

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository:<https://orca.cardiff.ac.uk/id/eprint/114738/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Pallett, M. A., Crepin, V. F., Serafini, N., Habibzay, M., Kotik, O., Sanchez-Garrido, J., Di Santo, J. P., Shenoy, A. R., Berger, C. N. and Frankel, G. 2017. Bacterial virulence factor inhibits caspase-4/11 activation in intestinal epithelial cells. *Mucosal Immunology* 10 , pp. 602-612. 10.1038/mi.2016.77

Publishers page: <http://dx.doi.org/10.1038/mi.2016.77>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See <http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



Bacterial Virulence Factor Inhibits Caspase-4/11 Activation in Intestinal Epithelial Cells

Mitchell A. Pallett¹, Valerie F. Crepin¹, Nicolas Serafini^{2,3}, Maryam Habibzay¹, Olga Kotik¹, Julia Sanchez-Garrido⁴, James P. Di Santo^{2,3}, Avinash R. Shenoy⁴, Cedric N. Berger¹, Gad Frankel^{1#}

¹Department of Life Sciences, and ⁴Department of Medicine, MRC Centre for Molecular Bacteriology and Infection, Imperial College London, UK; ²Innate Immunity Unit, Institut Pasteur, Paris, France; ³Inserm U668, Paris, France

The authors declare no conflict

Acknowledgements

We thank Guy Frankel for making the CR Δ *nleF* mutant. We are grateful to Dr. Bernhard Ryffel (TAAM-CDTA, Orelans, France) and Dr. Mohamed (Lamkanfi, [VIB Inflammation Research Center, Ghent University, Belgium](#)) for providing us with the *Casp1/11*^{-/-} and *Casp11*^{-/-} mice. This project was supported by grants to GF from the Biotechnology and Biological Sciences Research Council (BBSRC), the Wellcome Trust and the Medical Research Council (MRC). ARS acknowledges funds from the Royal Society (RG130811) and the Wellcome Trust (108246/Z/15/Z). JPD and NS are supported by grants from the Institut Pasteur, Inserm and Danone.

Key words: Caspase-4/11, *Citrobacter rodentium*, NleF, type III secretion system, intestinal epithelial cells

#Corresponding author

Gad Frankel, MRC CMBI, Flowers Building, Imperial College, London, SW7 2AZ

g.frankel@imperial.ac.uk

1 **Abstract**

2 The human pathogen enteropathogenic *Escherichia coli* (EPEC), as well as the mouse
3 pathogen *Citrobacter rodentium*, colonize the gut mucosa via attaching and effacing lesion
4 formation and cause diarrheal diseases. EPEC and *C. rodentium* type III secretion system
5 (T3SS) effectors repress innate immune responses and infiltration of immune cells.
6 Inflammatory caspases such as caspase-1 and caspase-4/11 are crucial mediators of host
7 defense and inflammation in the gut via their ability to process cytokines such as IL-1 β and
8 IL-18. Here we report that the effector NleF binds the catalytic domain of caspase-4 and
9 inhibits its proteolytic activity. Following infection of intestinal epithelial cells (IECs) EPEC
10 inhibited caspase-4 and IL-18 processing in an NleF-dependent manner. Depletion of
11 caspase-4 in IECs prevented the secretion of mature IL-18 in response to infection with
12 EPEC Δ nleF. NleF-dependent inhibition of caspase-11 in colons of mice prevented IL-18
13 secretion and neutrophil influx at early stages of *C. rodentium* infection. Neither wild-type *C.*
14 *rodentium* nor *C. rodentium* Δ nleF triggered neutrophil infiltration or IL-18 secretion in
15 *Cas11* or *Casp1/11* deficient mice. Thus, IECs play a key role in modulating early innate
16 immune responses in the gut via a caspase-4/11 - IL-18 axis, which is targeted by virulence
17 factors encoded by enteric pathogens.

18 **Introduction**

19 Central to the infection strategy of the extracellular pathogens enteropathogenic *Escherichia*
20 *coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC)⁽¹⁾ and *Citrobacter rodentium*⁽²⁾ is injection
21 of type III secretion system effectors into intestinal epithelial cells (IECs) where they target
22 diverse signalling pathways, particularly innate immune signaling. NleC and NleD are Zn-
23 dependent endopeptidases that specifically cleave and disable RelA (p65) and JNK,
24 respectively, thus blocking NF-κB and AP-1 activation⁽³⁾. NleE is a methyltransferase that
25 specifically modifies a cysteine in the zinc finger domain of TAB2 and TAB3 thus also
26 blocking NF-κB signalling⁽⁴⁾. NleB, which also inhibits NF-κB, has an N-acetylglucosamine
27 transferase activity that specifically modifies Arg 117 in the death domain of FADD^(5,6) and
28 NleH is a serine/threonine kinase that inhibits the RPS3/NF-κB pathway via phosphorylation
29 of CRKL (v-Crk sarcoma virus CT10 oncogene-like protein)⁽⁷⁾.

30 Inhibition of innate immunity by EPEC and EHEC is needed to counter its activation by the
31 T3SS, flagellins and lipopolysaccharides (LPS), which are readily detected by sensors and
32 receptors in mammalian hosts. In response to infection, some sensors assemble
33 macromolecular complexes called inflammasomes to stimulate the protease activity of
34 caspase-1. The proteolytic processing and release of interleukin (IL)-1β and IL-18, and the
35 induction of pyroptotic cell death triggered by caspase-1 can prevent the establishment and
36 spread of microbial pathogens^(8,9). In addition, the single mouse caspase-11 and the related
37 human caspase-4 and caspase-5 act as cytosolic receptors, which bind LPS directly via their
38 N-terminal caspase activation and recruitment domains (CARD, p22 domain). LPS binding
39 induces oligomerization and autoproteolytic activation of caspase-4/5/11 into their active
40 p20/p10 fragments and subsequent pyroptotic lysis of bacterially infected host cells⁽¹⁰⁾. In
41 human and mouse phagocytic cells LPS is detected by caspase-4/11, which stimulate

42 caspase-1-dependent maturation of IL-1 β and IL-18 via the NLRP3-ASC inflammasome⁽¹¹⁻
43 ¹³⁾. However, in IECs caspase-4/11 acts independently of NLRP3 and caspase-1 to directly
44 process IL-18 and induce pyroptosis during *Salmonella* infection⁽¹⁴⁾. Therefore the detection
45 of Gram-negative bacteria by IECs markedly contrasts that in myeloid cells. However, unlike
46 *Salmonella*, which are intracellular pathogens, extracellular pathogens use T3SS to prevent
47 death pathways in host cells to which they intimately adhere^(5,6,15). This suggests that EPEC,
48 EHEC and *C. rodentium* might manipulate caspase-4/11 and/or inflammasome pathways in
49 IECs.

50 Previous work on *C. rodentium* infections in mice showed that loss of inflammasome
51 signaling related genes such as *Nlrp3*, *Nlrc4*, *Casp1*, *Casp11*, *Il1 β* and *Il18* results in
52 enhanced morbidity and inflammatory disease, whereas wild-type mice clear the pathogen
53 within 14-21 days^(16,17). Detection of *C. rodentium*, EHEC and EPEC in myeloid cells has
54 also been studied previously, and a recent report identified the EPEC NleA T3SS effector
55 protein as an inhibitor of NLRP3-caspase-1 inflammasomes⁽¹⁸⁾. However, as IECs use non-
56 canonical, NLRP3- and caspase-1-independent mechanisms to detect bacteria, we
57 hypothesized that EPEC and *C. rodentium* subvert caspase-4/11 action in IECs upon initial
58 attachment. Here we report that bacterial T3SS effector NleF is a potent inhibitor of
59 mammalian caspase-4/11 and thus prevents IL-18 secretion from IECs *in vitro*, and blocks
60 caspase-11 – IL-18 mediated neutrophil influx during infection *in vivo*.

61 **Results**

62 **NleF binds human caspase-4**

63 The highly conserved effector NleF was previously reported to bind the active site and to
64 inhibit the activity of caspase-9, caspase-8 and caspase-4, however, whether NleF affects
65 inflammasome signaling and the innate immune response to bacterial infection *in vivo* has not
66 been tested⁽¹⁹⁾. By employing a yeast-2-hybrid screen (Table S1) and a direct yeast-2-hybrid
67 (DYH) assay (Fig. 1A) we confirmed that human caspase-4 is an interacting partner of EPEC
68 NleF (NleF_{EPEC}). Truncation analyses revealed an interaction between NleF_{EPEC} and the p30
69 catalytic domain of caspase-4 (Fig. 1B). Deletion of four C-terminal residues in NleF_{EPEC}
70 (NleF_{1-185_EPEC}) abrogates its binding to caspase-9⁽¹⁹⁾, and similar defects were seen in
71 binding to caspase-4 (Fig. 1B). Mutation of the substrate-binding pocket of caspase-4
72 (R152A, W313A and R314A) also abolished NleF-caspase-4 interaction (Fig. 1B). To
73 confirm that the binding is direct, the caspase-4 p20 subunit (22 kDa; His tagged), p10
74 subunit (10 kDa) and NleF_{EPEC} (65 kDa; MBP fusion) were co-expressed, purified by tandem
75 affinity chromatography and analyzed by gel filtration. Three chromatographic peaks
76 corresponding to free MBP-NleF_{EPEC}, free His-p20, and a complex containing NleF_{EPEC}, p20
77 and p10 subunits were observed (Fig. 1C). NleF_{EPEC} and caspase-4 subunits co-purified and
78 co-eluted as a macromolecular complex with an apparent molecular weight (MW) of ~230
79 kDa (Fig. 1C-D).

80 **NleF inhibits human caspase-4 and mouse caspase-11**

81 Recombinant caspase-4 underwent auto-proteolytic activation presumably as a consequence
82 of LPS binding when purified from *E. coli*. Wild-type caspase-4, but not a catalytic dead
83 mutant (caspase-4C285S), underwent auto-proteolysis to the active p20 form and hydrolyzed
84 the caspase-4 fluorogenic substrate peptide (Ac-LEVD-AFC; Fig. 2A). Recombinant
85 NleF_{EPEC} inhibited the activity of caspase-4 in a dose-dependent manner with an IC₅₀ of 5 nM

86 (Fig. 2B), comparable to 14 nM previously measured for NleF_{EHEC} by Blasche et al.⁽¹⁹⁾.
87 Despite not binding caspase-4 in DYH, NleF_{1-185_EPEC}, which was pulled down with caspase-
88 4 at low levels (data not shown), was able to inhibit caspase-4 activity although at an IC₅₀ of
89 25.5 nM (Fig. 2B). *C. rodentium* NleF (NleF_{CR}), which shares 84% amino acid identity with
90 NleF_{EPEC}, strongly inhibited the proteolytic activity of mouse caspase-11 (IC₅₀ of 13 nM; Fig.
91 2C-D) revealing an evolutionarily conserved functional property. Importantly, we found that
92 NleF_{EPEC} inhibits caspase-4 more efficiently than NleF_{CR} (Fig. 2C), while NleF_{CR} inhibits
93 caspase-11 more efficiently than caspase-4 (Fig. 2F).

94 **NleF inhibits h-caspase-4 activation during infection**

95 To investigate if NleF_{EPEC} targets caspase-4 during infection of human IECs, Caco-2 cells
96 were infected with the wild-type (WT) EPEC and EPEC Δ *nleF*; both strains adhered to the
97 cultured cells equally (Fig. 3A). However, while secreted caspase-4 was absent following
98 infection with WT EPEC, the active p30 fragment of caspase-4 was found in the supernatants
99 of cells infected with EPEC Δ *nleF* (Fig. 3B). Addition of the caspase-4 inhibitor Ac-LEVD-
100 CHO complemented the EPEC Δ *nleF* phenotype in a dose dependent manner (Fig. 3B).

101 NleF_{EPEC} did not affect the expression of pro-IL-18, which was similar in uninfected cells and
102 those infected with all the EPEC strains (Fig. 3C). While secretion of pro-IL-18 was detected
103 upon infection with WT EPEC and EPEC Δ *nleF*, pro-IL-18 was only processed into the active
104 form following infection with EPEC Δ *nleF* (Fig. 3D). Secretion of mature IL-18 (mIL-18),
105 induced by EPEC Δ *nleF*, was not detected when this strain was complemented with a plasmid
106 encoding NleF_{EPEC} (pNleF_{EPEC}) (Fig. 3D).

107 To confirm that inhibition of caspase-4 by NleF was sufficient to block processing of IL-18,
108 we generated Caco-2 cells depleted of caspase-4 using miRNA30E based stable shRNA
109 expression (Fig. 4A). EPEC Δ *nleF* infection of Caco-2 cells silenced for caspase-4 expression

110 (C4) did not secrete mIL-18, as measured by both western blotting (Fig. 4B) and ELISA (Fig.
111 4C), clearly pointing to a requirement of caspase-4 in IL-18 processing during EPEC
112 infection of IECs. Importantly, no cell death was detectable by measuring LDH release or PI
113 uptake following infection of control or caspase-4-depleted Caco-2 cells (Fig. 4D); this is
114 likely due to EPEC T3SS effectors (e.g. NleH, NleB), which inhibit cell death^(5,6,15). Thus, in
115 human IECs, pro-IL-18 processing during EPEC infection is caspase-4 dependent and the
116 bacterially injected NleF specifically inhibits this process.

117 ***C. rodentium* inhibits IL-18 secretion *in vivo* in an NleF_{CR}-dependent manner**

118 To test the role of NleF during infection *in vivo* we infected C57BL/6 mice with WT *C.*
119 *rodentium*, *C. rodentium* Δ *nleF* or *C. rodentium* Δ *nleF* complemented with *pnleF*_{CR}.
120 Colonization (Fig. 5A) and colonic crypt hyperplasia (Fig. 5B) were similar between the
121 different *C. rodentium* strains (Fig. 5). We quantified levels of IL-18 and IL-1 β secreted from
122 colonic explants, and the inflammasome-independent chemokine CXCL1 as a control, on
123 days 4 and 8 post-infection (p.i.). On day 4 post infection of C57BL/6 mice with *C.*
124 *rodentium* Δ *nleF* we detected a significantly increased colonic secretion of IL-18, while
125 mock-infected (PBS) or WT *C. rodentium*-infected colons released similarly low levels of IL-
126 18 (Fig. 5C). Complementing the *C. rodentium* Δ *nleF* mutant with a plasmid encoding
127 NleF_{CR} restored the inhibition of IL-18 secretion (Fig. 5C); secreted IL-1 β was below the
128 detectable limit (data not shown). Secretion of CXCL1 was similar in colons extracted from
129 mice treated with PBS or infected with WT *C. rodentium* or *C. rodentium* Δ *nleF* (Fig. 5D).
130 Complementing the *C. rodentium* Δ *nleF* mutant with a plasmid encoding NleF_{CR} resulted in a
131 significantly increased CXCL1 secretion (Fig. 5D), which is consistent with our recent
132 finding that over expression of NleF_{EPEC} activates NF- κ B in cultured cells⁽²⁰⁾. Importantly,
133 NleF-dependent inhibitory effects were only observed early during infection (day 4 p.i.), and

134 IL-18 secretion was similar following WT *C. rodentium* or *C. rodentium* $\Delta nleF$ infection on
135 day 8 p.i. (Fig. 5E).

136 To validate that NleF_{CR} inhibits IL-18 secretion via the inflammasomes, we first infected
137 *Casp1/11* deficient mice with *C. rodentium* and *C. rodentium* $\Delta nleF$. As expected, loss of
138 *Casp1* and *Casp11* abolished IL-18 secretion from colonic explants after infection with WT
139 *C. rodentium* or *C. rodentium* $\Delta nleF$ (Fig. 5C); CXCL1 secretion was similar in *Casp1/11*^{-/-}
140 mice infected with the two strains (data not shown). In order to confirm that the phenotype
141 was due to caspase-11, we next infected *Casp11*^{-/-} mice with *C. rodentium* or *C. rodentium*
142 $\Delta nleF$. This showed that while WT *C. rodentium* and *C. rodentium* $\Delta nleF$ colonized the
143 *Casp11*^{-/-} mice at comparable levels (Fig. 5F), secretion of IL-18 was extremely low and
144 similar to that in *Casp1/11*^{-/-} mice (Fig. 5C). We therefore concluded that caspase-11 is
145 responsible for secretion of IL-18 following infection with *C. rodentium* $\Delta nleF$.

146 **IL-18 is essential for the recruitment of neutrophils early during *C. rodentium* infection**

147 As IL-18 facilitates neutrophil and leukocyte recruitment to sites of inflammation⁽²¹⁾, we
148 investigated the effect of NleF_{CR} on immune cell recruitment. Infection of C57BL/6 mice for
149 4 days with *C. rodentium* $\Delta nleF$ resulted in a significant increase in neutrophil recruitment in
150 comparison to WT *C. rodentium*-infected or PBS-treated mice (Fig. 6B). Infection with the
151 *C. rodentium* $\Delta nleF$ *pnleF*_{CR} strain restored the inhibition of neutrophil recruitment (Fig. 6B).
152 No significant differences were observed for other myeloid or lymphocyte cell type analyzed,
153 including macrophages, ILC, B-cells and T-cells (data not shown). Furthermore, correlating
154 with similar IL-18 secretion, no difference in neutrophil recruitment was observed at day 8
155 post infection (Fig. 6C), suggesting that NleF_{CR} plays a specific role during early immune
156 responses to *C. rodentium*. Enhanced neutrophil influx was *Casp1/11* dependent; absence of
157 these caspases abolished the increase in neutrophil recruitment during infection with *C.*
158 *rodentium* $\Delta nleF$ (Fig. 6B). Similar results were obtained following infection of *Casp11*^{-/-}

159 mice (Fig. 6B). Thus NleF_{CR} is a virulence factor responsible for early inhibition of the host
160 inflammasomes, and that the inflammasome is essential for early neutrophil recruitment in
161 response to *C. rodentium* infection.

162 **Discussion**

163 Inflammasome dependent cytokines and pyroptosis have important antimicrobial
164 functions^(8,9). It is therefore not surprising that pathogenic bacteria have evolved mechanisms
165 to prevent inflammasome activation⁽²²⁾. For example, *Yersinia* uses YopK to prevent
166 detection of its T3SS⁽²²⁾, and bacteria modify their LPS to evade detection by caspase-11⁽²³⁾.
167 The *Shigella flexneri* effector OspC3 sequesters caspase-4 activity by binding the caspase-4
168 p20 subunit to prevent p10 binding and oligomerization⁽²⁴⁾. Here we demonstrate that a
169 virulence factor of A/E pathogens, NleF, targets the heterotetramer complex of caspase-4 via
170 its C-terminal motif, underlining the importance of caspase-4 inhibition during the course of
171 infection.

172 In agreement with our biochemical analyses, EPEC was able to inhibit caspase-4 in IECs in
173 an NleF-dependent manner, while recent reports showed that infection of cultured cells with
174 either *Salmonella* or EPEC led to caspase-4 activation⁽²⁴⁾ and caspase-4-dependent induction
175 of IL-18 release⁽¹⁴⁾. Taken together, our data suggest that while EPEC can initiate caspase-4
176 activation and IL-18 processing, NleF dampens this response. Previous studies have shown
177 that *Nlrp3*, *Nlrc4*, *Casp1* and *Casp11* are important in protection against *C. rodentium*
178 infection^(16,25). Loss of inflammasome-related genes results in significantly increased *C.*
179 *rodentium* bacterial load in the intestine late in infection, which may partly explain the
180 enhanced inflammation in inflammasome-deficient mice infected with *C. rodentium*. Loss of
181 inflammasome-dependent IL-1 β and IL-18 also results in enhanced bacterial burdens at late
182 stages of infection and susceptibility to *C. rodentium* infection of *Il1b*^{-/-} and *Il18*^{-/-} mice⁽¹⁶⁾.
183 Our studies establish that NleF functions at early stages of infection of mucosal surfaces by
184 inhibiting the inflammasome and preventing release of IL-18 by epithelial cells.

185 We also found that NleF_{CR} inhibited caspase-11-dependent neutrophil recruitment. IL-18 is a
186 key regulator of the adaptive immune response, stimulates the migration of innate and
187 adaptive immune cells^(21,26,27), and controls intestinal epithelial cell turnover and protects
188 against damage in the intestine⁽²⁸⁾. During the early stages of infection, IL-18 is largely
189 secreted by epithelial cells⁽¹⁷⁾. Current data⁽¹⁶⁾, including the secretion of IL-1 β which is not
190 expressed in non-hematopoietic cells⁽²⁹⁾, suggests that at later time points during *C.*
191 *rodentium* infection colonic IL-18 secretion may switch to be myeloid cell dependent⁽¹⁶⁾.
192 Therefore, myeloid cell secretion of the IL-1 family cytokines may not be subverted by
193 NleF_{CR} and would become the pre-dominant source of IL-18 and IL-1 β at the peak of
194 infection. Similarly secretion of IL-22 is switched from ILC3 at early phase of infection to
195 IL-22-producing T cells at later time points (> 7 days)⁽³⁰⁾.

196 The study demonstrates a pathway during infection of IECs, which leads to the activation of
197 caspase-11, secretion of IL-18 and recruitment of neutrophil. In addition, we show that
198 inhibition of caspase-11 by bacterial NleF blocks this pathway in the host. Our findings are
199 consistent with the recent study on the epithelial cell caspase-11–IL-18 axis during
200 *Salmonella* infection, which reported significant neutrophil influx in infected gall bladder
201 epithelia of wild-type mice, but no neutrophil influx in *Casp11*^{-/-} mice⁽³¹⁾.

202 Recent studies have revealed the contribution of non-inflammasome and inflammasome-
203 forming NLRs in the non-hematopoietic compartment for intestinal homeostasis and the host
204 mediated clearance and protection against enteric pathogens⁽³⁵⁾. Mice deficient in NLRP6
205 have impaired goblet cell mucus exocytosis and display a microbiome exposed epithelial cell
206 layer and persistence of *C. rodentium* infection⁽³⁶⁾. Moreover, NLRP12 is a checkpoint for
207 non-hematopoietic non-canonical NF- κ B activation⁽³⁷⁾, and acts as a negative regulator of
208 colitis and colitis-associated colon cancer. Furthermore, IEC-expressed NLRC4 mediates
209 early innate immune responses against *C. rodentium* via an unknown mechanism independent

210 of IL-1 family cytokine secretion⁽³⁸⁾. Here we show that the caspase-4/11 dependent IECs
211 inflammasome is crucial for IL-18 cytokine maturation and the early innate immune response
212 to EPEC/ *C. rodentium*. Consistently with this, Song-Zhao et al.⁽¹⁷⁾ recently suggested, based
213 on studies of *Nlrp3*^{-/-} and *Asc*^{-/-} mice, that early protection to *C. rodentium* infection is
214 mediated by IECs independently of NLRP3 activation. Taken together, our study identifies a
215 fundamental and novel role for the T3SS effector NleF in the pathogenesis and virulence of
216 A/E pathogens through the inhibition of the newly characterized IECs caspase-4/11
217 dependent inflammasome.

218 **Methods**

219 **Strains, oligonucleotides, plasmids and antibodies**

220 Strains, plasmids and primers used in this study are listed in Tables S2-S3 respectively. *nleF*
221 was amplified from EPEC E2348/69 and *C. rodentium* ICC169 genomic DNA by PCR. Site-
222 directed mutagenesis was carried out by inverse PCR using KOD Hot Start polymerase and
223 mismatch primers. All constructs were confirmed by sequencing (GATC biotech). For
224 Western Blot, Mouse monoclonal anti-caspase-4 clone 4B9 (sc-56056; Santa Cruz), anti- α -
225 Tubulin clone DM1A (T6199), mouse polyclonal antibody anti-caspase-11 p20 clone A-2
226 (sc-374615; Santa cruz) and anti-pro-IL-18 (CPTC-IL18-1; DSHB), the rabbit monoclonal
227 anti-IL-18 (PM014; MBL), anti-caspase-5 (4429; Cell signalling) and the rabbit polyclonal
228 antibody anti-GFP (Ab290; Abcam) were used as primary antibodies. Horse radish
229 peroxidase (HRP)-conjugated goat anti-rabbit IgG (Fc fragment; catalog no.111-035-008;
230 Jackson immunoresearch) and HRP-conjugated goat anti-mouse IgG (Fc fragment; catalog
231 no, 115-035-008; Jackson immunoresearch) were used as secondary antibodies.

232 **Retroviral transductions and stable knockdown cell lines**

233 Micro-RNA30 based (miR-30; Table S1) gene silencing constructs were generated in pMX-
234 CMV-YFP using one-step sequence and ligation independent cloning (SLIC) (36) following
235 the optimized miR-30E vector design⁽³⁹⁾. Sequences were as follows: *CASP4* -
236 CGACTGTCCATGACAAGAT; and *LacZ* (non-targeting negative control)
237 ACGTCGTATTACAACGTCGTGA. The miR-30E plasmids were transfected using
238 Lipofectamine 2000 (Invitrogen), along with the packaging plasmids pVSV-G and pCMV-
239 MMLV-pack⁽⁴⁰⁾ into HEK293E cells to produce a VSV-G pseudotyped retroviral particles for
240 transduction. After 48 h supernatants were filtered through 0.45 μ m syringe filters and added
241 directly to pre-seeded Caco-2 TC7 cells. Transduced cells were selected by puromycin
242 (Gibco Invitrogen) at 10 μ g.ml⁻¹ and knockdown was confirmed by western blotting.

243 **EPEC infection, ELISA and Western blotting**

244 Caco-2 TC7 cells (ATCC) were seeded at 7.5×10^4 /ml and upon reaching confluence (7 days)
245 the medium was changed every day for 7 the following 7 days. Before infection the cells
246 were starved for 3 h in serum free DMEM. Monolayers were infected with primed EPEC⁽²⁰⁾
247 at an MOI of 1:10 for 3 h. The cells were then washed twice in PBS and the medium was
248 replaced with serum free DMEM-high glucose plus penicillin and streptomycin at 100 U/ml
249 and 100 µg/ml, respectively. After 1 h cells were washed and either processed for Western
250 Blot (total IL-18) or incubated for a further 17 h (secreted caspase-4 and IL-18) with or
251 without Ac-LEVD-CHO (Enzo Lifesciences). Supernatants were collected, cleared by
252 centrifugation at 13000 rpm at 4 °C for 10 min and precipitated for Western blotting with the
253 addition of 10 % (v/v) trichloroacetic acid for 17 h at 4 °C. The concentration of IL-18 in cell
254 supernatant (MBL) was determined by ELISA according to the manufacturer's protocol.

255 **Cell adhesion and cytotoxicity assays**

256 Caco-2 TC7 were infected with the WT EPEC, EPEC Δ *nleF* and the complemented strain
257 (*pnleFEPEC*) for 3 h. The monolayers were lysed in 1 % PBS/triton X-100 and EPEC
258 attachment was enumerated by serial dilution on LB-Agar and calculation of colony forming
259 units (CFU).

260 Supernatants of uninfected cells or cell infected with EPEC for 21 h were harvested and the
261 level of LDH release was measured using CytoTox 96® Non-Radioactive Cytotoxicity Assay
262 (Promega). As a control for total LDH, cell lysis buffer (1 % Triton-X100/ PBS) was added
263 for 30 min at 37 °C directly to the medium and cell layer. Absorbance was measured at 490
264 nm using the FluoStar Omega plate reader and results are displayed as percentage of total
265 release corresponding to the LDH measured in the supernatant divided by the total LDH.

266 Alternatively the media was removed and cell layers were incubated in 3.3 µg/ml propidium
267 iodide (Invitrogen) in warm PBS (PI/PBS) for 15 min and fluorescence was measured at an
268 excitation of 510 nm and emission of 610 nm using the FluoStar Omega plate reader. As a
269 control PI/PBS alone was measured or cell lysis buffer (0.05 % Triton X-100/PBS)
270 supplemented with 3.3 µg/ml propidium iodide was added for 15 min at 37°C. Results are
271 displayed as a percentage of total PI uptake.

272 **Yeast-2-hybrid screen and yeast direct hybrids**

273 A yeast-2-hybrid screen was conducted using pGKBT7-*nleF*_{EPEC} and the HeLa cell cDNA
274 Library following the manufacturer's Handbook (Clontech). AH109 were co-transformed
275 with pGBT9-*bait* and pGADT7-*prey* (Table S3) and plated onto Difco Yeast Nitrogen Base
276 without amino acids (SD) agar supplemented with 2% glucose, 20 mg/L adenine hemisulfate,
277 20 mg/L arginine HCl, 20 mg/L histidine HCl monohydrate, 30 mg/L isoleucine, 30 mg/L
278 lysine HCl, 20 mg/L methionine, 50 mg/L phenylalanine, 200 mg/L threonine, 30 mg/L
279 tyrosine, 20/L mg uracil, 150 mg/ml valine and lacking tryptophan and leucine (Double
280 Drop-out; DDO) for selection of transformed clones. Clones positive for both plasmids were
281 re-streaked on to SD DDO and SD QDO /-His/-Ade supplemented with 40 mg/L x-α-gal (SD
282 QDO) for selection of positive interactions.

283 **Recombinant Protein expression and purification**

284 *E. coli* BL21 Star expressing pET28-NleF_{EPEC} (pICC1659), pET28-NleF_{1-185-EPEC} (pICC1660)
285 and pET28-NleF_{CR} (pICC1839) were cultured for 16 h in LB at 37 °C at 200 rpm. Bacteria
286 were sub-cultured at 1:100 into 1 L LB supplemented with 50 µg/ml kanamycin and
287 incubated at 37°C at 200 rpm until OD₆₀₀ of 0.4-0.6. Cultures were then induced with 0.5
288 mM IPTG for 18 h at 18°C. Cells were harvested by centrifugation at 10000 rpm for 20 min
289 and re-suspended in 30 ml ice cold His-lysis buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl,

290 and 5 mM Imidazole). The cells were lysed by Emulsiflex following the manufacturer's
291 instructions (Emulsiflex-B15; Avestin) and centrifuged at 14000 rpm for a further 30 mins at
292 4⁰C. Supernatant was removed and applied to 5 ml His resin (Novagen) pre-charged in 5 mM
293 NiSO₄ and pre-equilibrated in His-lysis buffer and rocked at 4⁰C for 1.5 h. Samples were
294 applied to a Poly-Prep Chromatography column (Qiagen) and flow-through was collected.
295 The column was washed twice with 20 ml His-lysis buffer and once in 20 ml wash buffer
296 (Tris-HCl pH 7.9, 0.5 M NaCl, and 60 mM Imidazole). His-tagged fusion proteins were
297 eluted with 10 x 1 ml elution buffer (His-lysis buffer supplemented with 1 M Imidazole).
298 Fractions containing His-purified NleF were checked by SDS-PAGE gel electrophoresis and
299 further purified by size exclusion (Akta prime) with a Superdex75 column (GE Healthcare;
300 10/300GL).

301 **Co-purification of the caspase-4-NleF_{EPEC} complex**

302 BL21 Star cells were co-transformed with pACYC-DUET-1-CASP4^{C258S} His-p20/p10 and
303 pMAL-c2x-nleF_{EPEC}. Bacterial pellets were re-suspended in 20mM Tris-HCl pH 7.4, 250
304 mM NaCl and lysed by sonication and purified by amylose affinity chromatography.
305 Bacterial lysates were incubated with amylose resin for 1.5 h at 4 °C and then washed with
306 50 ml wash buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl and eluted with wash buffer
307 supplemented with 10 mM maltose. The co-elute was dialysed and then purified further by
308 IMAC talon affinity chromatography, as described previously⁽⁴¹⁾. Complex formation was
309 analysed by size exclusion (Akta prime) with a Superdex200 column (GE Healthcare) using
310 the Gel Filtration Markers Kit for Protein Molecular Weights 12,000-200,000 Da (Sigma-
311 Aldrich) to determine complex size. Size exclusion fractions were verified by SDS PAGE gel
312 and confirmed by Mass spectrometry.

313 **Caspase activity assays**

314 BL21 star were transformed with pET28a-empty, pET28a-CASP4, pET28a-CASP4 C258S or
315 pET28a-Casp11. Soluble lysates at 200 µg/ml were incubated with or without 50 µM Ac-
316 LEVD-AFC (Enzo Life Sciences) in 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM
317 EDTA, 0.1% CHAPS, 10% sucrose pH 7.2 or 20 mM Tris, 250 mM NaCl pH 7.4 for
318 caspase-11 and caspase-4, respectively. Purified recombinant His-NleF derivatives were
319 added at varying concentrations from 50 nM to 1 pM. Fluorescence was measured in 5 min
320 intervals at 37 °C using an excitation of 410 nm and emission of 520 nm using the FLUOstar
321 Omega plate reader (BMG Labtech).

322 **Construction of *C. rodentium* mutant**

323 *C. rodentium* strain ICC169 $\Delta nleF$ (ICC1129) was generated using a modified version of the
324 lambda red-based mutagenesis system⁽⁴²⁾. Briefly, the *nleF* gene and its flanking regions were
325 PCR-amplified from WT *C. rodentium* ICC169 genomic DNA using the primers pair NleF-
326 up-Fw/NleF-down-Rv and cloned into pC-Blunt-TOPO vector (Invitrogen). The *nleF* gene
327 was then excised using inverse-PCR (primers NleF-up-Rv-BamHI/NleF-down-Fw-BamHI)
328 and the resulting linear product was BamHI digested, allowing insertion of the non-polar
329 *aphT*⁽⁴³⁾ cassette, resulting in plasmid pICC1674. After verifying for correct orientation of
330 the kanamycin cassette, the insert was PCR-amplified using NleF-up-Fw and NleF-down-Rv
331 primers. The PCR products were electroporated into wild type *C. rodentium* expressing the
332 lambda red recombinase from pKD46 plasmid. The deletion was confirmed by PCR and
333 DNA sequencing amongst the kanamycin resistant clones (primers NleF-up-Fw-check and
334 NleF-down-Fw-check).

335 **Oral infection of mice**

336 Pathogen-free female C57BL/6 mice were either purchased from Charles River or sourced
337 from BIME Institut Pasteur. *Casp1/11^{-/-}* mice were generously provided by Bernhard Ryffel
338 (TAAM-CDTA, Orelans, France) and *Casp11^{-/-}* were generously provided by Mohamed
339 Lamkanfi (Ghent University, Belgium). All animals were housed in individually HEPA-
340 filtered cages with sterile bedding and free access to sterilized food and water. Independent
341 infection experiments for wild-type C57BL/6, *Casp1/11^{-/-}* and *Casp11^{-/-}* mice were performed
342 using 3 to 8 mice per group. Mice were infected and followed for shedding as described⁽⁴⁴⁾.
343 Briefly, mice were infected via oral gavage with 10⁹ WT *C. rodentium* or *C. rodentium* Δ *nleF*
344 as described previously. For control, mice were gavaged with sterile PBS. The number of
345 viable bacteria used as inoculum was determined by retrospective plating onto LB agar
346 containing antibiotics. Stool samples were recovered aseptically at various time points after
347 inoculation and the number of viable bacteria per gram of stool was determined by plating
348 onto LB agar⁽⁴⁴⁾.

349 **Sample collection and colonic crypt hyperplasia measurement**

350 Segments of the terminal colon (0.5 cm) of each mouse were collected, flushed and fixed in
351 10% neutral buffered formalin. Formalin fixed tissues were then processed, paraffin-
352 embedded, sectioned at 5 μ m and stained with haematoxylin and eosin (H&E) using standard
353 techniques. H&E stained tissues were evaluated for colonic crypt hyperplasia microscopically
354 without knowledge of the treatment condition used in the study and the length of at least 100
355 well-oriented crypts from each section from all of the mice per treatment group (n=4-6) were
356 evaluated. H&E stained tissues were imaged with an Axio Lab.A1 microscope (Carl Zeiss
357 MicroImaging GmbH Germany), images were acquired using an Axio Cam ERc5s colour
358 camera, and computer-processed using AxioVision (Carl Zeiss MicroImaging GmbH,
359 Germany).

360 **Sample collection for cytokine analysis and flow cytometry**

361 Isolation of colonic cells and flow cytometry were performed as described⁽⁴⁴⁾. After a PBS
362 wash, the 5th cm of the distal colon was incubated in RPMI containing penicillin,
363 streptomycin, gentamicin and FBS at 37°C for 24 h. The concentrations of IL-18
364 (eBioscience, #BMS618/3), IL-1 β and KC (CXCL1; R&D Systems) were determined by
365 ELISA according to the manufacturer's protocols.

366 **Statistics**

367 All data was analyzed using GraphPad Prism software, using the Mann-Whitney test and
368 represented as the mean +/- standard error of mean or standard deviation. A P value less than
369 0.05 (P<0.05) was considered statistically significant.

370 **References**

371

372 (1) Croxen, M.A., Law, R.J., Scholz, R., Keeney, K.M., Wlodarska, M. & Finlay, B.B.
373 Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clin. Microbiol. Rev.*
374 26, 822-880 (2013).

375 (2) Collins, J.W., Keeney, K.M., Crepin, V.F., Rathinam, V.A., Fitzgerald, K.A., Finlay,
376 B.B., *et al.* *Citrobacter rodentium*: infection, inflammation and the microbiota. *Nat. Rev.*
377 *Microbiol.* 12, 612-623 (2014).

378 (3) Baruch, K., Gur-Arie, L., Nadler, C., Koby, S., Yerushalmi, G., Ben-Neriah, Y., *et al.*
379 Metalloprotease type III effectors that specifically cleave JNK and NF-kappaB. *EMBO J.* 30,
380 221-231 (2011).

381 (4) Zhang, L., Ding, X., Cui, J., Xu, H., Chen, J., Gong, Y., *et al.* Cysteine methylation
382 disrupts ubiquitin-chain sensing in NF-κB activation. *Nature* 481, 204-208 (2011).

383 (5) Pearson, J.S., Riedmaier, P., Marchès, O., Frankel, G. & Hartland, E.L. A type III effector
384 protease NleC from enteropathogenic *Escherichia coli* targets NF-κB for degradation. *Mol.*
385 *Microbiol.* 80, 219-230 (2011).

386 (6) Li, S., Zhang, L., Yao, Q., Li, L., Dong, N., Rong, J., *et al.* Pathogen blocks host death
387 receptor signalling by arginine GlcNAcylation of death domains. *Nature* 501, 242-246
388 (2013).

389 (7) Pham, T.H., Gao, X., Singh, G. & Hardwidge, P.R. *Escherichia coli* virulence protein
390 NleH1 interaction with the v-Crk sarcoma virus CT10 oncogene-like protein (CRKL)

391 governs NleH1 inhibition of the ribosomal protein S3 (RPS3)/nuclear factor kappaB (NF-
392 kappaB) pathway. *J. Biol. Chem.* 288, 34567-34574 (2013).

393 (8) von Moltke, J., Ayres, J.S., Kofoed, E.M., Chavarria-Smith, J. & Vance, R.E. Recognition
394 of bacteria by inflammasomes. *Annu. Rev. Immunol.* 31, 73-106 (2013).

395 (9) Eldridge, M.J. & Shenoy, A.R. Antimicrobial inflammasomes: unified signalling against
396 diverse bacterial pathogens. *Curr. Opin. Microbiol.* 23, 32-41 (2015).

397 (10) Shi, J., Zhao, Y., Wang, Y., Gao, W., Ding, J., Li, P., *et al.* Inflammatory caspases are
398 innate immune receptors for intracellular LPS. *Nature* 514, 187-192 (2014).

399 (11) Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., *et al.*
400 Non-canonical inflammasome activation targets caspase-11. *Nature* 479, 117-121 (2011).

401 (12) Casson, C.N., Yu, J., Reyes, V.M., Taschuk, F.O., Yadav, A., Copenhaver, A.M., *et al.*
402 Human caspase-4 mediates noncanonical inflammasome activation against gram-negative
403 bacterial pathogens. *Proc. Natl. Acad. Sci. U. S. A.* 112, 6688-6693 (2015).

404 (13) Rathinam, V.A., Vanaja, S.K., Waggoner, L., Sokolovska, A., Becker, C., Stuart, L.M.,
405 *et al.* TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-
406 negative bacteria. *Cell* 150, 606-619 (2012).

407 (14) Knodler, L.A., Crowley, S.M., Sham, H.P., Yang, H., Wrande, M., Ma, C., *et al.*
408 Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses
409 against enteric bacterial pathogens. *Cell. Host Microbe* 16, 249-256 (2014).

- 410 (15) Hemrajani, C., Berger, C.N., Robinson, K.S., Marches, O., Mousnier, A. & Frankel, G.
411 NleH effectors interact with Bax inhibitor-1 to block apoptosis during enteropathogenic
412 *Escherichia coli* infection. *Proc. Natl. Acad. Sci. U. S. A.* 107, 3129-3134 (2010).
- 413 (16) Liu, Z., Zaki, M.H., Vogel, P., Gurung, P., Finlay, B.B., Deng, W., *et al.* Role of
414 inflammasomes in host defense against *Citrobacter rodentium* infection. *J. Biol. Chem.* 287,
415 16955-16964 (2012).
- 416 (17) Song-Zhao, G.X., Srinivasan, N., Pott, J., Baban, D., Frankel, G. & Maloy, K.J. Nlrp3
417 activation in the intestinal epithelium protects against a mucosal pathogen. *Mucosal Immunol.*
418 7, 763-774 (2014).
- 419 (18) Yen, H., Sugimoto, N. & Tobe, T. Enteropathogenic *Escherichia coli* Uses NleA to
420 Inhibit NLRP3 Inflammasome Activation. *PLoS Pathog.* 11, e1005121 (2015).
- 421 (19) Blasche, S., Mortl, M., Steuber, H., Siszler, G., Nisa, S., Schwarz, F., *et al.* The *E. coli*
422 Effector Protein NleF Is a Caspase Inhibitor. *PLoS ONE* 8, e58937 (2013).
- 423 (20) Pallett, M.A., Berger, C.N., Pearson, J.S., Hartland, E.L. & Frankel, G. The type III
424 secretion effector NleF of enteropathogenic *Escherichia coli* activates NF-kappaB early
425 during infection. *Infect. Immun.* 82, 4878-4888 (2014).
- 426 (21) Dinarello, C.A. & Fantuzzi, G. Interleukin-18 and host defense against infection. *J.*
427 *Infect. Dis.* 187 Suppl 2, S370-84 (2003).
- 428 (22) Brodsky, I.E., Palm, N.W., Sadanand, S., Ryndak, M.B., Sutterwala, F.S., Flavell, R.A.,
429 *et al.* A *Yersinia* effector protein promotes virulence by preventing inflammasome
430 recognition of the type III secretion system. *Cell. Host Microbe* 7, 376-387 (2010).

- 431 (23) Hagar, J.A., Powell, D.A., Aachoui, Y., Ernst, R.K. & Miao, E.A. Cytoplasmic LPS
432 activates caspase-11: implications in TLR4-independent endotoxic shock. *Science* 341, 1250-
433 1253 (2013).
- 434 (24) Kobayashi, T., Ogawa, M., Sanada, T., Mimuro, H., Kim, M., Ashida, H., *et al.* The
435 *Shigella* OspC3 effector inhibits caspase-4, antagonizes inflammatory cell death, and
436 promotes epithelial infection. *Cell. Host Microbe* 13, 570-583 (2013).
- 437 (25) Gurung, P., Malireddi, R.K., Anand, P.K., Demon, D., Vande Walle, L., Liu, Z., *et al.*
438 Toll or interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-beta
439 (TRIF)-mediated caspase-11 protease production integrates Toll-like receptor 4 (TLR4)
440 protein- and Nlrp3 inflammasome-mediated host defense against enteropathogens. *J. Biol.*
441 *Chem.* 287, 34474-34483 (2012).
- 442 (26) Verri, W.A., Jr, Cunha, T.M., Ferreira, S.H., Wei, X., Leung, B.P., Fraser, A., *et al.* IL-
443 15 mediates antigen-induced neutrophil migration by triggering IL-18 production. *Eur. J.*
444 *Immunol.* 37, 3373-3380 (2007).
- 445 (27) Canetti, C.A., Leung, B.P., Culshaw, S., McInnes, I.B., Cunha, F.Q. & Liew, F.Y. IL-18
446 enhances collagen-induced arthritis by recruiting neutrophils via TNF-alpha and leukotriene
447 B4. *J. Immunol.* 171, 1009-1015 (2003).
- 448 (28) Dupaul-Chicoine, J., Yeretssian, G., Doiron, K., Bergstrom, K.S., McIntire, C.R.,
449 LeBlanc, P.M., *et al.* Control of intestinal homeostasis, colitis, and colitis-associated
450 colorectal cancer by the inflammatory caspases. *Immunity* 32, 367-378 (2010).

- 451 (29) Daig, R., Rogler, G., Aschenbrenner, E., Vogl, D., Falk, W., Gross, V., *et al.* Human
452 intestinal epithelial cells secrete interleukin-1 receptor antagonist and interleukin-8 but not
453 interleukin-1 or interleukin-6. *Gut* 46, 350-358 (2000).
- 454 (30) Basu, R., O'Quinn, D.B., Silberger, D.J., Schoeb, T.R., Fouser, L., Ouyang, W., *et al.*
455 Th22 cells are an important source of IL-22 for host protection against enteropathogenic
456 bacteria. *Immunity* 37, 1061-1075 (2012).
- 457 (31) Knodler, L.A., Crowley, S.M., Sham, H.P., Yang, H., Wrande, M., Ma, C., *et al.*
458 Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses
459 against enteric bacterial pathogens. *Cell. Host Microbe* 16, 249-256 (2014).
- 460 (32) Verri, W.A., Jr, Cunha, T.M., Ferreira, S.H., Wei, X., Leung, B.P., Fraser, A., *et al.* IL-
461 15 mediates antigen-induced neutrophil migration by triggering IL-18 production. *Eur. J.*
462 *Immunol.* 37, 3373-3380 (2007).
- 463 (33) Canetti, C.A., Leung, B.P., Culshaw, S., McInnes, I.B., Cunha, F.Q. & Liew, F.Y. IL-18
464 enhances collagen-induced arthritis by recruiting neutrophils via TNF-alpha and leukotriene
465 B4. *J. Immunol.* 171, 1009-1015 (2003).
- 466 (34) Spehlmann, M.E., Dann, S.M., Hruz, P., Hanson, E., McCole, D.F. & Eckmann, L.
467 CXCR2-dependent mucosal neutrophil influx protects against colitis-associated diarrhea
468 caused by an attaching/effacing lesion-forming bacterial pathogen. *J. Immunol.* 183, 3332-
469 3343 (2009).
- 470 (35) Elinav, E., Henao-Mejia, J. & Flavell, R.A. Integrative inflammasome activity in the
471 regulation of intestinal mucosal immune responses. *Mucosal Immunol.* 6, 4-13 (2013).

- 472 (36) Wlodarska, M., Thaiss, C.A., Nowarski, R., Henao-Mejia, J., Zhang, J.P., Brown, E.M.,
473 *et al.* NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating
474 goblet cell mucus secretion. *Cell* 156, 1045-1059 (2014).
- 475 (37) Allen, I.C. Non-Inflammasome Forming NLRs in Inflammation and Tumorigenesis.
476 *Front. Immunol.* 5, 169 (2014).
- 477 (38) Nordlander, S., Pott, J. & Maloy, K.J. NLRC4 expression in intestinal epithelial cells
478 mediates protection against an enteric pathogen. *Mucosal Immunol.* 7, 775-785 (2014).
- 479 (39) Fellmann, C., Hoffmann, T., Sridhar, V., Hopfgartner, B., Muhar, M., Roth, M., *et al.*
480 An optimized microRNA backbone for effective single-copy RNAi. *Cell. Rep.* 5, 1704-1713
481 (2013).
- 482 (40) Shenoy, A.R., Wellington, D.A., Kumar, P., Kassa, H., Booth, C.J., Cresswell, P., *et al.*
483 GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals. *Science* 336,
484 481-485 (2012).
- 485 (41) Kotik-Kogan, O., Valentine, E.R., Sanfelice, D., Conte, M.R. & Curry, S. Structural
486 analysis reveals conformational plasticity in the recognition of RNA 3' ends by the human La
487 protein. *Structure* 16, 852-862 (2008).
- 488 (42) Datsenko, K.A. & Wanner, B.L. One-step inactivation of chromosomal genes in
489 *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97, 6640-6645
490 (2000).
- 491 (43) Galan, J.E., Ginocchio, C. & Costeas, P. Molecular and functional characterization of
492 the *Salmonella* invasion gene *invA*: homology of *InvA* to members of a new protein family.
493 *J. Bacteriol.* 174, 4338-4349 (1992).

494 (44) Crepin, V.F., Habibzay, M., Glegola-Madejska, I., Guenot, M., Collins, J.W. & Frankel,
495 G. Tir Triggers Expression of CXCL1 in Enterocytes and Neutrophil Recruitment during
496 *Citrobacter rodentium* Infection. *Infect. Immun.* 83, 3342-3354 (2015).

497

498 **Acknowledgements**

499 We thank Guy Frankel for making the *CRΔnleF* mutant. We are grateful to Dr. Bernhard
500 Ryffel (TAAM-CDTA, Orelans, France) and Dr. Mohamed (Lamkanfi, [VIB Inflammation](#)
501 [Research Center, Ghent University, Belgium](#)) for providing us with the *Casp1/11^{-/-}* and
502 *Casp11^{-/-}* mice. This project was supported by grants to GF from the Biotechnology and
503 Biological Sciences Research Council (BBSRC), the Wellcome Trust and the Medical
504 Research Council (MRC). ARS acknowledges funds from the Royal Society (RG130811) and
505 the Wellcome Trust (108246/Z/15/Z). JPD and NS are supported by grants from the Institut
506 Pasteur, Inserm and Danone.

507

508

509 **Conflict of interest**

510 The authors declared no conflict of interest.

511

512

513 **Author Contribution**

514 MAP, VFP, NS and CNB - plan and conducted experiments and wrote the paper

515 MH, OK and JSG - plan and conducted experiments

516 JPDS, ARS and GF - plan experiments and wrote the paper

517 **Figure legends**

518 **Fig. 1. NleF binds caspase-4.** (A) A direct yeast two hybrid assay revealed that NleF_{EPEC},
519 but not NleF_{1-185_EPEC}, interacts with full-length and p30 caspase-4. (B) Substitution of amino
520 acids R152A, W313A and R314A within the putative caspase-4 substrate domain abrogated
521 the interaction with NleF_{EPEC}. (C) Fractions of size exclusion of the chromatographic profile
522 of MBP-NleF_{EPEC} and His-p20/p10 caspase-4 purified by amylose and talon affinity
523 chromatography and (D) analyzed by SDS-PAGE gel electrophoresis, revealed that NleF_{EPEC}
524 and caspase-4 subunits co-purified and co-eluted as a macromolecular complex at a MW of
525 ~230 kDa.

526

527 **Fig. 2. NleF inhibits caspase-4 activity.** (A) Recombinant caspase-4, but not caspase-4^{C258S},
528 is auto-activated (western blot) and cleaves the reporter Ac-LEVD-AFC. Results are plotted
529 as relative fluorescence units (RLU) minus background (No Ac-LEVD-AFC) over time
530 (min). (B) Dose-dependent inhibition of caspase-4 Ac-LEVD-AFC cleavage by recombinant
531 NleF_{EPEC}, and NleF_{1-185_EPEC} (shown by Coomassie stained gel). (C) NleF_{EPEC} (10 nM)
532 inhibits the activity of caspase-4 more efficiently than NleF_{CR} (10nM) after 30 min incubation
533 in the presence of Ac-LEVD-AFC. (D) Recombinant caspase-11 is auto-activated (western
534 blot) and cleaves the reporter Ac-LEVD-AFC. (E) Dose dependent inhibition of caspase-11
535 activity by recombinant NleF_{CR} (shown by Coomassie stained gel). (F) NleF_{CR} (50 nM)
536 inhibits the activity of caspase-11 more efficiently than NleF_{EPEC} (50nM) after 30 min
537 incubation in the presence of Ac-LEVD-AFC. Results are expressed as a percentage of wild-
538 type caspase-4 or caspase-11 RLU/min from at least two independent experiments. *
539 indicates P<0.05.

540

541 **Fig. 3. NleF inhibits secretion of caspase-4 and IL-18 during EPEC infection.** (A)
542 Infection of polarized Caco-2 cells with WT EPEC, EPEC Δ nleF or the complemented strain
543 (*pnleF_{EPEC}*) revealed similar levels of cell adhesion (3 h post infection). (B) Caco-2 cells
544 were infected with WT EPEC or EPEC Δ nleF in the absence or presence of the inhibitor Ac-
545 LEVD-CHO (total 21 h). Immunoblotting of supernatants (SN) revealed that EPEC inhibits
546 secretion of active caspase-4 (~28 kDa) in an NleF_{EPEC}-dependent manner, assessed by
547 western blots (upper panel) and quantified by densitometry of multiple experiments (lower
548 panel). (C) Infection of Caco-2 cells with WT EPEC, EPEC Δ nleF or complemented
549 EPEC Δ nleF (*pnleF_{EPEC}*) had no effect on the levels of total IL-18 at 4 h p.i. (D) NleF is
550 essential for inhibition of IL-18 secretion from infected Caco-2 cells (21 h post infection).

551

552 **Fig. 4. NleF_{EPEC} inhibits IL-18 secretion in an caspase-4 dependent manner.** (A) Western
553 blots showing knockdown of caspase-4, but not capsapse-5, by miRNA30E. (B) Infection of
554 Caco-2 cells (21 h) depleted of caspase-4 (C4) revealed that it is essential for IL-18
555 processing in response to infection with EPEC Δ nleF, assessed by western blots (upper panel)
556 and quantified by densitometry of two independent experiments (lower panel). (C) ELISA
557 from two biological repeats showing specific secretion of IL-18 from control (YFP), but not
558 from C4, Caco-2 cells infected for 21 h with EPEC Δ nleF. (D) EPEC does not trigger LDH
559 release or PI uptake during infection (21 h) of control or C4 Caco-2 cells, results are
560 represented as a percentage of total uptake or total release and are an average of two
561 biological repeats carried out in triplicate. * indicates P<0.05.

562

563 **Fig. 5. NleF_{CR} inhibits colonic IL18 secretion 4 days p.i.** WT *C. rodentium*, *C.*
564 *rodentium* Δ nleF and the complemented strain (Δ nleF *pnleF_{CR}*) similarly colonized and

565 triggered colonic hypoplasia in C57BL/6 mice (**A and B**). Each dot in B represents an
566 individual measurement of crypt length (from at least 20 measurements per section per
567 mouse), and horizontal bars represent mean values. Significant increase in secreted IL-18,
568 measured by ELISA, was seen specifically following infection of C57BL/6 with *C.*
569 *rodentium* Δ *nleF* (day 4), but not following infection of either *Casp1/11*^{-/-} or *Casp11*^{-/-} mice
570 (day 4) (**C**) or C57BL/6 (day 8) (**E**). Secreted CXCL1 was found in similar levels, except for
571 the complemented strain, which triggered greater secretion of CXCL1 (**D**). No difference in
572 colonization of *Casp11*^{-/-} mice was seen following infection with WT *C. rodentium* or *C.*
573 *rodentium* Δ *nleF* (**E**). * indicates P<0.05.

574

575 **Fig. 6. NleF_{CR} inhibits colonic neutrophil recruitment 4 days p.i.** C57BL/6, *Casp1/11*^{-/-}
576 and *Casp11*^{-/-} mice were infected with WT *C. rodentium*, *C. rodentium* Δ *nleF* or
577 complemented *C. rodentium* Δ *nleF* (Δ *nleF pnleF*_{CR}). (**A**) Representative image of flow
578 cytometry gating strategy for neutrophils (CD11b+Ly6G+) of control (PBS) and infected
579 C57BL/6 mice. The number of neutrophils (CD11b+Ly6G+) present within the myeloid gate
580 was counted from C57BL/6 (**B-C**, days 4 and 8 post infection), *Casp1/11*^{-/-} or *Casp11*^{-/-} (**B**,
581 day 4 post infection) mice (at least six mice per condition). * indicates P<0.05.