Bacterial virulence factor inhibits caspase-4/11 activation in intestinal epithelial cells

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

INTRODUCTION

Central to the infection strategy of the extracellular pathogens enteropathogenic *Escherichia coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC)1 and *Citrobacter rodentium*2 is injection of type III secretion system effectors into intestinal epithelial cells (IECs) where they target diverse signaling pathways, particularly innate immune signaling. NleC and NleD are Zn-dependent endopeptidases that specifically cleave and disable RelA (p65) and JNK, respectively, thus blocking NF-κB and AP-1 activation.3 NleE is a methyltransferase that specifically modifies a cysteine in the zinc finger domain of TAB2 and TAB3, thus also blocking NF-κB signaling.4 NleB, which also inhibits NF-κB, has an N-acetylglucosamine transferase activity that specifically modifies Arg 117 in the death domain of FADD5,6 and NleH is a serine/threonine kinase that inhibits the RPS3/NF-κB pathway via phosphorylation of CRKL (v-Crk sarcoma virus CT10 oncogene-like protein).7 Inhibition of innate immunity by EPEC and EHEC is needed to counter its activation by the type III secretion system (T3SS), flagellins, and lipopolysaccharides (LPS), which are readily detected by sensors and receptors in mammalian hosts. In response to infection, some sensors assemble macromolecular complexes called inflammasomes to stimulate the protease activity of caspase-1. The proteolytic processing and release of interleukin (IL)-1β and IL-18, and the induction of pyroptotic cell death triggered by caspase-1 can prevent the establishment and spread of microbial pathogens.8,9 In addition, the single mouse caspase-11 and the related human caspase-4 and caspase-5 act as cytosolic receptors, which bind LPS directly via their N-terminal caspase activation and recruitment domains (p22 domain). LPS binding induces oligomerization and auto-proteolytic activation of caspase-4/5/11 into their active p20/p10 fragments and subsequent pyroptotic lysis of bacterially infected host cells.10 In human and mouse
phagocytic cells, LPS is detected by caspase-4/11, which stimulate caspase-1-dependent maturation of IL-1β and IL-18 via the NLRP3-ASC inflammasome.11–13 However, in IECs caspase-4/11 acts independently of NLRP3 and caspase-1 to directly process IL-18 and induce pyroptosis during Salmonella infection.14 Therefore, the detection of Gram-negative bacteria by IECs markedly contrasts that in myeloid cells. However, unlike Salmonella, which are intracellular pathogens, extracellular pathogens use T3SS to prevent death pathways in host cells to which they intimately adhere.5,6,15 This suggests that EPEC, EHEC, and C. rodentium might manipulate caspase-4/11 and/or inflammasome pathways in IECs.

Previous work on C. rodentium infections in mice showed that loss of inflammasome signaling-related genes such as Nlrp3, Nlrc4, Casp1, Casp11, Il1β, and Il18 results in enhanced morbidity and inflammatory disease, whereas wild-type (WT) mice clear the pathogen within 14–21 days.16,17 Detection of C. rodentium, EHEC, and EPEC in myeloid cells has also been studied previously, and a recent report identified EPEC NleA TSSS effector protein as an inhibitor of NLRP3-caspase-1 inflammasomes.18 However, as IECs use non-canonical, NLRP3-, and caspase-1-independent mechanisms to detect bacteria, we hypothesized that EPEC and C. rodentium subvert caspase-4/11 action in IECs upon initial attachment. Here we report that bacterial T3SS effector NleF is a potent inhibitor of mammalian caspase-4/11 and thus prevents IL-18 secretion from IECs in vitro, and blocks caspase-11—IL-18 mediated neutrophil influx during infection in vivo.

RESULTS

NleF binds human caspase-4
The highly conserved effector NleF was previously reported to bind the active site and to inhibit the activity of caspase-9, caspase-8, and caspase-4, however, whether NleF affects inflammasome signaling and the innate immune response to bacterial infection in vivo has not been tested.19 By employing a yeast-2-hybrid screen (Supplementary Table S1 online) and a direct yeast-2-hybrid assay (Figure 1a) we confirmed that human caspase-4 is an interacting partner of EPEC NleF (NleFePEC). Truncation analyses revealed an interaction between NleFePEC and the p30 catalytic domain of caspase-4 (Figure 1b). Deletion of four C-terminal residues in NleFePEC (NleF1–185_EPEC) abrogates its binding to caspase-9,19 and similar defects were seen in binding to caspase-4 (Figure 1b). Mutation of the substrate-binding pocket of caspase-4 (R152A, W313A, and R314A) also abolished NleF-caspase-4 interaction (Figure 1b). To confirm that the binding is direct, the caspase-4 p20 subunit (22 kDa; His tagged), p10 subunit (10 kDa), and NleFePEC (65 kDa; maltose-binding protein fusion) were co-expressed, purified by tandem affinity chromatography and analyzed by gel filtration. Three chromatographic peaks corresponding to free maltose-binding protein-NleFePEC, free His-p20, and a complex containing NleFePEC, p20, and p10 subunits were observed (Figure 1c).

NleFePEC and caspase-4 subunits co-purified and co-eluted as a macromolecular complex with an apparent molecular weight of ≈230 kDa (Figure 1c and d).

NleF inhibits human caspase-4 and mouse caspase-11
Recombinant caspase-4 underwent auto-proteolytic activation presumably as a consequence of LPS binding when purified from E. coli. WT caspase-4, but not a catalytic dead mutant (caspase-4C285S), underwent auto-proteolysis to the active p20 form and hydrolyzed the caspase-4 fluorogenic substrate peptide (Ac-LEVD-AFC; Figure 2a). Recombinant NleFePEC inhibited the activity of caspase-4 in a dose-dependent manner with an IC50 of 5 nM (Figure 2b), comparable to 14 nM previously measured for NleF1_185_EPEC by Blasche et al.19 Despite not binding caspase-4 in direct yeast-2-hybrid, NleF1–185_EPEC which was pulled down with caspase-4 at low levels (data not shown), was able to inhibit caspase-4 activity although at an IC50 of 25.5 nM (Figure 2b). C. rodentium NleF (NleF_Cr), which shares 84% amino-acid identity with NleFePEC, strongly inhibited the proteolytic activity of mouse caspase-11 (IC50 of 13 nM; Figure 2c and d) revealing an evolutionarily conserved functional property. Importantly, we found that NleFePEC inhibits caspase-4 more efficiently than NleF_Cr (Figure 2c), whereas NleF_Cr inhibits caspase-11 more efficiently than caspase-4 (Figure 2f).

NleF inhibits human caspase-4 activation during infection
To investigate if NleFePEC targets caspase-4 during infection of human IECs, Caco-2 cells were infected with the WT EPEC and EPECΔnleF; both strains adhered to the cultured cells equally (Figure 3a). However, whereas secreted caspase-4 was absent following infection with WT EPEC, the active p30 fragment of caspase-4 was found in the supernatants of cells infected with EPECΔnleF (Figure 3b). Addition of the caspase-4 inhibitor Ac-LEVD-CHO complemented the EPECΔnleF phenotype in a dose-dependent manner (Figure 3b).

NleFePEC did not affect the expression of pro-IL-18, which was similar in uninfected cells and those infected with all the EPEC strains (Figure 3c). Although secretion of pro-IL-18 was detected upon infection with WT EPEC and EPECΔnleF, pro-IL-18 was only processed into the active form following infection with EPECΔnleF (Figure 3d). Secretion of mature IL-18, induced by EPECΔnleF, was not detected when this strain was complemented with a plasmid encoding NleFePEC (pNleFePEC) (Figure 3d).

To confirm that inhibition of caspase-4 by NleF was sufficient to block processing of IL-18, we generated Caco-2 cells depleted of caspase-4 using miRNA30E-based stable short hairpin RNA expression (Figure 4a). EPECΔnleF infection of Caco-2 cells silenced for caspase-4 expression (C4) did not secrete mature IL-18, as measured by both western blotting (Figure 4b) and ELISA (enzyme-linked immunosorbent assay) (Figure 4c), clearly pointing to a requirement of caspase-4 in IL-18 processing following EPEC infection of IECs. Importantly, no cell death was detectable by measuring lactate dehydrogenase (LDH) release or propidium iodide (PI) uptake following infection of control or caspase-4-depleted
Caco-2 cells (Figure 4d); this is likely due to EPEC T3SS effectors (e.g., NleH, NleB), which inhibit cell death.\(^5,6,15\) Thus, in human IECs, pro-IL-18 processing during EPEC infection is caspase-4 dependent and the bacterially injected NleF specifically inhibits this process.

**C. rodentium** inhibits IL-18 secretion in vivo in an NleFCR-dependent manner

To test the role of NleF during infection in vivo we infected C57BL/6 mice with WT *C. rodentium*, *C. rodentiumΔnleF*, or *C. rodentiumΔnleF* complemented with p*nleFCR*. Colonization (Figure 5a) and colonic crypt hyperplasia (Figure 5b) were similar between the different *C. rodentium* strains (Figure 5). We quantified levels of IL-18 and IL-1β secreted from colonic explants, and the inflammasome-independent chemokine CXCL1 as a control, on days 4 and 8 post infection. On day 4 post infection of C57BL/6 mice with *C. rodentium ΔnleF* we detected a significantly increased colonic secretion of IL-18, whereas mock-infected (phosphate-buffered saline, PBS) or WT *C. rodentium* infected colons released similarly low levels of IL-18 (Figure 5c). Complementing the *C. rodentium ΔnleF* mutant with a plasmid encoding NleFCR restored the inhibition of IL-18 secretion (Figure 5c); secreted IL-1β was below the detectable limit (data not shown). Secretion of CXCL1 was similar in colons extracted from mice treated with PBS or infected with WT *C. rodentium* or *C. rodentiumΔnleF* (Figure 5d). Complementing the *C. rodentium ΔnleF* mutant with a plasmid encoding NleFCR resulted in a significantly increased CXCL1 secretion (Figure 5d), which is consistent with our recent finding that over expression of NleF\textsubscript{EPEC} activates NF-κB in cultured cells.\(^20\) Importantly, NleF-dependent inhibitory effects were only observed early during infection (day 4 post infection), and IL-18 secretion was similar following WT *C. rodentium* or *C. rodentium ΔnleF* infection on day 8 post infection (Figure 5e).

To validate that NleFCR inhibits IL-18 secretion via the inflammasomes, we first infected Casp1/11-deficient mice with *C. rodentium* and *C. rodentiumΔnleF*. As expected, loss of Casp1 and Casp11 abolished IL-18 secretion from colonic explants after infection with WT *C. rodentium* or *C. rodentium ΔnleF* (Figure 5c); CXCL1 secretion was similar in Casp1/11\(^{-/-}\) mice infected with the two strains (data not shown). In order to
confirm that the phenotype was due to caspase-11, we next infected Casp11−/− mice with C. rodentium or C. rodentium ΔnleF. This showed that whereas WT C. rodentium and C. rodentium ΔnleF colonized the Casp11−/− mice at comparable levels (Figure 5f), secretion of IL-18 was extremely low and similar to that in Casp1/11−/− mice (Figure 5c). We therefore concluded that caspase-11 is responsible for secretion of IL-18 following infection with C. rodentium ΔnleF.

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

As IL-18 facilitates neutrophil and leukocyte recruitment to sites of inflammation,21 we investigated the effect of NleF_{CR} on immune cell recruitment. Infection of C57BL/6 mice for 4 days with C. rodentium ΔnleF colonized the Casp11−/− mice at a significantly higher level (Figure 6b). Infection with the C. rodentium ΔnleF pmlnleFCR strain restored the inhibition of neutrophil recruitment (Figure 6b). No significant differences were observed for other cell types analyzed, including macrophages, innate lymphoid cells, B cells and T cells (data not shown). Furthermore, correlating with similar IL-18 secretion, no difference in neutrophil recruitment was observed at day 8 post infection (Figure 6c), suggesting that NleF_{CR} has a specific role during early immune responses to C. rodentium. Enhanced neutrophil influx was Casp1/11 dependent; absence of these caspases abolished the increase in neutrophil recruitment during infection with C. rodentium ΔnleF (Figure 6b). Similar results were obtained following infection of Casp11−/− mice (Figure 6b). Thus, NleF_{CR} is a virulence factor responsible for early inhibition of the host inflammasomes, and that the inflammasome is essential for early neutrophil recruitment in response to C. rodentium infection.

**DISCUSSION**

Inflammasome-dependent cytokines and pyroptosis have important antimicrobial functions.8,9 It is therefore not surprising that pathogenic bacteria have evolved mechanisms to prevent inflammasome activation.22 For example, Yersinia uses YopK to prevent detection of its T3SS,22 and bacteria modify their LPS to evade detection by caspase-11.23 The Shigella flexneri effector OspC3 sequesters caspase-4 activity by binding the caspase-4 p20 subunit to prevent p10 binding and oligomerization.24 Here we demonstrate that a virulence factor of A/E pathogens, NleF, targets the heterotetramer complex of caspase-4 via its C-terminal motif, underlining the importance of caspase-4 inhibition during the course of infection.

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**Figure 2** NleF inhibits caspase-4 activity. (a) Recombinant caspase-4, but not caspase-4^{C258S}, is auto-activated (western blot) and cleaves the reporter Ac-LEVD-AFC. Results are plotted as relative fluorescence units (RFU) minus background (No Ac-LEVD-AFC) over time (min). (b) Dose-dependent inhibition of caspase-4 Ac-LEVD-AFC cleavage by recombinant NleF_{EPEC} and NleF_{1-185,EPEC} (shown by Coomassie stained gel). (c) NleF_{EPEC} (10 nm) inhibits the activity of caspase-4 more efficiently than NleF_{CR} (10 nm) after 30 min incubation in the presence of Ac-LEVD-AFC. (d) Recombinant caspase-11 is auto-activated (western blot) and cleaves the reporter Ac-LEVD-AFC. (e) Dose-dependent inhibition of caspase-11 activity by recombinant NleF_{CR} (shown by Coomassie stained gel). (f) NleF_{CR} (50 nm) inhibits the activity of caspase-11 more efficiently than NleF_{EPEC} (50 nm) after 30 min incubation in the presence of Ac-LEVD-AFC. Results are expressed as a percentage of wild-type caspase-4 or caspase-11 RLU min⁻¹ from at least two independent experiments. * indicates P<0.05.
In agreement with our biochemical analyses, EPEC was able to inhibit caspase-4 in IECs in an NleF-dependent manner, whereas recent reports showed that infection of cultured cells with either Salmonella or EPEC led to caspase-4 activation\(^2\) and caspase-4-dependent induction of IL-18 release.\(^1\) Taken together, our data suggest that whereas EPEC can initiate caspase-4 activation and IL-18 processing, NleF dampens this response. Previous studies have shown that Nlrp3, Nlrp4, Casp1, and Casp11 are important in protection against C. rodentium infection.\(^1\) Loss of inflammasome-related genes results in significantly increased C. rodentium bacterial load in the intestine late in infection, which may partly explain the enhanced inflammation in inflammasome-deficient mice infected with C. rodentium. Loss of inflammasome-dependent IL-1\(\beta\) and IL-18 also results in enhanced bacterial burdens at late stages of infection and susceptibility to C. rodentium infection of IL1\(\beta\)\(^{-/-}\) and IL18\(^{-/-}\) mice.\(^2\) Our studies establish that NleF functions at early stages of infection of mucosal surfaces by inhibiting the inflammasome and preventing release of IL-18 by epithelial cells.

We also found that NleFCR inhibited caspase-11-dependent neutrophil recruitment. IL-18 is a key regulator of the adaptive immune response, stimulates the migration of innate and adaptive immune cells,\(^1\) and controls IEC turnover and protects against damage in the intestine.\(^2\) During the early stages of infection, IL-18 is largely secreted by epithelial cells.\(^1\) Current data,\(^1\) including the secretion of IL-1\(\beta\), which is not expressed in non-hematopoietic cells,\(^2\) suggests that at later time points during C. rodentium infection colonic IL-18 secretion may switch to be myeloid cell dependent.\(^1\) Therefore, myeloid cell secretion of the IL-1 family cytokines may not be subverted by NleFCR and would become the predominant source of IL-18 and IL-1\(\beta\) at the peak of infection. Similarly secretion of IL-22 is switched from innate lymphoid cells\(^3\) at early phase of infection to IL-22-producing T cells at later time points (> 7 days).\(^3\)

The study demonstrates a pathway during infection of IECs, which leads to the activation of caspase-11, secretion of IL-18, and recruitment of neutrophil. In addition, we show that inhibition of caspase-11 by bacterial NleF blocks this pathway in the host. Our findings are consistent with the recent study on the epithelial cell caspase-11–IL-18 axis during Salmonella infection, which reported significant neutrophil influx in

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Figure 3  NleF inhibits secretion of caspase-4 and IL-18 during EPEC infection. (a) Infection of polarized Caco-2 cells with WT EPEC, EPEC\(\Delta\)nleF, or the complemented strain (pnlE\(_{\text{EPEC}}\)) revealed similar levels of cell adhesion (3 h post infection). (b) Caco-2 cells were infected with WT EPEC or EPEC\(\Delta\)nleF in the absence or presence of the inhibitor Ac-LEVD-CHO (total 21 h). Immunoblotting of supernatants (SN) revealed that EPEC inhibits secretion of active caspase-4 (\(\sim\) 28 kDa) in an NleF\(_{\text{EPEC}}\)-dependent manner, assessed by western blots (upper panel) and quantified by densitometry of multiple experiments (lower panel). (c) Infection of Caco-2 cells with WT EPEC, EPEC\(\Delta\)nleF, or complemented EPEC\(\Delta\)nleF (pnlE\(_{\text{EPEC}}\)) had no effect on the levels of total IL-18 at 4 h p.i. (d) NleF is essential for inhibition of IL-18 secretion from infected Caco-2 cells (21 h post infection).
infected gall bladder epithelia of wild-type mice, but no neutrophil influx in Casp11−/− mice.14,26,27,31 Recent studies have revealed the contribution of non-inflammasome and inflammasome-forming NOD-like receptors (NLRs) in the non-hematopoietic compartment for intestinal homeostasis and the host-mediated clearance and protection against enteric pathogens.32 Mice deficient in NLRP6 have impaired goblet cell mucus exocytosis and display a microbiome-exposed epithelial cell layer and persistence of C. rodentium infection.33 Moreover, NLRP12 is a checkpoint for non-hematopoietic non-canonical NF-κB activation,34 and acts as a negative regulator of colitis and colitis-associated colon cancer. Furthermore, IEC-expressed NLRC4 mediates early innate immune responses against C. rodentium via an unknown mechanism independent of IL-1 family cytokine secretion.35 Here we show that the caspase-4/11-dependent IECs inflammasome is crucial for IL-18 cytokine maturation and the early innate immune response to EPEC/C. rodentium. Consistently with this, Song-Zhao et al.17 recently suggested, based on studies of Nlrp3−/− and Asc−/− mice, that early protection to C. rodentium infection is mediated by IECs independently of NLRP3 activation. Taken together, our study identifies a fundamental and novel role for the T3SS effector NleF in the pathogenesis and virulence of A/E pathogens through the inhibition of the newly characterized IECs caspase-4/11-dependent inflammasome.

**METHODS**

**Strains, oligonucleotides, plasmids, and antibodies.** Strains, plasmids, and primers used in this study are listed in Supplementary Tables S2–S3, respectively. nleF was amplified from EPEC E2348/69 and C. rodentium ICC169 genomic DNA by PCR. Site-directed

Figure 4 NleF_EPEC inhibits IL-18 secretion in a caspase-4-dependent manner. (a) Western blots showing knockdown of caspase-4, but not caspase-5, by miRNA30E. (b) Infection of Caco-2 cells (21 h) depleted of caspase-4 (C4) revealed that it is essential for IL-18 processing in response to infection with EPEC ΔnleF, assessed by western blots (upper panel) and quantified by densitometry of two independent experiments (lower panel). (c) ELISA from two biological repeats specific secretion of IL-18 from control (YFP), but not from C4, Caco-2 cells infected for 21 h with EPEC ΔnleF. (d) EPEC does not trigger LDH release or PI uptake during infection (21 h) of control or C4 Caco-2 cells, results are represented as a percentage of total uptake or total release and are an average of two biological repeats carried out in triplicate. * indicates P<0.05.
Figure 5  NleFCR inhibits colonic IL-18 secretion 4 days p.i. WT C. rodentium, C. rodentiumΔnleF, and the complemented strain (ΔnleF pnleFCR) similarly colonized and triggered colonic hypoplasia in C57BL/6 mice (a and b). Each dot in (b) represents an individual measurement of crypt length (from at least 20 measurements per section per mouse), and horizontal bars represent mean values. Significant increase in secreted IL-18, measured by ELISA, was seen specifically following infection of C57BL/6 with C. rodentiumΔnleF (day4), but not following infection of either Casp1/11−/− or Casp11−/− mice (day 4) (c) or C57BL/6 (day 8) (e). Secreted CXCL1 was found in similar levels, except for the complemented strain, which triggered greater secretion of CXCL1 (d). No difference in colonization of Casp11−/− mice was seen following infection with WT C. rodentium or C. rodentiumΔnleF (e). * indicates *P<0.05.
mutagenesis was carried out by inverse PCR using KOD Hot Start polymerase and mismatch primers. All constructs were confirmed by sequencing (GATC Biotech, Konstanz, Germany). For western blot, mouse monoclonal anti-caspase-4 clone 4B9 (sc-56056; Santa Cruz, Heidelberg, Germany), anti-α-tubulin clone DM1A (T6199), mouse polyclonal antibody anti-caspase-11 p20 clone A-2 (sc-374615; Santa Cruz), and anti-pro-IL-18 (CPTC-IL18-1; DSHB, Iowa City, IA), the rabbit monoclonal anti-IL-18 (PM014; MBL, Woburn, MA), anti-caspase-5 (4429; Cell Signalling, Leiden, The Netherlands), and the rabbit polyclonal antibody anti-GFP (Ab290; Abcam, Cambridge, UK) were used as primary antibodies. Horse radish peroxidase-conjugated goat anti-rabbit IgG (Fc fragment; catalog no. 111-035-008; Jackson Immunoresearch, West Grove, PA) and horse radish peroxidase-conjugated goat anti-mouse IgG (Fc fragment; catalog no, 115-035-008; Jackson Immunoresearch) were used as secondary antibodies.

Retroviral transductions and stable knockdown cell lines. MicroRNA30 based (miR-30; Supplementary Table S1) gene silencing constructs were generated in pMX-CMV-YFP using one-step sequence and ligation independent cloning following the optimized miR-30E vector design. Sequences were as follows: CASP4—CGACTGTC-CATGACAAAGAT; and LacZ (non-targeting negative control) ACGTGTCATATACCGTGCAG. The miR-30E plasmids were transfected using Lipofectamine 2000 (Invitrogen), along with the packaging plasmids pVSV-G and pCMV-MMLV-pack into HEK293E cells to produce a VSV-G pseudotyped retroviral particles for transduction. After 48 h supernatants were filtered through 0.45 μm syringe filters and added directly to pre-seeded Caco-2 TC7 cells. Transduced cells were selected by puromycin (Gibco, Invitrogen, Paisley, UK) at 10 μg ml⁻¹ and knockdown was confirmed by western blotting.

EPEC infection, ELISA, and western blotting. Caco-2 TC7 cells (ATCC, Teddington, UK) were seeded at 7.5 × 10⁴ ml⁻¹ and upon reaching confluence (7 days) the medium was changed every day for the following 7 days. Before infection the cells were starved for 3 h in serum-free Dulbecco’s Modified Eagle’s medium. Monolayers were infected with primed EPEC at a multiplicity of infection of 1:10 for 3 h. The cells were then washed twice in PBS and the medium was replaced with serum-free Dulbecco’s Modified Eagle’s medium-high glucose plus penicillin and streptomycin at 100 U ml⁻¹ and 100 μg ml⁻¹, respectively. After 1 h cells were washed and either processed for western blot (total IL-18) or incubated for a further 17 h (secreted caspase-4 and IL-18) with or without Ac-LEVD-CHO (Enzo Lifesciences, Farmingdale, NY). Supernatants were collected, cleared by centrifugation at 13,000 rpm at 4 °C for 10 min and precipitated for western blotting with the addition of 10% (v/v) trichloroacetic acid for Figure 6 NleF_cr inhibits colonic neutrophil recruitment 4 days p.i. C57BL/6, Casp11⁻ /⁻ and Casp11⁻ /⁻ mice were infected with WT C. rodentium, C. rodentium ΔnleF or complemented C. rodentium ΔnleF ΔnleF p(nleF_cr). (a) Representative image of flow cytometry gating strategy for neutrophils (CD11b + Ly6G + ) of control (PBS) and infected C57BL/6 mice. The number of neutrophils (CD11b + Ly6G + ) present within the myeloid gate was counted from C57BL/6 (b–c, days 4 and 8 post infection), Casp11⁻ /⁻ or Casp11⁻ /⁻ (b, day 4 post infection) mice (at least six mice per condition). * indicates P < 0.05.
17 h at 4 °C. The concentration of IL-18 in cell supernatant (MBL) was determined by ELISA according to the manufacturer’s protocol.

**Cell adhesion and cytotoxicity assays.** Caco-2 TC7 were infected with the WT EPEC, EPECΔnleF and the complemented strain (plasmid expressing NleF-EPEC for 3 h). The monolayers were lysed by 1% PBS/triton X-100 and EPEC attachment was enumerated by serial dilution on Luria-Bertani (LB)-Agar and calculation of colony forming units.

Supernatants of uninfected cells or cell infected with EPEC for 21 h were harvested and the level of LDH release was measured using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Southampton, UK). As a control for total LDH, cell lysis buffer (1% Triton-X-100/ PBS) was added for 30 min at 37 °C directly to the medium and cell layer. Absorbance was measured at 490 nm using the Fluostar Omega plate reader and results are displayed as percentage of total release corresponding to the LDH measured in the supernatant divided by the total LDH.

Alternatively, the media was removed and cell layers were incubated in 3.3 μg ml⁻¹ PI (Invitrogen) in warm PBS (PI/PBS) for 15 min and fluorescence was measured at an excitation of 510 nm and emission of 610 nm using the Fluostar Omega plate reader. As a control PI/PBS alone was measured or cell lysis buffer (0.05% Triton X-100/PBS) supplemented with 3.3 μg ml⁻¹ PI was added for 15 min at 37°C. Results are shown as a percentage of total PI uptake.

** Yeast-2-hybrid screen and yeast direct hybrids. **A yeast-2-hybrid screen was conducted using pGBKTT7-nleF<sub>EPEC</sub> and the HeLa cell cDNA Library following the manufacturer’s Handbook (Clontech). AH109 were co-transformed with pGBT9-bait and pGADT7-prey (Supplementary Table S3) and plated onto Difco Yeast Nitrogen Base without amino acids (SD) agar supplemented with 2% glucose, 20 mg l⁻¹ arginine HCl, 20 mg l⁻¹ histidine HCl, 20 mg l⁻¹ leucine, 20 mg l⁻¹ tryptophan, 50 mg l⁻¹ phenylalanine, 200 mg l⁻¹ threonine, 30 mg l⁻¹ tyrosine, 20 mg l⁻¹ uracil, 150 mg l⁻¹ valine and lacking tryptophan and leucine (DDO; Double Drop-out) for selection of transformed clones. Clones positive for both plasmids were re-streaked on to SD DDO and SD QDO/-His/-Ade supplemented with 40 mg l⁻¹ x-gal (SD QDO) for selection of positive interactions.

**Recombinant protein expression and purification.** E. coli BL21 Star cells expressing pET28-NleF<sub>EPEC</sub> (pIC1659), pET28-NleF<sub>EPEC</sub>-185 (pIC1660), and pET28-NleF<sub>CR</sub> (pIC1839) were cultured for 16 h in LB at 37°C at 200 rpm. Bacteria were sub-cultured at 1:100 into 1 L LB supplemented with 50 μg ml⁻¹ kanamycin and incubated at 37°C at 200 rpm until OD₆₀₀ was 0.4–0.6. Cultures were then induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside for 18 h at 18°C.

**Construction of C. rodentium mutant.** C. rodentium strain ICC169 ΔnleF (ICC1129) was generated using a modified version of the lambda red-based mutagenesis system. In brief, the nleF gene and its flanking regions were PCR-amplified from WT C. rodentium ICC169 genomic DNA using the primers pair NleF-up-Fw/NleF-down-Rv and cloned into plc-Bin-TOPO vector (Invitrogen). The nleF gene was then excised using inverse PCR (primers NleF-up-Rv-BamHI/NleF-down-Fw-BamHI) and the resulting linear product was BamHI digested, allowing insertion of the non-polar aph<sub>T</sub> cassette, resulting in plasmid pIC1674. After verifying for correct orientation of the kanamycin cassette, the insert was PCR-amplified using NleF-up-Fw and NleF-down-Rv primers. The PCR products were electroeluted into wild-type C. rodentium expressing the lambda red recombinase from pKD46 plasmid. The deletion was confirmed by PCR and DNA sequencing among the kanamycin resistant clones (primers NleF-up-Fw-check and NleF-down-Fw-check).

**Oral infection of mice.** Pathogen-free female C57BL/6 mice were either purchased from Charles River (Harlow, England, UK) or sourced from BIME Institut Pasteur, Casp1/11<sup>−/−</sup> mice were generously provided by Bernhard Ryffel (TAAM-CDTA, Orelsans, France) and Casp11<sup>−/−</sup> mice were generously provided by Mohamed Lamkanfi (Ghent University, Belgium). All animals were housed in individually high-efficiency particulate arrestance -filtered cages with sterile bedding and free access to sterilized food and water. Independent infection experiments for wild-type C57BL/6, Casp1/11<sup>−/−</sup> and Casp11<sup>−/−</sup> mice were performed using 3–8 mice per group. Mice were infected and followed for shedding as described. In brief, mice were gavaged via oral gavage with 10<sup>7</sup> WT C. rodentium or C. rodentium ΔnleF as described previously. For control, mice were gavaged with sterile PBS. The number of viable bacteria used as inoculum was determined by retrospective plating onto LB agar containing antibiotics. Stool samples were recovered aseptically at various time points after inoculation and the number of viable bacteria per gram of stool was determined by plating onto LB agar.

**Sample collection and colonic crypt hyperplasia measurements.** Segments of the terminal colon (0.5 cm) of each mouse were collected, flushed, and fixed in 10% neutral buffered formalin. Formalin fixed tissues were then processed, paraffin-embedded, sectioned at 5 μm and stained with H&E (haematoxylin and eosin) using standard techniques. H&E-stained tissues were evaluated for colonic crypt...
hyperplasia microscopically without knowledge of the treatment condition used in the study and the length of at least 100 well-oriented crypts from each section from all of the mice per treatment group (n = 4–6) were evaluated. H&E-stained tissues were imaged with an Axio Lab.A1 microscope (Carl Zeiss MicroImaging, Germany), images were acquired using an AxioCam ERC5s color camera, and computer-processed using AxioVision (Carl Zeiss MicroImaging, Berlin, Germany).

Sample collection for cytokine analysis and flow cytometry. Isolation of colonic cells and flow cytometry were performed as described. After a PBS wash, the 5th cm of the distal colon was incubated in Rosewell Park Memorial Institute medium containing penicillin, streptomycin, gentamicin, and fetal bovine serum at 37°C for 24 h. The concentrations of IL-18 (eBioscience, San Diego, CA, #BMS618/3), IL-1β, and KC (CXCL1; R&D Systems, Minneapolis, MN) were determined by ELISA according to the manufacturer’s protocols.

Statistics. All data were analyzed using GraphPad Prism software, using the Mann–Whitney test and represented as the mean ± s.e.m. or s.d. A P-value < 0.05 (P < 0.05) was considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

ACKNOWLEDGMENTS

We thank Guy Frankel for making the CR3ΔnleF mutant. We are grateful to Dr Bernhard Ryffel (TAAM-CDTA, Orelans, France) and Dr Mohamed Lamkanfi (VIB Inflammation Research Center, Ghent University, Belgium), Bernhard Ryffel (TAAM-CDTA, Orelans, France) and Dr Mohamed We thank Guy Frankel for making the CR

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AUTHOR CONTRIBUTIONS

M.A.P., V.F.P., N.S., and C.N.B.—plan and conducted experiments and wrote the paper. M.H., O.K., and J.S.G.—plan and conducted experiments. J.P.D., A.R.S., and G.F.—plan experiments and wrote the paper.

DISCLOSURE

The authors declare no conflict of interest.

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