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EspL is a bacterial cysteine protease effector that cleaves RHIM proteins to block necroptosis and inflammation

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Cell death signalling pathways contribute to tissue homeostasis and provide innate protection from infection. Adaptor proteins such as RIPK1, RIPK3, TRIF and ZBP1/DAI that contain receptor-interacting protein (RIP) homotypic interaction motifs (RHIM) play a key role in cell death and inflammatory signalling¹⁻³. RHIM-dependent interactions help drive a caspase-independent form of cell death termed necroptosis^{4,5}. Here we report that the bacterial pathogen enteropathogenic *Escherichia coli* (EPEC) uses the type III secretion system (T3SS) effector EspL to degrade the RHIM containing proteins, RIPK1, RIPK3, TRIF and ZBP1/DAI during infection. This required a previously unrecognised tripartite cysteine protease motif in EspL (Cys⁴⁷, His¹³¹, Asp¹⁵³) that cleaved within the RHIM of these proteins. Bacterial infection and/or ectopic expression of EspL led to rapid inactivation of RIPK1, RIPK3, TRIF and ZBP1/DAI and inhibition of TNF, LPS or poly(I:C)-induced necroptosis and inflammatory signalling. Furthermore, EPEC infection inhibited TNF-induced phosphorylation and plasma membrane localization of MLKL. *In vivo*, EspL cysteine protease activity contributed to persistent colonization of mice by the EPEC-like mouse pathogen *Citrobacter rodentium*. The activity of EspL defines a family of T3SS cysteine protease effectors found in a range of bacteria and reveals a mechanism by which gastrointestinal pathogens directly target RHIM-dependent inflammatory and necroptotic signalling pathways.

RHIM containing proteins, including RIPK1, RIPK3, TRIF and ZBP1/DAI, play essential roles in the regulation of inflammatory and cell death-signalling pathways⁵. RIPK1 is a key regulator of the NF-κB signalling pathway in response to TNF/TNFR1 stimulation, and may induce apoptosis through formation of a cytosolic complex containing TRADD/FADD and caspase-8⁶. However, upon inhibition of caspase-8 activity, RIPK1 binds RIPK3 through RHIM-RHIM interactions leading to phosphorylation of RIPK3 and the recruitment and phosphorylation of MLKL by

activated RIPK3⁷⁻¹¹. Phosphorylated oligomeric MLKL translocates to the plasma membrane, which leads to the caspase independent form of cell death termed necroptosis^{12,13}. Necroptosis may also result from TRIF or DAI/ZBP1 interactions with RIPK1 and RIPK3, which are also mediated by RHIM-RHIM interactions^{14,15}. In addition, RIPK3 can promote NLRP3 inflammasome activation independently of necroptosis that is thought to be triggered by RHIM-RHIM amyloid formation¹⁶.

Infection of intestinal epithelial cells with the attaching and effacing enteropathogen, EPEC, leads to rapid inhibition of host inflammatory and apoptosis signalling pathways due to the activity of T3SS effectors¹⁷. While studying the effect of EPEC infection on assembly of the TNFR1 receptor complex, we observed that RIPK1 was rapidly degraded during wild type EPEC infection (E2348/69) but not during infection with the T3SS mutant (*ΔescN*) (Supplementary Figure 1a). By testing derivatives of EPEC lacking the genomic islands *PP4* alone (*ΔPP4*) or *PP4* and *IE6* (*ΔPP4/IE6*), we identified *IE6* and subsequently the gene encoding the effector EspL as essential for T3SS-dependent RIPK1 degradation (Fig. 1a; Supplementary Figure 1b). *espL* is located upstream of the T3SS effector genes, *nleB1* and *nleE*, which encode known inhibitors of apoptosis and NF-κB activation respectively (Fig. 1a)¹⁸⁻²⁰. We confirmed that EspL was translocated by the T3SS using the TEM1 β-lactamase reporter²¹ and that deletion of *espL* had no impact on actin accretion by EPEC, a measure of adherence and T3SS activity (Supplementary Figure 1c-e).

In mammalian cells, RIPK1 may be removed by K48-linked ubiquitylation and proteosomal degradation or by caspase-mediated cleavage²²⁻²⁴. However, neither caspase nor proteasome inhibitors, z-VAD-FMK (z-VAD) and MG132 respectively, prevented EspL-dependent loss of RIPK1 (Fig. 1b). Therefore, we speculated that EspL might mediate direct degradation of RIPK1. Although amino acid sequence analysis failed to uncover any canonical protease motifs, alignment

of EspL with homologues identified by BLAST²⁵ from a range of bacterial pathogens revealed a putative conserved cysteine protease motif with the possible catalytic residues Cys⁴⁷, His¹³¹ and Asp¹⁵³ (Fig. 1c, d, 2a; Supplementary Figure 2). Despite lacking primary amino acid sequence similarity with known cysteine proteases, the secondary structure of EspL predicted by Phyre²⁶ showed N-terminal similarity to the CA clan of papain-like cysteine proteases, which includes the unrelated T3SS effector YopT from *Yersinia* spp. (Fig. 1c)²⁷. Despite this, the broad spectrum cysteine protease inhibitors, antipain and Z-FA-FMK had no or only weak effect on EspL activity (Supplementary Figure 1f). Complementation of EPEC strain $\Delta PP4/IE6$ or the $\Delta espL$ mutant with native EspL expressed *in trans* restored RIPK1 degradation. However, alanine substitution of Cys⁴⁷, His¹³¹ and Asp¹⁵³ but not Cys⁴⁰ abrogated EspL-induced RIPK1 degradation, confirming the crucial role of these amino acids in EspL activity (Fig. 1d).

To determine the specificity of EspL for RIPK1 degradation, we examined the effect of EPEC infection on human as well as murine RIP kinases by immunoblot. In addition to RIPK1, catalytically active EspL also induced loss of RIPK3, which shares a high degree of similarity with RIPK1²⁸ (Fig. 1d, Supplementary Figure 3a). Levels of RIPK2 were unaffected by EspL. Using an antibody generated to residues 385-650 of RIPK1, we detected a ~14 kDa cleavage product following ectopic expression of codon optimised Flag-EspL in HEK293T cells, suggesting that EspL removed the C-terminus of RIPK1 which encompasses the RHIM (Supplementary Figure 3b). To test the ability of EspL to cleave all mammalian RHIM containing proteins directly, we incubated purified recombinant EspL with the purified RHIM-containing regions of RIPK1, RIPK3, TRIF and ZBP1 and observed cleavage by catalytically active EspL for all RHIM proteins (Fig. 2b). Intact mass spectrometry and N-terminal sequencing of the cleavage products from RIPK3 and TRIF identified the cleavage site as QxGxx↓N (P5-P4-P3-P2-P1-P1') (Fig. 2b, c, Supplementary Figure 3c, d). Substitution of V⁴⁴⁸, Q⁴⁴⁹, I⁴⁵⁰ and G⁴⁵¹ with alanine abrogated the ability of EspL to

107 cleave RIPK3 during EPEC infection suggesting that this conserved RHIM sequence was important
108 for substrate recognition by EspL (Supplementary Figure 3e). EspL also possessed the ability to
109 cleave the viral RHIM containing protein M45 from MCMV (Supplementary Figure 3f).

110
111 Given the observed cleavage of RIPK1 and RIPK3, we hypothesised that EspL would prevent
112 RIPK1/RIPK3-dependent necroptosis. Mouse dermal fibroblasts (MDF) were used to create stable,
113 doxycycline inducible cell lines expressing EspL or EspL_{C47S}. Induction of catalytically active EspL
114 was coincident with loss of RIPK1 and RIPK3 and this effect was reversible upon removal of
115 induction (Supplementary Figure 4a, b). Cells expressing EspL were protected from necroptotic cell
116 death, as measured by PI uptake, when induced by treatment with TNF, QVD or z-VAD (as caspase
117 inhibitors) and the Smac-mimetic IAP antagonist, compound A (Cp.A) as an inhibitor of NF-κB
118 activation²⁹. These conditions are known to induce cell death by necroptosis¹³. This protection
119 required EspL activity (Supplementary Figure 4c, 5a, b). EspL expression in MDF cells also
120 prevented MLKL oligomerization and membrane translocation (Supplementary Figure 5c), two
121 hallmarks of necroptosis¹³. During infection, EPEC blocked MLKL phosphorylation,
122 oligomerization and membrane translocation and consequently necroptosis in HT-29 cells in an
123 EspL dependent manner (Fig. 3, Supplementary Figure 6).

124
125 Apart from EspL, the T3SS effector NleB1 from EPEC can block TNF induced necroptosis by
126 modifying a conserved arginine in the death domain of RIPK1 with *N*-acetyl glucosamine
127 (GlcNAc)¹⁹. Consistent with partial redundancy in EspL and NleB1 function, only EPEC
128 derivatives lacking both *espL* and *nleB1* (*ΔPP4/IE6* or *ΔespLnleBE*) were unable to inhibit TNF-
129 induced necroptosis (Fig. 3b; Supplementary Figure 6). In addition, complementation of *ΔPP4/IE6*
130 with either active EspL or NleB but not NleE, restored EPEC-mediated inhibition of necroptosis

whereas inactive EspL (EspL_{C47S}) or NleB1 (NleB1_{AAA})^{18,19} did not (Fig. 3, Supplementary Figure 6).

Consistent with loss of RIPK1, ectopic expression of EspL, but not inactive EspL, blocked TNF-induced expression of an NF- κ B dependent luciferase reporter (Supplementary Figure 7a). In addition, EspL delivered by the T3SS in the EPEC mutant background Δ PP4/IE6 resulted in reduced IL-8 production by infected HT-29 cells (Supplementary Figure 7b). EspL dependent loss of TRIF following EPEC infection or ectopic expression resulted in impaired interferon- β (*Inf β*) expression and necroptosis induced by the TLR3 and TLR4 ligands, poly(I:C) and LPS, respectively (Fig. 4a, b, Supplementary Figure 7c). Using immortalised bone marrow derived macrophages (iBMDM), we observed that EspL blocked NLRP3/RIPK3-dependent caspase-1 activation induced by treatment with Cp.A/QVD¹⁶, whereas activation of the canonical NLRP3 inflammasome by nigericin was unaffected (Supplementary Figure 7d).

Although EspL possessed the ability to cleave all mammalian RHIM-containing proteins, a time course comparing RIPK1 and RIPK3 cleavage suggested that RIPK1 was the preferred target during EPEC infection (Supplementary Figure 8a). In addition, cleavage likely occurred before amyloid formation as RHIM fibrils were only inefficiently cleaved by EspL compared to the monomeric proteins (Supplementary Figure 8b, c). Amyloid fibrils form the signalling scaffold of the necrosome and arise from RHIM-RHIM interactions between RIPK1 and RIPK3⁴.

The targeted inhibition of necroptosis and RHIM-dependent inflammatory signalling by EspL during EPEC infection suggested that the activity of EspL might aid mucosal immune evasion. Here we assessed the ability of derivatives of the EPEC-like mouse pathogen, *C. rodentium* to colonise wild type C57BL/6 mice. *C. rodentium* is a murine attaching and effacing pathogen that carries all

the conserved T3SS effector genes present in EPEC, including *espL*. We confirmed that EspL from *C. rodentium* (CREspL) cleaved RIPK1, whereas inactive CREspL_{C42S} did not (Fig. 4c). In wild type C57BL/6 mice, we observed that an *espL* mutant of *C. rodentium* was attenuated for intestinal colonization in the resolving phase of infection suggesting that EspL promoted bacterial persistence in the gut, similar to previous findings³⁰ (Supplementary Figure 9). Complementation of the *espL* mutant with *espL* but not *espL*_{C47S} restored intestinal colonization by *C. rodentium* (Fig. 4d) suggesting that the cysteine protease activity of EspL was critical to its virulence function. Given the semi-redundant activities of EspL and NleB1 that we observed in the inhibition of necroptosis, and the fact that *nleB* mutants of *C. rodentium* also exhibit a colonization defect^{18,19}, further work should examine the relative contribution of each effector in vivo using an *espL/nleB* double mutant of *C. rodentium* complemented with active and inactive forms of NleB and EspL.

Here we have defined EspL from EPEC as the prototypic member of a family of T3SS cysteine protease effectors and identified the targets of EspL as host RHIM-containing proteins. EspL inactivated inflammatory, inflammasome and necroptotic signalling by cleaving within the RHIM, thereby disrupting a range of host mucosal defence pathways. EspL adds to the arsenal of bacterial T3SS effectors that subvert host cell signalling and the presence of, as yet uncharacterised, EspL homologues in a broad range of bacterial pathogens suggests that this family of cysteine protease effectors constitutes a widespread virulence mechanism.

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191 J.V. and M.S. designed and performed the experiments. S.L.M., J.M., G.N.S., C.V.O., V.F.C. and
192 G.F contributed reagents and expertise. L.F.D. and A.I.W. performed mass spectrometry analyses.
193 J.S.P. C. G, J.S. and E.L.H. designed the experiments and wrote the manuscript.

194

METHODS

Bacterial strains, plasmids, cell lines and growth conditions

The bacterial strains, plasmids and oligonucleotide primers used in this study are listed in Table S1. Bacteria were grown at 37 °C in Luria-Bertani (LB) medium, Dulbecco's Modified Eagle's medium (DMEM) with GlutaMAX (Gibco, NY), or Roswell Park Memorial Institute medium (RPMI) with GlutaMAX (Gibco) where indicated and supplemented with ampicillin (100 µg/mL), kanamycin (100 µg/mL), nalidixic acid (50 µg/mL) or chloramphenicol (25 µg/mL) where necessary.

Mouse dermal fibroblasts (MDFs) were isolated from the dermis of adult mice and immortalised with SV40 large T antigen³¹. Bone marrow derived macrophages from C57BL/6 mice were immortalized to generate a macrophage cell line (iBMDM) with CreJ2 virus as described previously³². All other cell lines were sourced from and authenticated by either the ATCC Global Bioresource Centre, or the ECACC via Sigma-Aldrich. HeLa cells, HEK293T cells, Caco-2 cells, iBMDMs, MDFs and MEFs were grown in DMEM GlutaMax (Gibco) supplemented with 10% FCS (Sigma) at 37 °C with 5% CO₂. HT-29 cells were grown in Roswell Park Memorial Institute medium (RPMI) GlutaMAX (Gibco) with 10% FCS (Sigma) at 37 °C with 5% CO₂.

Construction of EspL expression vectors

For expression in bacteria, the *espL* gene was amplified from EPEC E2348/69 genomic DNA by PCR using the primer pair EspL_F/EspL_R for cloning into pTrc99A. PCR amplification consisted of an initial denaturation step at 95 °C for 10 min, followed by 30 cycles of 94 °C for 44 sec, 55 °C for 45 sec and 70 °C for 2 min followed by a final elongation step of 70°C for 10 min. The PCR product was digested with KpnI and EcoRI and ligated into pTrc99A to produce pEspL.

For expression in mammalian cells, the gene encoding EspL from either EPEC E2348/69 or *C. rodentium* ICC169 was codon-optimised (DNA2.0), amplified using the primer pair EspL_{COF}/EspL_{COR} or EspL_{CRCOF}/EspL_{CRCOR} and ligated into KpnI/BamHI digested p3XFlag-*Myc*-

220 CMV-24 to generate N-terminal 3xFlag fusions of EspL (pFlag-EspL or pFlag-CREspL). For
221 construction of the lentiviral plasmid to generate stable inducible cell lines, codon optimised Flag-
222 EspL was amplified from pFlag-EspL and ligated into pF TRE3G PGK puro^{10,29} using
223 BamHI/XbaI.

224 Genes encoding residues 2-549 of EPEC E2348/68 EspL and EspL_{C47S} were amplified by
225 PCR using pEspL and pEspL_{C47S} as template DNA respectively, using the primer pair
226 EspL_{GEXF}/EspL_{GEXR}. PCR products were digested with BamHI and NotI and ligated into the vector
227 pGEX-2T-TEV, as previously described³³ to enable bacterial expression with an in-frame N-
228 terminal GST fusion. Insert sequences were verified by Sanger sequencing (Micromon, Monash
229 University, Australia).

230 **Site-directed mutagenesis**

231 Site-directed mutants were generated using the Stratagene QuikChange II Site-Directed
232 Mutagenesis Kit according to manufacturer's protocol. pEspL_{C40S}, pEspL_{C47S}, pEspL_{H131A},
233 pEspL_{D153A} were generated using pEspL as template DNA and primer pairs EspL_{(C40S)F}/EspL_{(C40S)R},
234 EspL_{(C47S)F}/EspL_{(C47S)R}, EspL_{(H131A)F}/EspL_{(H131A)R} and EspL_{(D153A)F}/EspL_{(D153A)R} respectively. pFlag-
235 EspL_{C40S}, pFlag-EspL_{C47S}, pFlag-EspL_{H131A}, pFlag-EspL_{D153A} were generated using pFlag-EspL as
236 template DNA and primer pairs EspL_{(C40S)COF}/EspL_{(C40S)COR}, EspL_{(C47S)COF}/EspL_{(C47S)COR},
237 EspL_{(H131A)COF}/EspL_{(H131A)COR} and EspL_{(D153A)COF}/EspL_{(D153A)COR} respectively. pF TREG-Flag-
238 EspL_{C47S} was generated using pF TREG-Flag-EspL as template DNA and primer pair
239 EspL_{(C47S)COF}/EspL_{(C47S)COR}. pFlag-CREspL_{C42S} was generated using pFlag-CREspL as template
240 DNA and primer pair EspL_{C42S(CO)F}/EspL_{C42S(CO)R}. pGEX-EspL_{C47S} was generated by PCR-
241 amplification of a cDNA encoding EPEC E2348/69 EspL_{C47S} (pTrec-EspL_{C47S}) using primers
242 EspL_{GEXF}/EspL_{GEXR} bearing restriction sites (5' BamHI, 3' NotI), followed by restriction digest and
243 ligation into the vector, pGEX-2T-TEV (pGEX-EspL_{C47S}). pGFP-mRIPK3_{AAAA} was generated
244 using pGFP-mRIPK3 as template DNA and primer pair mRIPK3_{AAAA-F}/mRIPK3_{AAAA-R}.

Purification of GST-EspL

600 mL Super broth cultures containing 100 µg/mL ampicillin were inoculated with *E. coli* BL21 Codon Plus transformed with GST-EspL or GST-EspL_{C47S} expression constructs and cultured at 37 °C with shaking to OD₆₀₀ of 0.6-0.8. Cultures were then cooled to 18 °C, protein expression induced by addition of 1 mM IPTG with continued shaking and incubation at 18 °C overnight. Cell pellets were resuspended in lysis buffer (200 mM NaCl, 20 mM HEPES pH 7.5, 5% w/v glycerol, 0.5 mM TCEP), before lysis by sonication, elimination of debris by centrifugation at 45000 g, 0.45 µm filtration of the lysate and incubation with glutathione agarose (UBP Bio) at 4 °C with agitation for 1-2 h. Beads were collected and washed with lysis buffer before incubation with 200 µg TEV protease at 20 °C for 2 h on rollers. Supernatant containing cleaved EspL or EspL_{C47S} was concentrated by centrifugal ultrafiltration and loaded on to Superdex S200 gel filtration column pre-equilibrated with gel filtration buffer (200 mM NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol). Fractions containing purified EspL or EspL_{C47S}, as assessed by SDS-PAGE, were pooled, concentrated by centrifugal ultrafiltration to 5 mg/mL, aliquoted, snap frozen in liquid nitrogen and stored at -80 °C until required.

RHIM domain protein constructs, expression and purification

Synthetic genes encoding RHIM-containing regions of human RIPK1 (Q13546; residues 497-583), human RIPK3 (Q9Y572; residues 387-518), human DAI/ZBP1 (Q9H171; residues 170-429), human TRIF (Q8IUC6; residues 601-712) with flanking BamHI and EcoRI restriction sites were purchased from Genscript, digested with these restriction enzymes, purified by agarose gel electrophoresis and ligated individually into the pHUE vector cut with the same two restriction enzymes³⁴. All expressed fusion proteins therefore consist of His6-ubiquitin-RHIM region. Successful cloning was confirmed by sequencing at AGRF (Westmead Institute) Sydney. Proteins were expressed in BL21(DE3) grown at 37 °C to an OD₆₀₀ of 0.6-0.8, and induced with 0.5 mM IPTG for 3 h. Cell pellets were lysed in 6M GuHCl, 100 mM NaH₂PO₄, 20 mM Tris.Cl, 5 mM β

mercaptoethanol, pH 8.0, and soluble material further purified on Ni-NTA agarose under denaturing conditions, with exchange into 8 M urea, 100 mM NaH₂PO₄, 20 mM Tris, 5 mM β mercaptoethanol at pH 6.0 for washing and pH 4.0 for elution from the Ni-NTA agarose.

Generation of stable inducible cell lines in mouse dermal fibroblasts (MDF) and immortalized bone marrow derived macrophages (iBMDM)

HEK293T cells were seeded at 2×10^5 cells per 10 cm culture dishes and 24 h later were co-transfected with 10 µg of either pF TRE3G-Flag-EspL or pF TRE3G-Flag-EspL_{C47S} along with the helper plasmids pMDL-RRE, pRSV-REV and pVSV-g (5, 2.5 and 3 µg, respectively) using Effectine Transfection Reagent (QIAGEN) for a further 24 h. Culture media was then changed and the cells were incubated for a further 48 h for virus production. Polybrene (5 µg/mL) (Sigma) was added to the cell culture dishes and the virus-containing supernatant was collected and passed through a 0.45 µm filter. Virus-containing supernatant was added to either MDF monolayers or iBMDM monolayers and incubated at 37 °C with 5% CO₂ for 24 h. Infected cells were selected for using increasing concentrations of up to 5 µg/mL of puromycin (Sigma) for at least one week. Expression of Flag-EspL or Flag-EspL_{C47S} in either MDFs or iBMDMs was tested by adding 20 ng/mL doxycycline at varying time points followed by immunoblot using anti-Flag antibodies.

Construction of *Citrobacter rodentium* *espL* mutant

A 325 bp upstream region of *espL* was amplified using primer pair Up-EspL-Fw/BamHI-Up-EspL-Rv, and a 500 bp downstream region of *espL* was amplified using primer pair BamHI-Down-EspL-Fw/Down-EspL-Rv. Both fragments were then digested with BamHI, ligated together, and cloned into pGEMT. The non-polar *aphT* cassette³⁵ was then inserted into the BamHI site between the two fragments and the orientation of the *aphT* cassette ascertained by PCR. This construct was then amplified using the primer pair Up-EspL-Fw/Down-EspL-Rv. The PCR products were electroporated into ICC169 containing pKD46 encoding lambda red recombinase³⁶.

294 Transformants were selected on kanamycin agar plates and *espL* deletion confirmed by PCR
295 (Check-EspL-UP-Fw /Down-EspL-Rv) and DNA sequencing.

296 ***cis* complementation of the *Citrobacter rodentium espL* mutant**

297 The *Citrobacter rodentium espL* mutant was *cis* complemented with either WT *espL* or
298 *espL_{C42S}* using the transgene insertion method previously described³⁷. Briefly, the *espL* gene with
299 its native promoter was amplified from *C. rodentium* ICC169 genomic DNA template by PCR
300 using primers EspL_{CRF1}/EspL_{CRR1}, and then ligated into the XmaI/XhoI restriction sites of the
301 pGRG36 vector. The pGRG36-EspL_{C42S} construct was generated by site-directed mutagenesis. The
302 pGRG36-EspL construct was used as template DNA and amplified by PCR using primers
303 EspL_{C42SF}/EspL_{C42SR}. The resulting plasmid was digested with DpnI at 37 °C overnight before
304 transformation into the appropriate *E. coli* strain.

305 The pGRG36-EspL and pGRG36-EspL_{C42S} constructs were confirmed by PCR using
306 primers Tn7_F/Tn7_R, then electroporated into electrocompetent *C. rodentium espL* mutant cells and
307 selected for using 100 ug/mL ampicillin and incubated at 30 °C overnight. Transformants were
308 streaked out once, then grown overnight in LB without antibiotics at 30 °C. Dilutions were prepared
309 and plated on LB and grown overnight at 42 °C. Transposition of the Tn7:*espL/espL_{C42S}* into the
310 *attTn7* insertion site in the *C. rodentium espL* mutant chromosome was confirmed by the absence of
311 the ampicillin resistance marker and PCR using primers CRseq_F/CRseq_R.

312 **Construction of EPEC single and triple deletion mutants**

313 To construct the EPEC E2348/69 *espL* deletion mutant strain, 342-bp and 331-bp fragments
314 were amplified from 5' and 3' flanking sites of *espL* using oligonucleotides EspL_{5'F}/ EspL_{5'R} and
315 EspL_{3'F}/ EspL_{3'R}. The plasmid pKD4 was used as template DNA to amplify the kanamycin cassette
316 using oligonucleotides pKD3-4_F and pKD3-4_R. Overlapping PCR was used to assemble the *espL*
317 flanking regions with the kanamycin cassette construct using oligonucleotides EspL_{5'F} and EspL_{3'R}.
318 Lambda red mediated recombination³⁶ was used to replace the wild type allele with the kanamycin

319 resistance cassette. The cassette was electroporated into wild-type EPEC E2348/69 and positive
320 clones were selected for on LB agar with 25 µg/mL kanamycin. To construct the EPEC E2348/69
321 *espL**nleBE* deletion mutant, a 368-bp fragment was amplified from 5' flanking site of *nleB* using
322 oligonucleotides NleB_{5'F}/ NleB_{5'R} and 550-bp fragment was amplified from 3' flanking site of *nleE*
323 using oligonucleotides NleE_{3'F}/ NleE_{3'R}. The plasmid pKD3 was used as template DNA to amplify
324 the chloramphenicol cassette using oligonucleotides pKD3-4_F and pKD3-4_R. Overlapping PCR was
325 used to assemble the *nleB* and *nleE* flanking regions with the chloramphenicol cassette construct
326 using oligonucleotides NleB_{5'F} and NleE_{3'R}. Lambda red mediated recombination³⁶ was used to
327 replace the wild type allele with the chloramphenicol resistance cassette. The cassette was
328 electroporated into EPEC E2348/69 *espL* mutant cells and positive clones were selected for on LB
329 agar with 5 µg/mL chloramphenicol. Deletions were confirmed by PCR with a combination of
330 primers from outside and inside the altered region. Attachment and pedestal formation by parental
331 and mutant strains were confirmed using fluorescence actin staining.

332 **EPEC infection**

333 Cell lines of HeLa, Caco-2, MDF and HT-29 cells are maintained in our laboratory and
334 regularly tested for mycoplasma contamination. Two days prior to infection HeLa, Caco-2, MDF or
335 HT-29 cell monolayers were seeded into 24 well tissue culture trays. One day prior to infection
336 derivatives of EPEC were inoculated into LB broth and grown with shaking at 37 °C overnight. On
337 the day of infection, overnight cultures of EPEC were sub-cultured 1:75 in DMEM GlutaMAX
338 (Gibco) or RPMI GlutaMAX (Gibco) and grown statically for 3 h at 37 °C with 5% CO₂. Where
339 necessary, cells were induced with 1 mM isopropyl-B-D-thiogalactopyranoside IPTG (Sigma) 30
340 min prior to infection. Cells were washed twice with PBS and infected with EPEC grown to an
341 OD_{600nm} of 0.03 for 1-3 h (depending on the experiment). When required, the inhibitors MG132
342 (Sigma) (10 µM), antipain (Sigma) (10, 20 or 40 µg/mL), z-VAD-FMK (Abcam) (25 µM), or z-FA-

343 FMK (Abcam) (10, 20 or 40 μ M) were added to the cells 1 hr prior to infection and kept on for the
344 duration of the infection.

345 **Transfection**

346 All transfections were performed in HEK293T cells using Eugene® 6 (Promega)
347 transfection reagent. Cells were seeded into 24 well tissue culture trays and transfected 24 h later
348 with 1 μ g DNA for a period of ~18 h.

349 **Fluorescent actin stain**

350 HeLa cells were seeded on coverslips and infected as previously described. After infection,
351 cells were washed with PBS, fixed in 4 %PFA in PBS for 30 min and permeabilised in 1% Triton
352 X-100 for another 30 min. Cells were then washed twice with PBS and stained with 4',6-diamidino-
353 2-phenylindole (DAPI, Invitrogen) at 0.5 mg/mL and Phalloidin-Tetramethylrhodamine B
354 isothiocyanate (Sigma) in 3% BSA/PBS for 30 min. Coverslips were mounted onto microscope
355 slides with Prolong Gold anti-fade reagent (Invitrogen). Images were acquired using a Zeiss
356 confocal laser scanning microscope with a 1003/EC Epiplan-Apochromat oil immersion objective.

357 **Immunoblot analysis**

358 For immunoblot analysis following EPEC infection, transfection or induction of stable cell
359 lines, cells were collected and lysed in cold lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH
360 7.4, 1 mM EDTA, 150 mM NaCl) with Complete Protease Inhibitor (Roche), 2 mM Na₃VO₄, 10
361 mM NaF, 1 mM PMSF and incubated on ice for 10 min to complete lysis. Samples were then
362 pelleted at 4 °C by centrifugation and the supernatants added to 4×Bolt® LDS Sample Buffer
363 (Thermo Fisher), heated to 70 °C for 10 min and resolved on Bolt® 4-12% Bis-Tris Plus Gels
364 (Thermo Fisher) by PAGE. Proteins were transferred to nitrocellulose membranes using an iBlot2
365 Gel Transfer Device (Thermo Fisher) and probed with one of the following primary antibodies:
366 mouse monoclonal anti-RIPK1 (38/RIP) (BD Transduction Laboratories), mouse monoclonal anti-
367 RIPK2/RICK (25/RIG-G) (BD Transduction Laboratories), rabbit polyclonal anti-RIPK3 (Abcam)

(for HT-29 cells), rabbit polyclonal anti-RIPK3 (ProSci) (for MDF cells) or rabbit polyclonal anti-TRIF (Cell Signaling), mouse monoclonal anti-Flag M2-HRP (Sigma), mouse monoclonal anti-GFP (7.1 and 13.1) (Roche), mouse monoclonal anti- β -actin (AC-15) (Sigma), mouse monoclonal anti-TRADD (7G8) (Cell Signaling), rabbit polyclonal anti-TRAF2 (Cell Signaling), monoclonal rat anti-mouse MLKL (WEHI-3H1) (WEHI, made in-house), mouse monoclonal anti-TEM1 β -lactamase (8A5.A10) (QED Bioscience) diluted in TBS with 5% BSA (Sigma) and 0.1% Tween (Sigma). Proteins were detected using anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase (PerkinElmer) diluted in TBS with 5% BSA (Roche) and 0.1% Tween (Sigma) and developed with enhanced chemiluminescence (ECL) western blotting reagent (Amersham). Images were visualised using an MFChemibis imaging station (DNR, Israel). At least three biological replicates were performed for all experiments.

Cell viability assays (MTT and propidium iodide staining)

For analysis of cell viability using MTT assays, immortalised mouse bone marrow-derived macrophages (iBMDM) stably expressing either EspL or EspL_{C47S} were seeded into 24 well tissue culture plates (Corning) for 18-24 h before being left untreated or treated with 20 ng/mL of LPS (*E. coli* 0111:B4) (Sigma) or 50 μ g/mL Poly I:C (for iBMDMs) (Sigma) or 10 μ g/mL high molecular weight Poly I:C (InvivoGen, CA, USA) and 10 μ M z-VAD-FMK (Abcam) for a further 18 h. The cells were washed once with PBS and replaced with DMEM containing 0.1 μ g/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) for 1 h, after which the medium was removed and 100 μ L of dimethyl-sulfoxide (DMSO; Sigma) was added to each well. After thorough mixing on an orbital shaker for 1 min, the absorbance at 540 nm for each well was obtained using a CLARIOstar microplate reader (BMG Labtech, Germany). Results were obtained from at least 3 independent experiments.

For analysis of cell viability by propidium iodide (PI) staining of HT-29 monolayers, cells were seeded into 24 well tissue culture plates with sterile glass coverslips and 48 h later were

infected with EPEC derivatives as previously mentioned for 2.5 h followed by 4 h incubation in media supplemented with 50 µg/mL gentamicin and 20 ng/mL TNF (Calbiochem), 500 nM compound A (Cp. A, Tetralogic), 25 µM z-VAD-FMK (Abcam). PI (50 µg/mL) (Sigma) was added for the final 15 min of treatment. Cells were then fixed in 3.7% (wt/vol) formaldehyde (Sigma) in PBS for 10 min and permeabilised with 0.2% Triton (Sigma) for 4 min. 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen) was applied at 0.5 µg/mL in PBS for 10 min. Cells were washed with PBS three times and coverslips were mounted onto microscope slides with Prolong Gold anti-fade reagent (Invitrogen). Images were acquired using a Zeiss confocal laser-scanning microscope with a 100x/EC Epiplan-Apochromat oil immersion objective. Duplicate coverslips were blinded for counting of PI positive cells, and results were obtained from at least 3 independent experiments.

For analysis of cell viability by PI staining and confocal microscopy in MDF cells, EspL and EspL_{C47S} expressing lines were induced with 20 ng/mL doxycycline for 2 h followed by 4 h incubation in media supplemented with 20 ng/mL TNF (Calbiochem), 500 nM Cp. A (Tetralogic), 25 µM z-VAD-FMK (Abcam). PI, DAPI staining and confocal microscopy were carried out as described above. Duplicate coverslips were blinded for counting of PI positive cells, and results were obtained from at least 3 independent experiments.

For analysis of cell viability by PI staining and flow cytometry, MDF-EspL and MDF-EspL_{C47S}, cell lines were induced with 10 ng/mL doxycycline for 1 h followed by 24 h incubation in media supplemented with 100 ng/mL hTNF-Fc produced in house (WEHI), 500 nM Cp. A (Tetralogic), 50 µM QVD-OPH (Abcam). Cell death was assessed with PI staining (1 µg/mL) and quantified using a BD FACSCalibur flow cytometer. Data was analysed using the WEASEL Flow Cytometry Software.

Monitoring MLKL complex formation using BN-PAGE

For MDF cells, 5 x 10⁵ cells (wild type, stably expressing inducible Flag-EspL or Flag-EspL_{C47S}) were used to seed each well of a 6 well tissue culture plate and allowed to attach

overnight. Cells were stimulated with 0.5 µg/mL doxycycline for 2 h to induce Flag-EspL expression prior to the addition of 100 ng/mL hTNF-Fc produced in house (WEHI), 500 nM Cp. A (Tetralogic), 25 µM z-VAD-FMK (Abcam) for a further 4 h to induce necroptosis. For HT29 cells, 5 x 10⁵ cells were plated in each well of a six well plate and allowed to attach for 48 h. Cells were infected with derivatives of EPEC E2348/69 for 2.5 h followed by stimulation with 20 ng/mL TNF (Calbiochem), 500 nM Cp. A (Tetralogic), 25 µM z-VAD-FMK (Abcam) for a further 5 h to induce necroptosis. Cells were harvested by scraping and permeabilised in MELB buffer (20 mM HEPES (pH 7.5), 100 mM KCl, 2.5 mM MgCl₂, 100 mM sucrose, 0.025% digitonin (BIOSYNTH, Staad, Switzerland) 2 µM N-ethyl maleimide, Complete Protease Inhibitor (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche)). Cytosolic and crude membrane fractions were separated by centrifugation and the crude membrane fraction further solubilized in MELB buffer containing 1% digitonin and clarified by centrifugation. Digitonin was added to the cytosolic fraction (final 1% w/v) and fractions were resolved on a 4-16% Bis-Tris Native PAGE gels (Thermo Fisher), transferred to PVDF and probed for rabbit anti-human phospho-MLKL (Abcam), monoclonal rat anti-mouse MLKL (WEHI-3H1) (WEHI, made in house), rabbit polyclonal anti-VDAC1 (Millipore) and rabbit polyclonal anti-GAPDH (Cell Signaling).

Inflammasome activation

iBMDMs (WT, EspL or EspL_{C47S}) were seeded in 12 well tissue culture treated plates and treated with 50 ng/mL ultra-pure LPS (Invivogen) for 2 h then 1 µg/mL doxycycline (Sigma) added for an additional 2 h. Cells were subsequently stimulated with 1 µM Cp.A (Tetralogic pharmaceuticals) and/or 15 µM QVD-Oph (RnD Systems) for 6 h or 10 µM Nigericin (Sigma) for 1 h. Cell supernatants and lysates were analysed by western blot. Primary antibodies used were mouse monoclonal anti-Flag M2-HRP (Sigma), mouse monoclonal caspase-1 (casper-1) (Adipogen), mouse monoclonal anti-RIPK1 (38/RIP) (BD Transduction), rabbit polyclonal RIPK3

(Axxora; PSC-2283-C100) and mouse monoclonal anti- β -actin (AC-15) (Sigma). All antibody dilutions were performed in 5% skim milk/0.1% PBS Tween.

IL-8 secretion assay

For analysis of IL-8 secretion, HT-29 cell monolayers were infected for 3 h before being incubated for 8-12 h in media supplemented with 50 μ g/mL gentamicin with or without 20 ng/mL TNF (Calbiochem, EMD4Biosciences, USA). Following this, the HT-29 cell supernatant was collected and either used immediately or stored at -20 °C for subsequent analysis of IL-8 secretion. IL-8 secretion was measured using the Human IL-8 ELISA MAX Deluxe Set (Biolegend, CA, USA) according to the manufacturer's instructions.

qRT-PCR

Samples for qRT-PCR experiments were DNase treated using Ambion TURBO DNA-free kit and cDNA synthesis was completed using the iScriptTM cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using SsoAdvancedTM Universal SYBR[®] Green Supermix (Bio-Rad) according to manufacturer's instructions and gene specific primers used are listed in Table S1. Samples were loaded onto MicroAmp[®] Optical 384-well reaction plates (Life technologies) in duplicate and run on the ABI Quant Studio 7 according to manufacturer's instructions. Melting curve analysis was used to ensure there were no primer dimers. Negative controls included both a no-reverse transcriptase control and a no cDNA control. Data were analysed by the threshold cycle method ($\Delta\Delta C_t$ method)³⁸ and normalised to *18S* abundance. All data are represented as fold induction relative to gene expression in uninduced, unstimulated cells or uninfected, unstimulated cells. All experiments were carried out in triplicate.

Beta-lactamase translocation assay

HeLa cells were seeded in black 96 well trays with transparent well bottom (Greiner Bio-One) for 16 to 24 h prior to infection. On the day of infection, EPEC strains with derivatives of pCX340 were cultured as previously described. 2.5 mM Probenecid (Sigma) and 1 mM IPTG

(Sigma) were added to bacterial cultures for the last 45 min before infection. HeLa cells were loaded with CCF2/AM substrate following manufacturer's instructions (Invitrogen) and incubated at room temperature in the dark for one hour. 15 minutes before infection, cells were transferred back to 37 °C 5% CO₂. Infection was carried out using 50 µl of bacterial culture with an OD₆₀₀ of 0.1 for 60 min at 37 °C in 5% CO₂. Translocation was measured as a ratio of Emission_{450nm}:Emission_{520nm} using a CLARIOstar Omega microplate reader (BMGLabtech) using triplicate wells for each strain.

Dual-luciferase reporter assay

For the NF-κB dual-luciferase assay, HeLa cells were seeded into 24-well trays (Corning) and co-transfected with derivatives of p3xFlag-*Myc*-CMV-24 (0.4 µg), 0.05 µg of pRL-TK (Promega, Madison WI, USA) and 0.2 µg of pNF-κB-Luc (Clontech, Palo Alto CA, USA). Approximately 24 h post-transfection, cells were left untreated or stimulated with 20 ng/mL TNFα (Calbiochem, La Jolla, CA) for 6 h. Firefly and Renilla luciferase levels were measured using the Dual-luciferase reporter assay system (Promega) in a CLARIOstar Omega microplate reader (BMGLabtech, Germany). The expression of firefly luciferase was normalised for Renilla luciferase measurements and Luciferase activity was expressed relative to unstimulated p3xFlag-*Myc*-CMV-24-transfected cells.

***In vitro* cleavage assays**

Purified proteins were diluted out of urea-containing buffer and incubated in 100 µl of reaction buffer (25 mM NaH₂PO₄, 150 mM NaCl, 0.5 mM DTT, pH 7.4) at a concentration of 20 µM (RHIM proteins) or 0.9 µM (EspL/EspL_{C47S}) for 1 hour at 37 °C. Sample buffer was then added to incubated proteins, before the samples were boiled and subjected to SDS-PAGE on Nu-PAGE 4-12% Bis-Tris polyacrylamide gels. The gels were stained with Coomassie Blue Stain and imaged with a GelMax imager (UVP, Analytik Jena, USA).

491 To compare the cleavage of RHIM-containing protein in monomeric and fibrillar forms,
492 10 µg EspL was added to 200 µL of 20 µM of His-Ub-RIPK3 in 25 mM NaH₂PO₄, 150 mM NaCl,
493 0.5 mM DTT, pH 7.4 immediately following dilution of His-Ub-RIPK3 from 8M urea-containing
494 buffer or after incubation of the diluted His-Ub-RIPK3 for 225 min at 37 °C to allow fibril
495 formation. Samples were subsequently incubated with EspL at 37 °C for 1 h. For both ‘monomer’
496 and ‘fibril’ samples, 100 µL was pelleted at 16,000g for 10 min. The supernatant was collected as
497 the soluble fraction and the pellet was resuspended with 100 µL of 8M urea, pH 4.0. Samples were
498 subjected to SDS-PAGE and stained with Coomassie Blue Stain for visualisation and imaging as
499 above.

500 **Thioflavin-T (ThT) assays**

501
502 Thioflavin-T (ThT) assays were used to monitor fibril formation. 20µM of recombinant
503 RHIM containing His-Ub-RIPK3 was incubated in buffer (25 mM NaH₂PO₄, 150 mM NaCl, 0.5
504 mM DTT, pH 7.4, 40 µM ThT) in a Costar 96-well plate (Corning) at 37 °C for 3.75 hours inside a
505 POLARstar Omega microplate reader (BMGLabtech). Samples were excited at 440 nm and ThT
506 fluorescence emission was measured at 480 nm every 60 seconds. Fibril formation was evident after
507 225 min. To test the cleavage of RHIM fibrils by EspL, 500 µl of 20 µM of His-Ub-RIPK3 was
508 dialysed against 1 L of dialysis buffer (25 mM NaH₂PO₄, 150 mM NaCl, 0.5 mM DTT, pH 7.4) for
509 24 h at room temperature. 100 µl of ‘fibril’ sample was then incubated with 0.9 µM purified EspL,
510 vortexed briefly and incubated at 37 °C for either 1 or 2 h. For both ‘monomer’ and ‘fibril’ samples,
511 100 µl was pelleted at 16,000 g for 10 min. The supernatant was collected as the soluble fraction
512 and the pellet was resuspended with 100 µl of 8M urea, pH 4.0. Samples were subjected to SDS-
513 PAGE and stained with Coomassie Blue Stain for visualisation and imaging as previously
514 mentioned.

515

516

517 **Reverse-phase HPLC**

518 200–1000 µL of purified RHIM proteins at a concentration of 20 µM were incubated with or
519 without 0.9 µM of purified EspL for 1 hour at 37 °C. Proteins were then analysed by RP-HPLC
520 using a C₈ VYDAC column running in MilliQ water containing 0.1% TFA, 10% methanol and
521 eluted with an increasing gradient of acetonitrile. Peaks were collected and lyophilized for further
522 analysis by N-terminal sequencing or LC-MS/MS.

523 **N-terminal sequencing**

524 N-terminal sequencing was performed by the Australian Proteome Analysis Facility (APAF)
525 at Macquarie University, New South Wales, Australia.

526 **Characterisation of His₆-Ub-RIPK3 and His₆-Ub-TRIF cleavage products by intact mass** 527 **spectrometry analysis**

528 Characterisation of RIPK3 and TRIF cleavage products by intact mass spectrometry were
529 performed on samples prepared by *in vitro* cleavage assay and purified by RP-HPLC as described
530 above.

531 *Time-of-flight tandem mass spectrometry*

532 Mass measurements of the intact protein ions and ETD and CID MS/MS were performed on the
533 high resolution, high mass accuracy quadrupole time-of-flight (qTOF) mass spectrometer (maXis II
534 UHR qTOF, Bruker Daltonics, Bremen, Germany) equipped with an electrospray ion (ESI) source
535 for the direct-infusion method. For the direct-infusion experiments a flow rate of 180 µL/min was
536 provided by a syringe pump (KdScientific, Holliston, MA, USA) using a 25 µL syringe (SGE
537 Analytical Sciences, VIC, AUS). The following settings were applied: capillary voltage of 4.5 kV,
538 end plate offset of 500 V, mass range of *m/z* 450 to 2500, dry gas of 4.0 L/min, and drying
539 temperature of 220 °C. Nano-ESI infusion was performed using HPLC fractions which were
540 reconstituted in 20 µl of water and diluted 1:10 with 50% acetonitrile: 50% water containing 1%
541 formic acid.

Data analysis

The MS and MS/MS spectra were analyzed using a dedicated top-down data analysis procedure. Briefly, precursor and product ion mass spectra were summed over the infusion time in each MS and MS/MS experiment with Data Analysis software version 4.3 (Bruker Daltonics). Automatic data analysis was performed by deisotoping keeping only the monoisotopic masses followed by charge state deconvolution (SNAP 2 algorithm). The obtained mass list of product ions was matched on the predicted cleaved mRIPK3 sequence to determine the product ion identity and the sequence coverage using the observed mass of the precursor ion.

Necroptosis experiments. The human epithelial cell line, HT-29, as well as mouse dermal fibroblasts (MDF) and immortalised bone marrow derived macrophages (iBMDMs) either infected with derivatives of EPEC E2348/69 or engineered to express EspL derivatives were used to examine the effect of EspL on necroptosis. Necroptosis was induced by treatment with 1) 20 ng/mL TNF, 500 nM Cp.A and 25 μ M z-VAD-FMK or 50 μ M QVD-OPH or 2) 20 ng/mL of LPS (*E. coli* 0111:B4) and 10 μ M z-VAD-FMK or 3) 50 μ g/mL Poly I:C (for iBMDMs) or 10 μ g/mL high molecular weight Poly I:C (for HT-29) and 10 μ M z-VAD-FMK. Cell viability was assessed by reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or by propidium iodide (PI) uptake. MLKL complex formation and membrane translocation were examined by Blue native PAGE of cell membrane and cytosolic fractions and immunoblotting using antibodies to MLKL and phospho-MLKL.

Protein sequence alignment and phylogenetic tree

RHIM domain alignment for human and mouse RIPK1, RIPK3, TRIF and DAI was performed by Clustal Omega and ESPript³⁹. Accession numbers (same order as displayed in Fig. 2c) NP_003795, NP_033094, NP_006862, NP_064339, NP_891549, NP_778154, NP_110403, NP_067369, NP_001153889, NP_001132991. For comparison of the cysteine protease motif, sequences were identified through BLAST using EspL from EPEC E2348/69 as a reference. A

567 section of the proteins identified by BLAST were aligned using Clustal Omega and presentation of
568 alignment performed using ESPript3³⁹. Accession numbers (same order as displayed in
569 Supplementary Figure Fig. 2) CAS10778, AIG70345, WP_012905388, WP_031942474,
570 WP_024259347, ENZ84489, WP_023263817, WP_015872003, WP_020957625, CCA83579,
571 WP_009667375, CDG86051, WP_004389152, WP_004714204, WP_019080404, AHK18540,
572 WP_002211641, AIN16488.

573 A set of protein sequences was curated based on the identification of homologues of EspL
574 from EPEC E2348/69. BLAST²⁵ was used to compare these protein sequences to the *nr* database
575 and *nt* database (downloaded on 31st July from <ftp://ftp.ebi.edu.au/pub/databases/ncbi/blast/db/>)
576 with blastp and tblastn respectively to identify additional proteins. The subsequent results were
577 filtered on the E scores of 0.0 and length, and reduced to unique accession (removing duplicates).
578 The nucleotide sequences were translated into amino acid sequences with EMBOSS⁴⁰ and the
579 protein sequences were aligned with Muscle⁴¹. The best fitting protein model was determined using
580 the Perl script ProteinModelSelection.pl available at <http://sco.hits.org/exelixis/web/software/raxml/>
581 for RAXML⁴². One hundred pseudo-replicate RAXML analyses were run three times using the best-
582 fitting substitution model, PROTGAMMA VF. The best scoring Maximum Likelihood tree was
583 selected and midpoint rooted in Dendroscope⁴³. The following accession numbers were used for
584 phylogenetic analyses; CAS10778.1, AIG70345.1, CBG87854.1, ACR69900, CP001064,
585 CP011417, LM996972, LM997319, CP001064, Z54194, WP_028120439, LM996116,
586 WP_028120664, WP_038348374, AJ303141 LM996576, AP010958, LM995478, LM995537,
587 LM996653, LM997233, LM997407, LM995613, LM997087, WP_001121612, WP_001121619,
588 WP_001121621, WP_033810450, WP_044863368, AAJV00000000, AIAN00000000, FM986650,
589 AP010960, LM996367, EHW09036, EHW21689, WP_001121623, WP_001121627,
590 WP_001121746, WP_001121747, WP_001121748, WP_021824236, WP_023981847,
591 WP_032272532, WP_032273780, AIHA00000000, WP_032349748, AIHB00000000,

592 AIHD00000000, AIHE00000000, AAJX00000000, AIBC00000000, LN554915, LM996042,
593 LM996071, LM996922, WP_001121620, WP_032210130, AKNI01000047, AIAL00000000,
594 AIAO00000000, AIGY00000000, AIGZ00000000, AIBX00000000, CP006262, CP007133,
595 LM996313, LM996458, CP007136, LM995993, LM996803, LM997001, WP_001121622,
596 AIAI00000000, AIAX00000000, AF453441, AIBD00000000, CP008805, CP001164, CP001368,
597 CP001925, CP010304, EHV10408, WP_001121626, WP_024256897, WP_032208682,
598 AIAQ00000000, LM995690, LM995751, LM995947, AJ277443, FM201463, LM995831,
599 LM996694, LM997125, EHW62569, WP_001121617, WP_032345473, WP_045889142,
600 AICF00000000, AIAC00000000, AIGK00000000, AIGN00000000, AIGO00000000,
601 AIAG00000000, AIGL00000000, AIGM00000000, AIBT00000000, AIBR00000000,
602 AIBV00000000, AIGX00000000, WP_001121628, CP001846, CP003109, WP_001121608,
603 WP_001121609, WP_001121624, AIAH00000000, AIAD00000000, ADUL01000000,
604 AIFD00000000, AIAE00000000, AIFS00000000, AIFT00000000, AIFU00000000,
605 AIFQ00000000, FM986652, LM996749, CDG86051, WP_038497945, WP_009667375,
606 CCA83579, FP885907, AEG72234, CBJ36034, CEJ16658, EUJ11993, WP_003265371,
607 WP_003274329, WP_013209029, WP_039553678, WP_042549988, WP_042592182,
608 WP_043947056, ENZ84489, CP000037, CP001062, WP_012421777, CP000035, CP006737,
609 ADA76828, EFP73031, EFW48342, WP_000608472, WP_005015229, WP_011379052,
610 AY879342, NG_035859, NG_035867, AF348706, AF386526, AL391753, AY879342, CP001384,
611 CP007038, Z54211, CAA90938, EIQ30821, WP_005058548, WP_005061014, WP_005065444,
612 WP_005115993, WP_005117432, WP_010921597, WP_010921642, WP_015060143,
613 WP_024259347, WP_025746267, WP_025748914, WP_025759433, WP_025766126,
614 WP_031942474, WP_039060413, WP_040234710, WP_047204882, WP_047204897, CP000039,
615 CP011423, HE616529, WP_005041841, WP_005138925, WP_024261348, CFB70006,
616 CFQ67145, CNC44257, CRE36360, WP_019080404, CFR06093, WP_004389152, CP000305,

617 CP001585, CP001589, CP001608, CP002956, CP009492, CP009704, CP009723, CP009785,
618 CP009836, CP009844, CP009973, CP009996, CP010023, CP010293, AE009952, AE017042,
619 AL590842, CP000308, CP001593, CP009840, CP009906, CP009991, KGA51839,
620 WP_002211641, WP_045123609, BX936398, CP001048, CP009757, CP009780, CP009786,
621 CP010067, CP000950, CP008943, CP009712, CP009759, CP009792, CFV36814, CNG55237,
622 CNJ16181, WP_011193035, WP_012104544, WP_012303595, ESJ22116.1, WP_032466541,
623 WP_038400874, WP_004714204, CP007230, CNC46702, WP_025381344, WP_006576111.1

624 **Mouse infection studies**

625 All animal experimentation was approved by the Melbourne University Animal Ethics
626 Committee. All mice used were of a C57BL/6 background and were age matched as best as possible
627 between 5-8 weeks pre-infection. No calculation was used to assess the number of animals required.
628 Male and female mice were allocated to experimental groups to ensure even distribution of age, sex
629 and weight and investigators were not blinded to the allocation. *Citrobacter rodentium* was cultured
630 in LB broth overnight before centrifugation and re-suspension in PBS to a concentration of $\sim 5 \times 10^9$
631 cells/mL. C57BL/6 (5- to 8 weeks old) were inoculated by oral gavage with 200 μ L of
632 approximately 1×10^9 c.f.u of *C. rodentium*. The viable count of the inoculum was determined
633 retrospectively by plating dilutions of the inoculum on plates with appropriate antibiotics. Mice
634 were weighed every 2 days and faeces collected every 2 or 4 days for enumeration of c.f.u. The
635 viable count per g of faeces was determined by plating serial dilutions of faeces onto media
636 containing selective antibiotics.

637 **Statistical analysis.** All statistical analyses were performed using GraphPad Prism version 6.0.
638 Statistical tests used were unpaired two-tailed Student's *t*-test for pairwise comparisons between
639 groups or One-way ANOVA with Holm-Sidak's Test for multiple comparisons where indicated.
640 Variance was similar in all comparisons. Differences in faecal counts of CR from mice and

641 diarrhoea and pathology scores were assessed using a Mann Whitney U test, where normal
642 distribution was not assumed. $P < 0.05$ was considered to be significant.

643 **Data availability.** The data that support the findings of this study are available from the
644 corresponding author upon request

645

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Legend to the figures

Figure 1. EspL is a T3SS cysteine protease that degrades RIPK1 and RIPK3. a, Schematic

representation of EPEC E2348/69 genomic integrative element 6 (IE6) harbouring *espL*, *nleB1*, and

nleE and immunoblot showing RIPK1 degradation in HT-29 cells infected with derivatives of

EPEC E2348/69 as shown. Representative immunoblot from at least 3 independent experiments.

Actin; loading control. **b,** Immunoblot showing RIPK1 degradation in HeLa cells uninfected or

infected with EPEC E2348/69; untreated, or treated with either MG132 or z-VAD-FMK (z-VAD).

Representative immunoblot from at least three independent experiments. Actin; loading control. **c,**

Schematic representation of cysteine protease motif and secondary structure predicted by Phyre in

YopT from *Y. pestis* KIM and EspL from EPEC E2348/69. **d,** Immunoblot showing levels of

RIPK1, RIPK2 and RIPK3 in HT-29 cells infected with derivatives of EPEC E2348/69.

Representative immunoblot from at least three independent experiments. Actin; loading control.

Figure 2. Distribution of EspL in Gram negative pathogens and substrate specificity. a,

Phylogeny of EspL homologues from a range of Gram negative pathogens which was midpoint

rooted. Different genera are highlighted by background colour and the tips are coloured by species

or pathotype. **b,** Coomassie Brilliant Blue stain of SDS PAGE gel showing *in vitro* cleavage of

purified RHIM-containing regions of RIPK1, RIPK3, TRIF and ZBP1 (expressed as recombinant

His₆-ubiquitin-tagged proteins) by purified recombinant EspL. Representative gel from at least two

independent experiments. Purified recombinant EspL_{C47S} was used as a negative control. White

arrows indicate cleavage products. Black arrows indicate EspL and the band corresponding to free

His₆-ubiquitin (His-Ub) Note, observed cleavage product derived from ZBP1/DAI was consistent

with EspL cleavage in first RHIM of ZBP1/DAI. **c,** EspL cleavage site indicated by an arrow in the

RHIM containing regions of RIPK1, RIPK3, TRIF and ZBP1/DAI. Alignment was performed using

Clustal Omega and ESPrpt3. Cleavage sites in RIPK3 and TRIF were determined experimentally by mass spectrometry and N-terminal sequencing.

Figure 3. EspL inhibits TNF-induced necroptosis. **a**, Cell death visualised by propidium iodide (PI) staining in HT-29 cells infected with derivatives of EPEC and treated with TNF, compound A (Cp.A) and z-VAD-FMK (z-VAD). Hoechst; stain for nucleic acid. Scale bar, 20 μ m. Representative images shown from at least three independent experiments. **b**, Quantification of PI staining from microscopic analysis in HT-29 cells infected with derivatives of EPEC E2348/69 and treated with TNF, Cp.A and z-VAD. Results are mean $\pm$ s.e.m. percentage of cells positive for PI staining from three independent experiments counting $\sim$ 200 cells in triplicate. $*P < 0.0001$ compared to EPEC E2348/69 infected cells, one-way ANOVA with Holm-Sidak multiple comparison. **c**, Blue native PAGE analysis of MLKL membrane translocation in HT-29 cells infected with derivatives of EPEC E2348/69 treated with TNF, Cp.A and z-VAD. Representative immunoblot from at least three independent experiments. GAPDH; cytosolic fraction loading control, VDAC; membrane fraction loading control.

Figure 4. EspL activity inhibits TLR3/4-mediated signalling and contributes to *in vivo* persistence. **a**, *Ifn β* expression in doxycycline-inducible iBMDMs stably expressing either Flag-EspL or Flag-EspL_{C47S} and treated with either LPS or Poly I:C for 3 h as indicated. Results are mean $\pm$ s.e.m of at least three independent experiments performed in triplicate. *Ifn β* expression relative to uninduced, unstimulated cells. $*P < 0.0005$ compared to Flag-EspL induced with doxycycline and treated with LPS or poly I:C, unpaired, two-tailed *t*-test. **b**, MTT reduction in doxycycline-inducible iBMDMs stably expressing either Flag-EspL or Flag-EspL_{C47S} and treated with either LPS/z-VAD or Poly I:C/z-VAD for 20 h. Results are mean $\pm$ s.e.m. of absorbance at 540 nm from three independent experiments performed in triplicate. $*P < 0.05$ compared to Flag-EspL_{C47S} induced with

doxycycline and treated with LPS or poly I:C, unpaired, two-tailed *t*-test. **c**, Immunoblot showing degradation of endogenous RIPK1 by EspL from *C. rodentium* (Flag-CREspL) but not EspL_{C42S} (Flag-CREspL_{C42S}) expressed ectopically in HEK293T cells. Representative immunoblot from at least three independent experiments. **d**, Bacterial load in the faeces of mice 16 days after infection with derivatives of *C. rodentium*, including wild type *C. rodentium* ICC169 (CR), an *espL* deletion mutant ($\Delta espL$) and $\Delta espL$ complemented with *espL* (EspL) or *espL*_{C42S} (EspL_{C42S}) by Tn7 transposition. Each data point represents log₁₀ c.f.u. per g faeces per individual animal (c.f.u., colony forming units). Mean $\pm$ s.e.m. are indicated. Data was combined from three independent experiments. *P* values from Mann–Whitney U-test.

a **b** **c**

a

+ TNF/Cp.A/z-VAD

b **c**