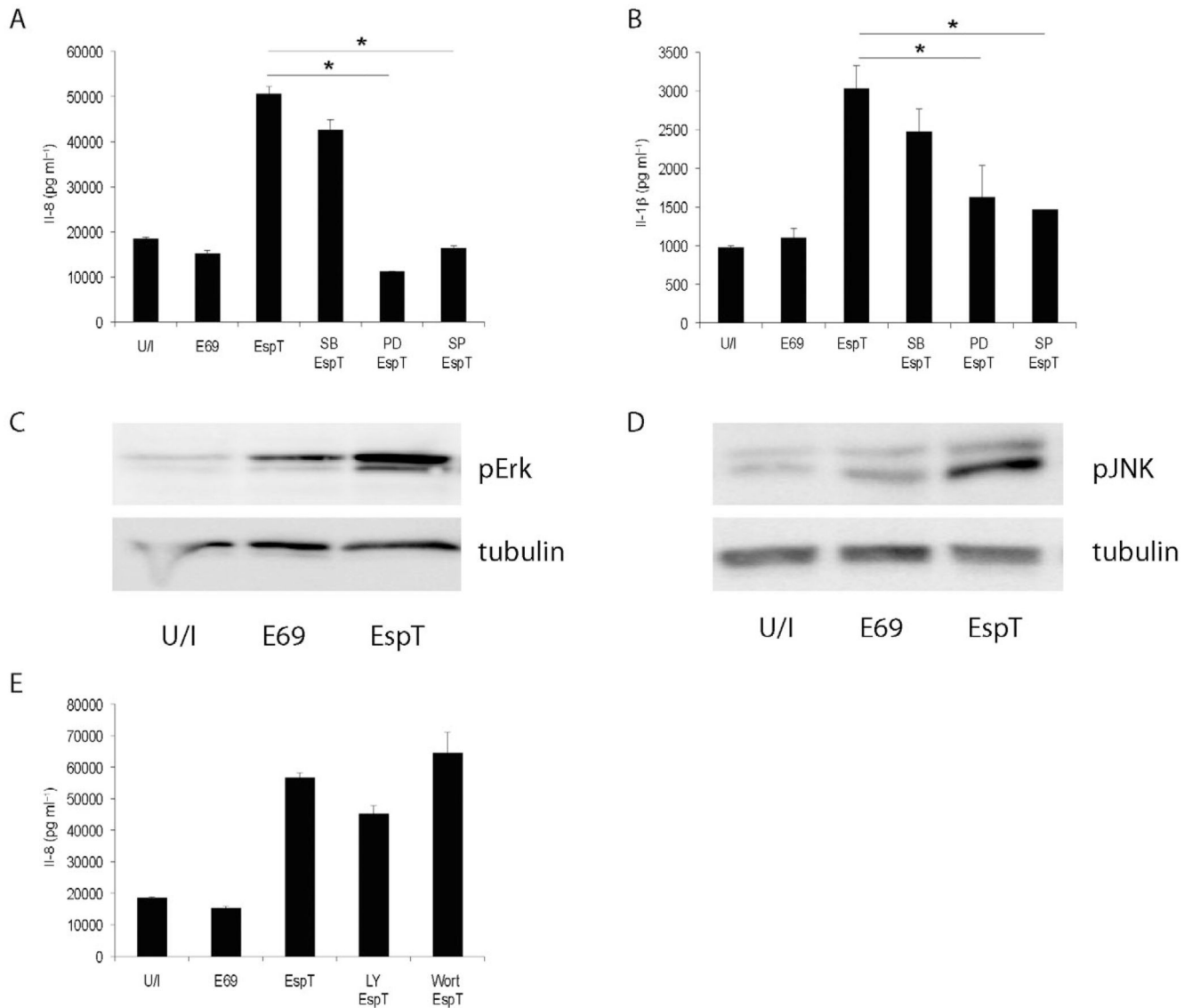
Fig. 3.
EspT induces Il-8 and Il-1β secretion via JNK and Erk activation. U937 cells were pre-treated with SB203580 (SB), PD98059 (PD) or SP600125 (SP) 1 h before infection. PD and SP significantly reduce the level of EspT-mediated Il-8 (A) and Il-1β (B) secretion. Western blot of whole-cell lysates probed with antibodies directed against phospho-Erk (pErk) (C) and phospho-JNK (pJNK) (D) and normalized using a-tubulin antibody (tubulin) show that EPEC expressing EspT triggers phosphorylation of both Erk and JNK MAPKs. Pre-treatment with Wortmannin (Wort) or LY294002 (LY) 1 h before infection shows no significant changes on the level of EspT-induced Il-8 secretion (E). Data are means + SD and are representative of at least three independent experiments; P value determined by Student's T-test (*$P < 0.05$) comparing EspT strain with all other conditions as indicated.
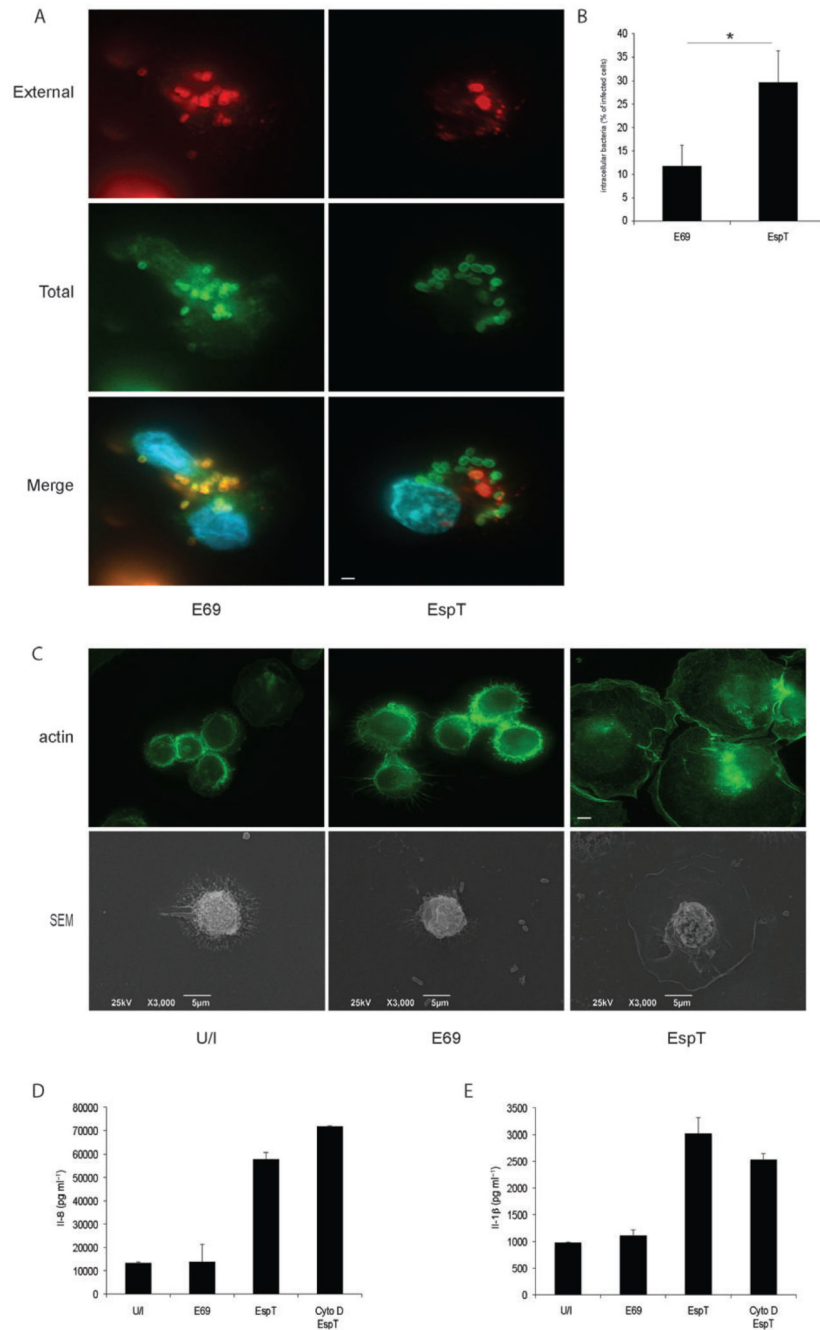
Fig. 4.
EspT induces Il-8 and Il-1β secretions independently of bacterial uptake.
A and B. U937 cells were either uninfected (U/I), or infected with the E2348/69 (E69) or
E2348/69 expressing EspT (EspT). After 3 h cells were washed and the number of
intracellular and extracellular bacteria, quantified by immunofluorescence using O127
antiserum (red – external, green – total) and Dapi (blue), revealed that EspT triggers EPEC
internalization into macrophages.

C. Immunofluorescence using AlexaFluor 488 phalloidin (green – upper section) and scanning electron microscopy (SEM – lower section) show that EspT triggers macrophage cell spreading. Scale bar 5 μm.

D and E. Pre-treatment of U937 with cytochalasin D (Cyto D) for 1 h before infection inhibited cell spreading but showed no significant reduction in EspT-mediated Il-8 (D) and Il-1β (E) secretions. Data are means + SD and are representative of at least three independent experiments.
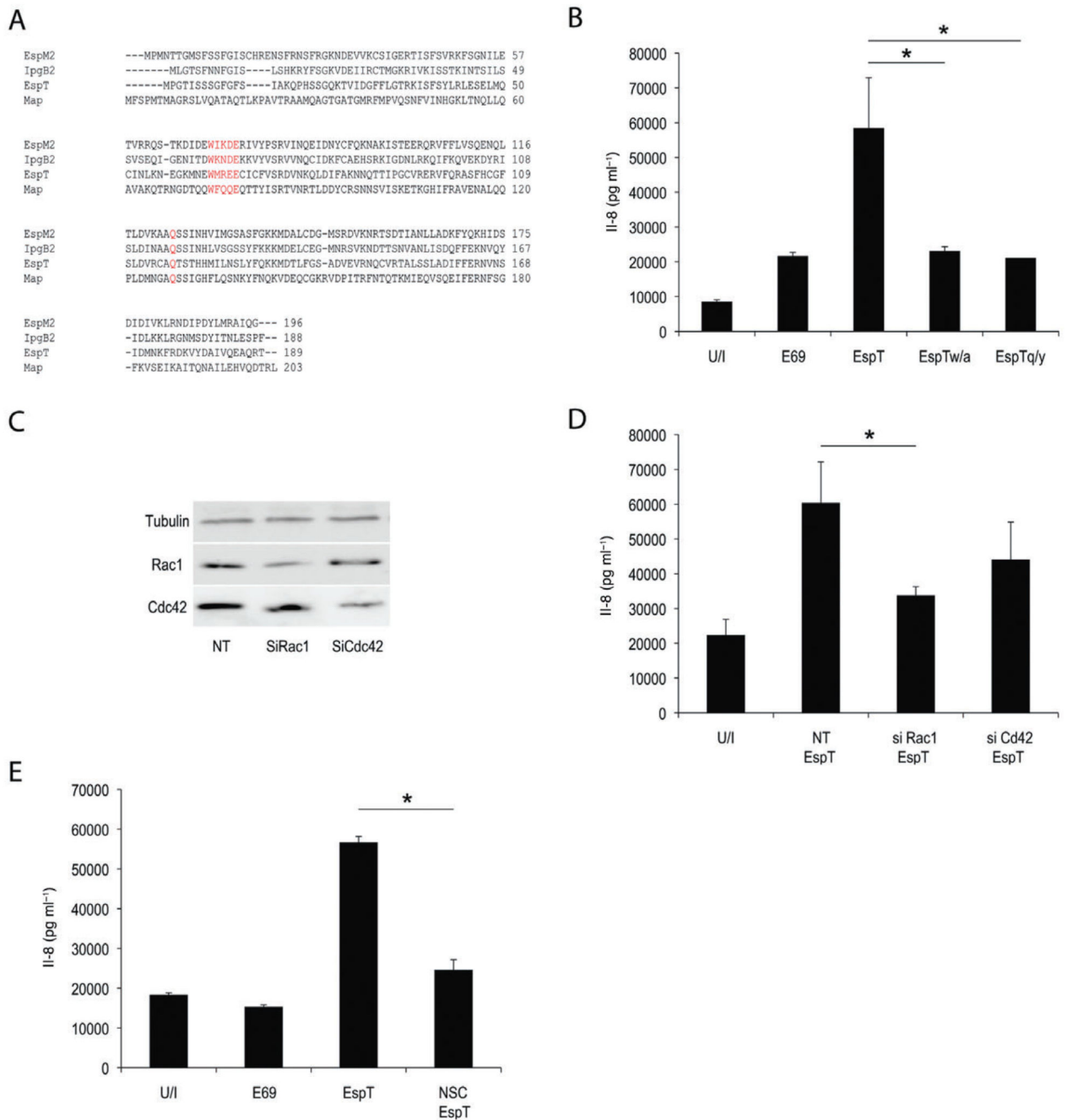
Fig. 5.

EspT-mediated Il-8 production is regulated via RAC1.

A. Alignment of IpgB1, Map EspM2 and EspT effector proteins; the point mutations at position W63 and Q117 are indicated in red.

B. U937 cells were infected with E2348/69 or E2348/69 expressing EspT$_{WT}$, EspT$_{W/A}$ or EspT$_{Q/Y}$. EspT$_{W/A}$ and EspT$_{Q/Y}$ mutants drastically reduced the level of EspT-mediated Il-8 secretion.

C and D. U937 cells were transfected with non-targeting (NT) siRNA or siRNA targeted against Rac1 (SiRac1) or Cdc42 (SiCdc42) for 48 h. Successful knock-down was confirmed by Western blotting (C). Transfected U937 cells were either uninfected (U/I) or infected with E2348/69 expressing EspT (EspT). siRac1 inhibits the level of EspT-mediated Il-8 secretion while siCdc42 showed no effect on the level of Il-8 secretion.

E. Pre-treatment with NSC23766 (NSC) for 1 h reduced the level of EspT-mediated Il-8 secretion. Data are means + SD and are representative of at least three independent experiments; P value determined by Student's T-test (*$P < 0.05$) comparing EspT strain with all other conditions as indicated.
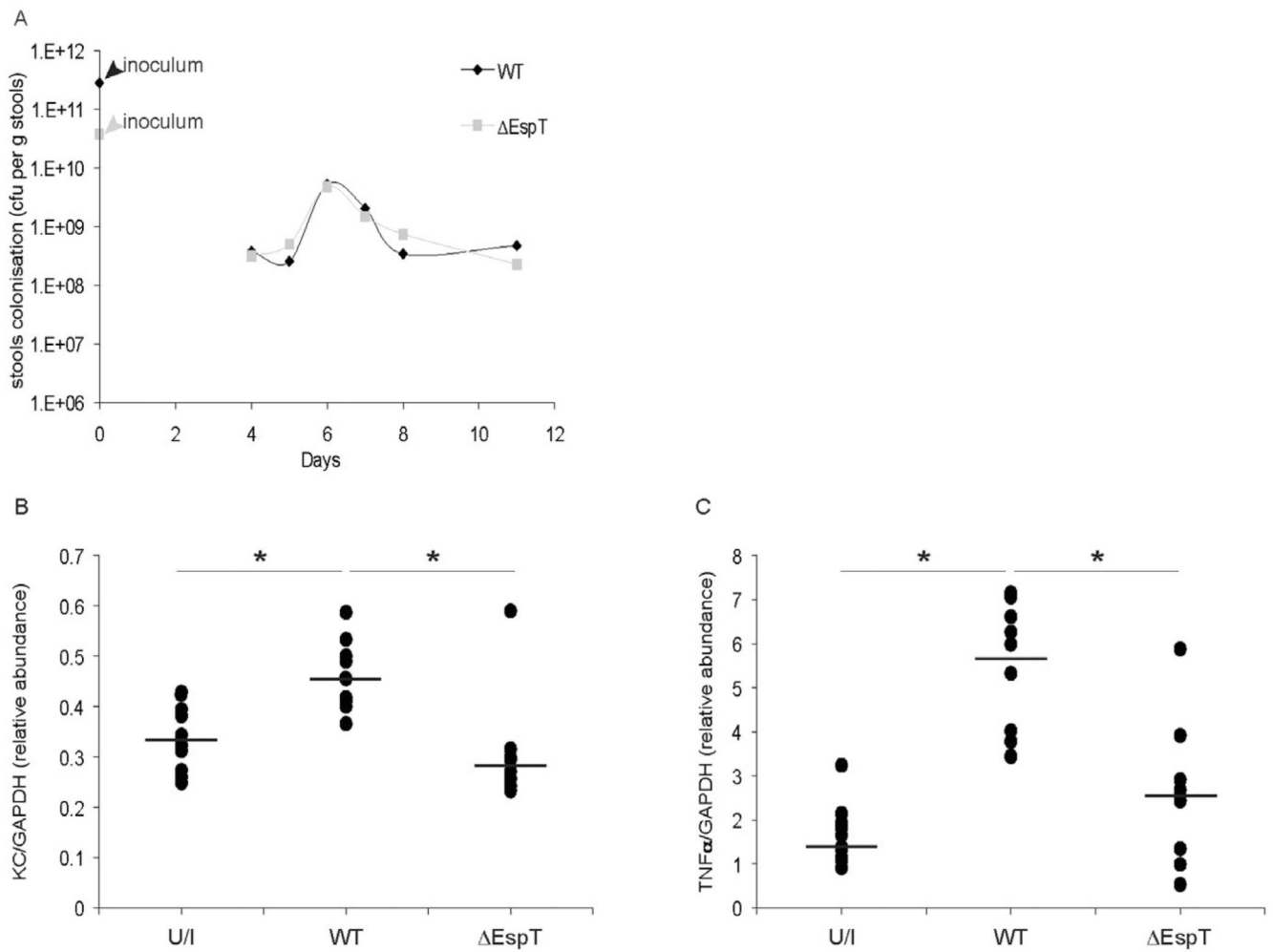
Fig. 6.
EspT induces mucosal expression of KC and TNFα. C57BI/6 mice were orally inoculated with PBS (PBS), wild-type *C. rodentium* (WT) or *C. rodentium espT* mutant (*espT*).
A. Colonization and clearance dynamics of *C. rodentium* strains shows the wild-type and mutant strains colonized at comparable level over a 12-day period.
B and C. At day 12, sections of colon were collected and *KC* (B) and *TNFα* (C) mRNA expressions were analysed and normalized with mRNA expression of *GAPDH*. Wild-type *C. rodentium* expressing EspT triggers significantly higher levels of *KC* and *TNFα* mRNA compared with *C. rodentium espT*. Data are means + SD and are representative of two independent experiments; *P* value determined by Mann–Whitney *U*-test (*$P < 0.05$) comparing wild-type with uninfected and *espT*.
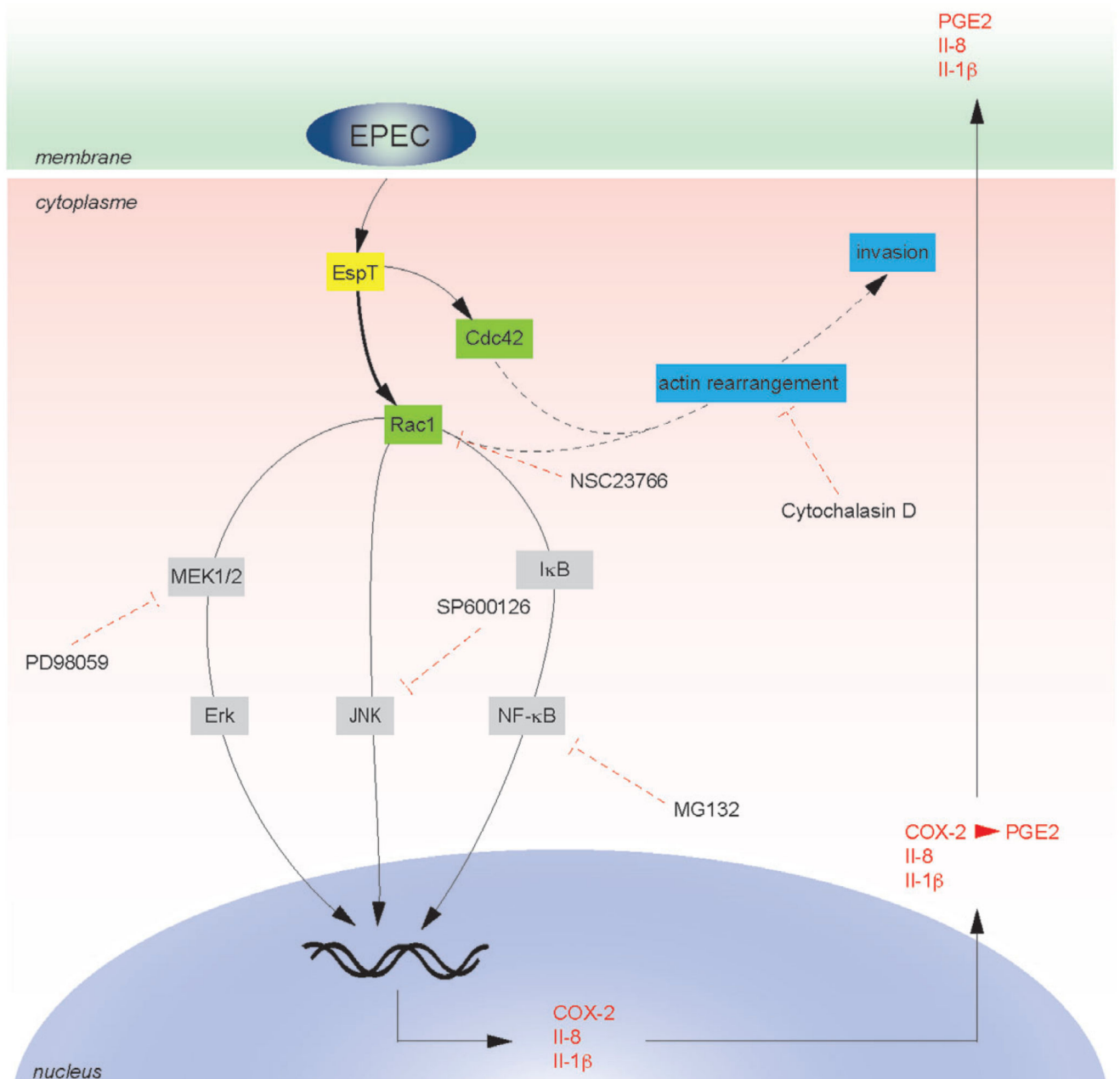
Fig. 7.
A model deciphering the signalling pathway hijacked by EspT leading to induction of inflammatory responses in macrophages. Translocated EspT activates Rac1 and Cdc42 leading to bacterial internalization in a microfilament (black dash lines)-dependent manner. Activation of Rac1 upregulates *COX-2, Il-8* and *Il-1β* genes expression and the secretions of PGE2 and Il-1β through Erk, JNK and NF-κB pathways. The dash red lines represent the targets of the specific inhibitors used in this study. EspT appears to antagonize the inhibitory impact of immunosuppressive effectors translocated by EPEC.