**Shigella flexneri** suppresses NF-κB activation by inhibiting linear ubiquitin chain ligation

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The linear ubiquitin chain assembly complex (LUBAC) is a multimeric E3 ligase that catalyses M1 or linear ubiquitination of activated immune receptor signalling complexes (RSCs). Mutations that disrupt linear ubiquitin assembly lead to complex disease pathologies including immunodeficiency and autoinflammation in both humans and mice, but microbial toxins that target LUBAC function have not yet been discovered. Here, we report the identification of two homologous *Shigella flexneri* type III secretion system effector E3 ligases IpaH1.4 and IpaH2.5, which directly interact with LUBAC subunit Heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1L) and conjugate K48-linked ubiquitin chains to the catalytic RING-between-RING domain of HOIL-1-interacting protein (HOIP). Proteasomal degradation of HOIP leads to irreversible inactivation of linear ubiquitination and blunting of NF-κB nuclear translocation in response to tumour-necrosis factor (TNF), IL-1β and pathogen-associated molecular patterns. Loss of function studies in mammalian cells in combination with bacterial genetics explains how *Shigella* evades a broad spectrum of immune surveillance systems by cooperative inhibition of receptor ubiquitination and reveals the critical importance of LUBAC in host defence against pathogens.

The Linear Ubiquitin chain Assembly Complex (LUBAC) is a multimeric E3 ligase composed of two accessory subunits Heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1L/RBCK1) and Shank-associated RH domain-interacting protein (SHARPIN), and a catalytic subunit HOIL-1-interacting protein (HOIP/RNF31). Recruitment of LUBAC to activated cytokine- or pattern recognition receptors (PRRs)1-3 results in the ligation of receptor-interacting protein kinase 1 (RIPK1) and NF-κB essential modulator (NEMO/IκKγ) with methionine 1 (M1)-linked linear ubiquitin (Ub) chains4. This ubiquitination event is crucial for the activation of transcription factors NF-κB and AP-1, which induce rapid gene expression in response to microbial infection5-7. Because of the ubiquitous requirement of M1-Ub in numerous cytokine signalling pathways, humans lacking functional LUBAC exhibit severe autoinflammatory diseases and are especially vulnerable to pyogenic bacterial infections including life-threatening enteroinvasive *Escherichia coli*8,9. Recent studies have elucidated many of the structural and biochemical properties of LUBAC function, but it remains unclear how pathogens avoid immunological detection propagated by M1-Ub chain ligation.

Gram-negative bacteria including *Yersinia*, *E. coli*, *Salmonella* and *Shigella* use type III secretion systems (T3SS) to dampen the innate immune response to infection10. These bacteria secrete between 10 and 50 effector proteins, many of which have unknown functions. Identifying the substrates targeted by individual effector proteins has been confounded by the functional redundancy found within the repertoire of secreted effectors and by the complexity of the host molecules that are assembled at activated immune receptors (for example, tumour-necrosis factor receptor (TNF-R1), interleukin-1 receptor (IL-1R) and toll like receptor (TLR)). Thus, new model systems are needed to elucidate the immune evasion strategies employed by these microbial pathogens. Here, we focus on the mechanisms of innate immune evasion by *Shigella flexneri*, an enteroinvasive pathogen that infects an estimated 165 million people, resulting in more than one million deaths annually11. *Shigella* spp., are closely related to enteroinvasive *E. coli*12. These pathogens invade intestinal epithelial cells and encode multiple T3SS effector proteins on a large virulence plasmid, some of which have been shown to target host defence systems12-17. We also sought to identify novel inhibitors of cytokine signalling by developing a simplified host cell system to examine effector mechanisms for NF-κB suppression (Fig. 1a).

Here, we show that the *S. flexneri* E3 ligase effectors IpaH1.4 and IpaH2.5 interact with HOIL-1L and HOIP subunits of LUBAC and direct K48-linked ubiquitination and proteasomal destruction of HOIP. Although LUBAC destruction is sufficient to suppress the immune responses induced by TNF, elimination of both M1- and K63-linked Ub chains is required to inhibit a range of receptors including PRRs and IL-1R during *Shigella* infection. Collectively, these results (1) establish an irreversible mechanism of host immune suppression through proteasome-dependent elimination of M1-linked linear ubiquitination, (2) reveal the requirement for cooperativity among T3SS effectors, and (3) provide alternative avenues for interrogating immune receptor activation in combined immunodeficiency and autoinflammatory diseases associated with LUBAC dysfunctions.

**Results**

**Shigella** modulators of innate immune signalling pathways. A simplified cell-based assay was used to characterize the anti-immunological function of *Shigella* T3SS effector proteins. As shown in Fig. 1a, ectopic expression of specific TNF-receptor signalling complex (RSC) components TRAF2, TAK1/Tab2 or LUBAC induced NF-κB mediated transcripational responses (Fig. 1a,b). Consistent with previous studies, NF-κB activation by the expression of TRAF2 E3 ligase was potently inhibited by *Shigella* OspI (Fig. 1b), a T3SS effector protein that deamidates Ubc13 required for TRAF2 directed K63-linked Ub chain conjugation of TNF–RSC17. We also found that OspZ, a T3SS effector that inactivates the TAK/Tab kinase complex, inhibited TRAF2 mediated NF-κB activation. These data are consistent with OspZ induced methylation of the high-affinity K63-Ub binding NZF domain of Tab2 (refs 18,19). Thus, *Shigella* can suppress signalling through K63-linked Ub chains at the level of receptor K63-ubiquitylation and/or K63-ub mediates recruitment of TAK/Tab.

Interestingly, we also observed a partial reduction of TRAF2 induced NF-κB activation in cells expressing IpaH1.4, but not other IpaH family members including IpaH9.8 (Fig. 1b). In contrast,
IpaH1.4 did not inhibit NF-κB activation in cells expressing TAK1/Tab2 (Fig. 1b). IpaH1.4 therefore targets a signalling component that depends on the formation of K63-linked Ub chains, but that does not depend on downstream phosphorylation steps in the pathway (Fig. 1a). The LUBAC is recruited to the TNF–RSC through binding to K63-linked Ub chains and conjugates M1-linked Ub chains to TNF–RSC (Fig. 1a)\(^2\),\(^3\). IpaH1.4 potently inhibited NF-κB stimulation by LUBAC, whereas OspI and OspZ had little effect (Fig. 1b).

IpaH1.4 belongs to a large family of bacterial E3 ligases characterized by a highly variable NH\(_2\)-terminal leucine rich repeat (LRR) domain and a nearly identical COOH-terminal catalytic domain (Supplementary Fig. 1a)\(^2\),\(^1\). LUBAC signalling was inhibited by both IpaH1.4 and IpaH2.5, which share 98% sequence homology\(^4\), but not other family members including IpaH4.5, IpaH7.8 and IpaH9.8, which share less than 80% homology (Supplementary Fig. 1b). The E3 ligase activity of IpaH1.4,2.5 (refs 21,22) was necessary to inhibit LUBAC-stimulated NF-κB activation (Supplementary Fig. 1c). Evaluation of the experiments in Fig. 1b also revealed a consistent downregulation of LUBAC-mediated NF-κB by Shigella kinase OspG. However, we found the kinase activity was unnecessary for LUBAC inhibition (Supplementary Fig. 1c) and an unbiased yeast two-hybrid screen failed to identify OspG substrates beyond its established activators Ub and Ube2L3 (UbCH7) E2-conjugating enzyme (Supplementary Fig. 2a)\(^1\).\(^6\),\(^2\),\(^3\). It is therefore likely that OspG inhibits LUBAC through non-specific sequestration of Ube2L3 ~Ub machinery required for linear ubiquitination under these experimental conditions\(^2\),\(^5\),\(^6\). Taken together, we have confirmed previous work on Shigella effector proteins OspI and OspZ that target K63-linked Ub chains and have demonstrated that Shigella may require additional effector proteins, and particularly IpaH1.4,2.5, to target M1-linked Ub chains to effectively suppress host defence mechanisms.

**IpaH1.4,2.5 directly interacts with LUBAC.** To elucidate the molecular mechanism of IpaH1.4,2.5 targeting of LUBAC, an unbiased yeast two-hybrid screen for IpaH2.5 substrates identified the ubiquitin-like domain (UBL) of the LUBAC subunit HOIL-1L (Supplementary Fig. 2a). IpaH2.5 also bound to full-length HOIL-1L in this assay (Supplementary Fig. 2b), suggesting a direct mechanism of LUBAC regulation. The UBL

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**Figure 1 | Screening S. flexneri T3SS effector proteins for disrupting the TNF-R signalling pathway.**

**a.** Schematic of the TNF-R signalling complex leading to NF-κB translocation to the nucleus. Components of the pathway expressed in HEK293T cells (in **b**) are coloured. **b.** NF-κB activation by cellular expression of components of the TNF-RSC is inhibited by Shigella effectors as measured using a NF-κB luciferase assay. *P < 0.05, **P < 0.01, ****P < 0.0001* (unpaired two-tailed t-test compared to GFP control). Data are presented as the mean ± s.d. of three independent experiments. Western blots show expression of Flag- or Myc-tagged TNF-RSC components and GFP-tagged effector proteins. **c.** GST pulldown shows that MBP-His\(_6\)-IpaH2.5 interacts with mini-LUBAC (GST-HOIL-1L, residues 557–1072, in complex with MBP-His\(_6\)-HOIL-1LUBL, residues 1–134). Top: western blot probed with anti-His antibodies. Middle: the same pulldown samples in Coomassie-stained SDS–PAGE gel. Bottom: input samples in Coomassie-stained SDS–PAGE gel. **d.** Schematic of Flag-HOIP fragments used in mapping assays. **e.** Immunoprecipitation using anti-GFP antibodies show that the region upstream of RING1 in HOIP is required for interaction with GFP-IpaH2.5. Arrows indicate fragments interacting with low affinity. Fl, full length. Results shown in **c** and **e** are representative of three independent experiments.
domain of HOIL-1L is required for LUBAC assembly through interaction with the UBA domain of HOIP<sup>25,26</sup>. We then asked if IpaH1.4/2.5 directly interacts with intact LUBAC, and if so, what protein regions are necessary and sufficient for substrate recognition. A ‘mini-LUBAC’ was generated by mixing a fragment of HOIL-1L encompassing the UBL domain (HOIL-1L<sub>UBL</sub>, residues 1–134) with a glutathione S-transferase (GST)-tagged fragment of HOIP encompassing the UBA and catalytic RING-between-RING (RBR) domain (HOIP<sub>UBA-RBR</sub>, residues 557–1,072). Mini-LUBAC precipitated recombinant MBP-tagged IpaH2.5, but not maltose binding protein (MBP) control (Fig. 1c, lanes 7 and 8), showing that the <i>Shigella</i> effector proteins directly interact with the pre-assembled E3 ligase complex. Unexpectedly, HOIP<sub>UBA-RBR</sub> precipitated IpaH2.5, even in the absence of HOIL-1L<sub>UBL</sub> (Fig. 1c, lane 6), revealing an additional interaction site for IpaH1.4/2.5 in HOIP.

Cell-based studies confirmed the interaction between full-length HOIP and catalytically inactive IpaH1.4/2.5<sub>C368S</sub>, but not other IpaH family members (Supplementary Fig. 2c–e). Truncation analysis (Fig. 1d) revealed the binding site on HOIP, encompassing the HOIP UBA domain (for example, HOIP<sub>ΔN1</sub>) that binds HOIL-1L and the region between the UBA domain and the catalytic RBR domain (for example, HOIP<sub>ΔN2</sub>) (Fig. 1e, arrows). The combination of UBA and RBR consistently yielded the greatest interaction with IpaH2.5 (Fig. 1e, lane 4), further confirming the interaction between two E3 ligases of different species.

**IpaH1.4/2.5 mediates proteasomal degradation of HOIP.** LUBAC exists as a heterodimer, or heterotrimer, composed of HOIL-1L and/or SHARPIN in complex with the catalytic subunit HOIP (LUBAC I, II and III). Co-expression of IpaH1.4/2.5 with each of the three LUBAC forms in HeLa cells revealed a significant reduction in HOIP protein levels, with no corresponding reduction in either HOIL-1L or SHARPIN (Fig. 2a). Mutant IpaH2.5<sub>C368S</sub>, lacking E3 ligase function did not reduce HOIP protein stability. In addition, IpaH4.5, 7.8 and 9.8 did not target HOIP or any LUBAC subunit for degradation (Fig. 2a). HOIP protein stability was rescued in IpaH1.4 or IpaH2.5 expressing cells treated with proteasome inhibitor MG132, showing that IpaH1.4/2.5-mediated HOIP degradation is proteasome dependent (Fig. 2b). Finally, we identified residues 687–761 encompassing RING1 of the catalytic RBR domain<sup>27</sup> as the minimal HOIP fragment destabilized in cells expressing IpaH2.5 (Fig. 2c). RING1 is adjacent to the IpaH2.5 binding site on HOIP (Fig. 1d), indicating that IpaH1.4/2.5 directly interacts with LUBAC and targets HOIP for ubiquitin-mediated proteasomal degradation (see illustration in Fig. 2d).

We performed <i>Shigella</i> infection experiments to test the ability of IpaH1.4/2.5 to degrade endogenous HOIP in a human intestinal epithelial cell line (CaCo-2). An infection rate of ∼50% could be achieved without appreciable cell death (Supplementary Fig. 3), which set the limitation on the total HOIP degradation that could be monitored in bulk biochemical experiments. As shown in Fig. 2e, wild-type (WT) <i>Shigella</i> reduced endogenous HOIP by more
Figure 3 | IpaH1.4/2.5 post-translationally modifies Lys residues 735, 783 and 875 in HOIP. a, LUBAC (Flag-His6-SHARPIN, Myc-HOIP and Flag-HOIL-1L) was obtained from HeLa cell lysates by Ni-NTA affinity chromatography. The western blot shown was stained with anti-Myc followed by anti-Flag antibodies. b, Schematic of in vitro ubiquitination reaction in which purified LUBAC was mixed with recombinant human His6-UbE1, His6-UbcH5b, Flag-Ubiquitin, Shigella GST-IpaH and ATP. c, HOIP is specifically ubiquitinated in vitro reactions by IpaH1.4/2.5. Top: western blot probed with anti-Myc antibodies. Bottom: GST-IpaH input samples in Coomassie-stained SDS–PAGE gel. d, Tandem mass (MS/MS) spectra of IpaH2.5-treated HOIP sample. High confidence assignment of ubiquitination on Lys 735, 783 and 875 was based on sequence coverage from Y and B ions and miscleavage by trypsin due to GlyGly residues on Lys. In addition these lysines were not ubiquitinated in IpaH2.5ΔC/S treated samples (Supplementary Fig. 7). e, Lys to Arg mutations in residues 735, 783 and 875 (3xR) or 735, 737, 783, 784, 873, 875 (6xR) in Flag-His6-HOIP fragment ΔN3 (amino acids 687–1072) reduces degradation by IpaH2.5. Western blots from three independent experiments were quantified. Data are presented as mean ± s.d. *P < 0.05, **P < 0.01 (unpaired two-tailed t-test). f, IpaH2.5 can use WT ubiquitin and K48-only ubiquitin (all Lys residues mutated to Arg except for Lys48), but not K48R ubiquitin (Lys48 mutated to Arg), to ligate ubiquitin chains (Ubchain) to Flag-His6-HOIP fragment ΔN3. K48R ubiquitin can only be used for mono-ubiquitination (Ubmono) of HOIP Lys residues by IpaH2.5. Results in a, c, d and f are representative of three independent experiments.

than 50% (consistent with the rate of bacterial infection), but not HOIL-1L or β-actin control. HOIP degradation was not observed during infection with the ShigellaIpaH1.4/2.5 strain, and analysis of single mutations revealed IpaH1.4 is the essential bacterial E3 ligase necessary for targeting LUBAC in this cellular model of infection (Fig. 2e). The varying promoter sequences upstream of *ipaH1.4* and *ipaH2.5* genes (52.0% identity), but with nearly identical coding sequences (98.9% identity), suggest that although *ipaH1.4* and *ipaH2.5* encode...
identical proteins, expression may vary depending on the conditions encountered by Shigella during infection (Supplementary Fig. 4).

We were unable to complement the Shigella ΔipaH1.4 strain with an arabinose inducible copy of ipaH1.4 or ipaH2.5 encoded on a plasmid. To determine if IpaH1.4/2.5 targets LUBAC function at protein levels secreted by bacterial pathogens, we engineered an attenuated Listeria monocytogenes (Lm) strain to secrete IpaH2.5 during intracellular invasion (Supplementary Fig. 5a). Both Lm and Shigella have very similar intracellular lifestyles, yet Lm does not encode the IpaH homologue or induce HOIP degradation. We observed dose- and time-dependent degradation of endogenous HOIP in U2OS cells infected with Lm::ipaH2.5, but not infected with WT or IpaH2.5C368S secreting strains (Supplementary Fig. 5b–d). These data further confirm that IpaH1.4/2.5 specifically targets HOIP during infection.

**IpaH1.4/2.5 ubiquitinates the RBR domain of HOIP in vitro.**

Next, we reconstituted the ubiquitination reaction to determine whether IpaH1.4/2.5 directly modifies LUBAC. 6X-Histidine tagged Flag-SHARPIN was co-expressed with Myc-HOIP and Flag-HOIL-1L in HeLa cells and the intact LUBAC-III was purified by Ni-NTA affinity chromatography (Fig. 3a). LUBAC-III

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**Figure 4 | IpaH1.4 is the major E3 ligase regulator of cytokine RSCs.**

**a**, Degradation assay in HEK293T cells expressing GFP-tagged Shigella IpaH proteins in combination with TNF-R and IL-1R signalling components. Shown are representative western blots from three independent experiments. *S. flexneri* IpaH6 and IpaH7 are nearly identical to IpaH1 and IpaH4, respectively 30. IpaH1 is encoded by *S. flexneri* M90T ORF SF5M90T_2545, IpaH2 by SF5M90T_1825, IpaH3 by SF5M90T_1355, IpaH4 by SF5M90T_1966, IpaH5 by SF5M90T_2665, IpaH6 by SF5M90T_744 and IpaH7 by SF5M90T_2130. **b**, Western blotting shows that, compared to non-infected control cells (NI), endogenous HOIP protein levels are reduced in CaCo-2 cells infected for 5 h with *S. flexneri* M90T (WT) at an MOI of 150. No reduction in stability (in same samples) of endogenous TRAF2, NEMO, p65, HOIL-1L, TRADD, TAK1 or IKKa was detected. Western blots from three independent experiments were quantified and presented as the mean ± s.d. *P* < 0.05, **P** < 0.001; (unpaired two-tailed *t*-test). NI, not infected.
was then mixed with recombinant human UbE1(E1), UbcH5b(E2), Flag-Ub and ATP (Fig. 3b). Addition of either recombinant IpaH1.4 or IpaH2.5 induced a large (over 150 kDa) and time-dependent shift in HOIP mobility compared to untreated samples (Fig. 3c, lanes 1 and 2; Supplementary Fig. 6a,b). This mobility shift depended on both catalytic Cys368 in IpaH2.5 (Fig. 3c, lane 7) and the presence of the E1 ubiquitin activating enzyme UbE1 (lane 8), indicating that IpaH1.4/2.5 directly ubiquitinates HOIP. The isolated E3 ligase domain (NEL, residues 265–575) did not ubiquitinate HOIP, suggesting that, similar to IpaH family member SspH1 from Salmonella19, the LRR domain of IpaH1.4/2.5 defines its substrate selectivity (lane 6). Consistent with this notion, IpaH4.5, IpaH7.8 and IpaH9.8, which encode highly variable LRRs yet identical E3 ligase domains, were unable to catalyse HOIP ubiquitination (lanes 3–5). Finally, neither HOIL-1L nor SHARPIN were modified by IpaH1.4/2.5 (Supplementary Fig. 6a), demonstrating that HOIP is the primary target of the bacterial E3 ligase machinery.

Mass spectrometry analysis identified K735, K783 and K875 of HOIP as the primary residues ubiquitinated by IpaH2.5 in vitro (Fig. 3d and Supplementary Fig. 7). K735 is located in the RING1 domain, K783 in the IBR domain and K875 in the RING2 domain of HOIP. These sites of modification are in agreement with the IpaH2.5 binding site near RING1 (Fig. 1d), the RING1 domain itself being required for degradation in cells (Fig. 2d) and the RBR domain being directly ubiquitinated by IpaH1.4/2.5 in vitro (Supplementary Fig. 6c, lanes 1–3). IpaH2.5 induced cellular degradation (Fig. 3e) and ubiquitination of HOIP was reduced significantly by arginine (R) substitutions in K735, K783 and K875 (ΔipaH1.4/2.5 (3×R; Supplementary Fig. 6c, lanes 4–6) and further by mutations of three adjacent residues K737, K784 and K873 (6×R; lanes 7–9). Finally, IpaH2.5 catalyses K48-Ub conjugation of HOIP, which is recognized by the 26S proteasome subunit that mediates protein degradation (Fig. 3f)29.

IpaH1.4/2.5 is the major E3 ligase regulator of cytokine RSCs. S. flexneri encodes 12 IpaH effector proteins, including IpaH4.5, IpaH9.8 and IpaH0722 (IpaH5), which have been reported to target NF-κB subunit p65, NEMO and TRAF2, respectively, for degradation3,13,30,31. To directly test the contributions of chromosomal and plasmid encoded IpaH-type E3 ligases on innate immune evasion, a cellular degradation assay was
performed with all major components of TNF and IL-1 RSCs. To first demonstrate the specificity of this assay, *Shigella* and *Salmonella* E3 ligases were expressed together with PKN1, a known *Salmonella* Spsh1 substrate. As expected, Spsh1 induced the cellular degradation of PKN1, whereas other IpaH family members had no effect (Supplementary Fig. 8a,b). Contrary to previous studies, the reported targets of family members had no effect (Supplementary Fig. 8a,b). Induced the cellular degradation of PKN1, whereas other IpaH effectors arrests innate immune signalling during infection. Elimination of M1- and K63-linked ubiquitination by IpaH1.4 and OspI is required for complete suppression of signalling in a cytokine RSC such as IL-1R.

**IpaH1.4 and OspI cooperate to limit RSC signalling during *Shigella* infection.** Our studies now indicate that IpaH1.4/2.5 is a highly specific inhibitor of M1-Ub chain assembly. In addition, no other signalling components tested were degraded in the presence of IpaH proteins (Fig. 4 and Supplementary Fig. 9). These data reveal the central importance of IpaH1.4/2.5 among IpaH family members for blocking cytokine RSCs.
during *Shigella* infection. To first determine whether HOIP ubiquitination and degradation suppresses immune system activation during *Shigella* infection, we quantified endogenous NF-kB (p65) nuclear translocation at single cell resolution (Fig. 5a). Less than half (44.3 ± 7.9%) of WT *Shigella*-infected U2OS cells exhibited nuclear NF-kB accumulation (Fig. 5b). In contrast, NF-kB translocated to the nucleus in 94.3% (±3.6) of U2OS cells infected with *ShigellaΔipaH1.4-2.5*, indicating that secretion of IpaH1.4/2.5 suppresses the host immune responses to pathogen associated molecular patterns (PAMPs) (Fig. 5a,b). A comparison of *ShigellaΔipaH1.4* and *ShigellaipaH2.5* mutant strains showed that IpaH1.4 is the essential T3SS effector protein required for blocking NF-kB activation (Fig. 5a,b), which is consistent with the role of IpaH1.4 in HOIP degradation during *Shigella* infection (Fig. 2e). Importantly, NF-kB activation by *ShigellaΔipaH1.4* was independent of the total bacterial load in infected cells and it was not due to alterations in bacterial intracellular growth characteristics (Fig. 5c and Supplementary Fig. 10). Interestingly, p65 readily accumulated in the nucleus of U2OS cells infected with *ShigellaΔospI* and *ShigellaipaH1.4ΔospI* strains, indicating that, similar to IpaH1.4, OspI secretion is required for immune suppression (Fig. 5a,b). These data indicate that IpaH1.4 and OspI perform non-redundant functions to inhibit PAMP-mediated NF-kB activation.

**Discussion**

Here, we have demonstrated that *S. flexneri* suppresses immune signal transduction by specifically recognizing the LUBAC machinery and targeting its enzymatic centre HOIP for proteosomal degradation. In biochemical and cell-based studies, the *Shigella* E3 ubiquitin ligase IpaH1.4/2.5 directly interacted with *L. monocytogenes* subunits HOIL-1L and HOIP and catalysed conjugation of K48-linked Ub chains to the RBR domain of HOIP. Proteasome-dependent degradation of HOIP by IpaH1.4/2.5 was required for suppression of immune receptor signalling in NF-kB-luciferase assays as well as during *Shigella* infection. *S. flexneri* effectors that target components of innate immune RSCs have been reported previously. However, except for OspI and OspZ, we have not been able to reproduce these results. Unexpectedly, we found that effectors IpaH4.5, IpaH9.8 and IpaH0722 had no effect on cytokine RSC signalling, as suggested by previous reports. These findings were confirmed in NF-kB activation assays (Fig. 1 and Supplementary Fig. 1), in proteasomal degradation studies (Fig. 4a and Supplementary Fig. 9) and by bacterial infection of mouse intestinal epithelial cells (Fig. 4b). In contrast, we found that the cooperative functions of IpaH1.4 and OspI (by elimination of linear and K63-linked ubiquitination) were necessary to suppress NF-kB activation in response to IL-1β, TNF and bacterial PAMPs. Linear ubiquitination has been associated with signalling through broad subsets of cytokine receptors (for example, TNF-R1 and IL-1R) and PRRs (for example, NOD2, TLR4 and NLRP3). In fact, human patients with loss of function mutations in human HOIL-1L and HOIP genes are severely immunocompromised and especially susceptible to pyogenic bacterial infections. Thus, the ability to deactivate LUBAC may increase the virulence of invasive enteric pathogens such as *Shigella* spp. in immunocompetent patients. *Shigella* spp. are transmitted via the faeco-oral route and invade the colon and rectal mucosa of humans. Although infection with *Shigella* leads to severe inflammation and mucosal destruction during late stages of infection, it is possible that LUBAC inactivation and suppression of NF-kB activation is important during early stages of *Shigella* invasion and infection of intestinal epithelial cells. Taken together, these findings increase our understanding of how bacterial pathogens target innate immune signalling pathways and open up new ways to probe complex immune pathologies caused by LUBAC dysfunction.

**Methods**

**NF-kB luciferase reporter assay.** *S. flexneri M90T* genes encoding OspI, OspG, IpaH1.4, IpaH2.5 and IpaH9.8 and the *Shigella dysenteriae* gene encoding OspZ were PCR-amplified and cloned into pEGFP-C2. pFlagCMV6b plasmids carrying Flag-TRA2, Flag-Tab2, Flag-Tab3 or Flag-HOIL-1L and pDNA3.1 plasmids carrying Myc-HOIP or Flag-His6-SHARPIN were transfected into indicated combinations, into HEK293T cells in a 48 well plate (10,000 cells per well) for 48 h. The cells were co-transfected with plasmids carrying NF-kB-luc (pNF-kB-luc and pNF-kB) to correct for transfection efficiency using a beta-galactosidase assay and with green fluorescent protein (GFP)-effector (pEGFP-C2) plasmids. After 48 h, cells were lysed and luciferase was measured according to the manufacturer’s protocol (Promega).

**Yeast 2-hybrid assay.** *S. flexneri M90T* genes encoding OspG, IpaH2.5 and IpaH4.5 were cloned into vector pLexNA. After the introduction of constructed plasmids into Saccharomyces cerevisiae L40, expression of OspG, IpaH2.5 and IpaH4.5 was confirmed by western blotting using anti-LexA antibodies (Millipore). In this Y2H screen, LexA-effector expressed in the yeast *S. cerevisiae* L40 served as the bait, and a library of mouse embryonic cDNA cloned in the vector pVP16 served as the prey, as...
previously described.4,12 The pLexNA plasmid provided L40 with the ability to grow on Trp−/Ura−/His− minimal medium (SD−W) and the pLexNA plasmid on the SD−E medium. An interaction between bait and prey allows LexA to be shuttled into the nucleus (via the nuclear localization signal of VP16), resulting in expression of LacZ and HIS3 genes, and growth in Trp−, Leu−, His− (SD−WHLUK) medium. Plasmids were isolated from yeast colonies growing on SD−WHLUK (+3 mM 3-amino-1,2,4-triazole) using phenol/chloroform and were electroporated into E. coli HB101 strain. The pVP16 and pLexNA plasmids were isolated from E. coli and inserts in pVP16 were sequenced using the pVP16 5′ primer. For hits, pVP16 plasmids were reintroduced together with pLexNA plasmids containing effectors into the L40 yeast strain. Drops of tenfold dilution of colonies picked from SD−UWL plates were plated on SD−UWL and SD−WHLUK plates.

GST pulldowns. HOIP, UBA_BBB fragment was fused to the C-terminus of GST (in pGEX6P-1) and GST purified using glutathione-coated beads. IpaH2.5 full length was fused to the C-terminus of His6-MBP-His6-FLAG (pET28b) and His-tag purified on Ni−NTA beads. GST beads were loaded with 20 µg GST or GST-HOIP. pGEX6P-1 and GST pulldowns were isolated from E. coli and inserts in pGEX6P-1 were sequenced using the pGEX6P-1 5′ primer, and confirmed by SD−PAGE and western blotting using anti-GST antibodies.

Immunoprecipitation (IP) assays. To test cellular interaction of HOIP with IpaH1.4/2.5, Cys to Ser mutations in IpaH proteins (IpaH1.4C68S, IpaH2.5C68S, IpaH4.C379S, IpaH7.C357S and IpaH9.C375S) were generated by Quickchange mutagenesis. GST-IpaH proteins were co-expressed in BL21 and purified using glutathione-coated beads. His6-UbcH5b and His6-UbE1 were expressed in E. coli HB101 strain and purified using Ni−NTA agarose beads. LUBAC purified from HeLa cells was then used in an Ub reaction, containing 0.1 µM Ube1I (2.5 µM UbCh5 (2E), 5 µM (Flag, HA or His6 tagged) Ub (BostonBiochem). 0.5 mM MgO4, 0.5 mM ATP, 20 units ml−1 inorganic pyrophosphatase (Sigma Aldrich), 1 mM dithiothreitol (DTT) and 50 nM GST-IpaH (IpA). After 0, 20, 60 or 90 min at 37 °C, a total volume of 2× SDS loading buffer (Biorad) was added to samples for SDS−PAGE and Western blotting with anti-Myc antibodies. WT Ub (Flag, HA, His6 tagged), K48 only (Ub (His6 tagged) and K48R only (His6 tagged) were purchased from BostonBiochem.

Mass spectrometry. To determine which lysines in HOIP are ubiquitinated by IpaH2.5, an Ub reaction (500 µl volume) was performed with GST-IpaH2.5 or GST-IpaH2.5.C68S, and after 90 min at 37 °C, Myc-HOIP was immunoprecipitated using anti-Myc antibodies (Covance). The IP fractions were then run on a SDS−PAGE gel (Biorad) and Coomassie-stained bands were excised and proteins were digested in gel with trypsin and run on a Q Exactive MS platform at the Mass Spectrometry Core at University of Texas Southwestern.

Infections of tissue culture cells and microscopy. For infections with S. flexneri MT90, strains were grown overnight at 30 °C in brain heart infusion (BHI) broth (BD Biosciences) followed by 1:30 dilution in BHI broth and an additional 2 h growth at 37 °C to an optical density at 600 nm of 0.15. Cells were infected at a multiplicity of infection (MOI) of 2.5 × 10^5 per well) were transfected with pcDNA3.1-Flag-HOIP, pcDNA3.1-Myc-HOIP or Flag-HOIP, 2 µg anti-Myc (Covance) or anti-Flag (Sigma Aldrich) and pcDNA3.1-Myc-HOIP or Flag-HOIP, 2 µg anti-Myc (Covance) or anti-Flag (Sigma Aldrich) and pcDNA3.1-Myc-HOIP or Flag-HOIP, 2 µg anti-Myc (Covance) or anti-Flag (Sigma Aldrich) and pcDNA3.1-Myc-HOIP or Flag-HOIP, 2 µg anti-Myc (Covance) or anti-Flag (Sigma Aldrich). Lysate was first pre-cleared with 15 µl Protein A agarose beads (Thermo Scientific) for 1 h. To IP Myc-HOIP or Flag-HOIP, 2 µg anti-Myc (Covance) or anti-Flag (Sigma Aldrich) antibody was added for 1 h followed by addition of 15 µl Protein A agarose beads for 1.5 h. Beads were then washed 10 times in lysis buffer and 50 µl 2× SDS loading buffer (Biorad) was added. For mapping experiments, IP was performed using 2 µg anti-TAK1 antibodies (Clontech).

Cellular degradation assays. To determine IpaH1.4/2.5-mediated degradation of HOIP as part of LUBAC I, II or III, HEK293T cells (seeded in a 6 well plate at 2.5 × 10^5 per well) were transfected with (0.35 µg per well) pEGFP−C2 plasmids carrying IpaH1.4 or IpaH2.5 genes together with (0.15 µg per well) pFlagCMV6b−HOIP. Cells were washed once in cold PBS and lysed in buffer containing 25 mM Tris−HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 and 1× protease inhibitor (Sigma Aldrich) for 20 min on ice. After 24 h, cells were lysed in 1% Triton-X100 in PBS, and serial dilutions were plated on LB plates. For western blotting, cells were washed once in PBS and lysed in 2× SDS loading buffer (Biorad).

For p65 translocation immunofluorescence experiments, cells on coverslips were washed twice in PBS, fixed in 10% formaldehyde for 10 min, washed twice in PBS, permeabilized (in 10% horse serum, 0.5% Triton X100) for 5 min, stained with primary antibody (anti-p65 from Cell Signaling in 10% horse serum, 0.5% Triton X100, PBS) for 45 min, washed three times in PBS, stained with secondary antibody (fluorescein-conjugated goat anti-rabbit from Pierce in 10% horse serum, 0.5% Triton X100, PBS + 4,6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 594 Phalloidin from Life Sciences), washed three times in PBS and once in H2O, followed by mounting of the coverslips on glass slides. A Zeiss Observer Z1 fluorescent microscope and Zeiss software Zen were used to obtain and process images. At least ten images per slide were obtained for each experiment. Quantification of the translocation of p65 to the nucleus was scored manually. In uninfected samples (and infections with Shigella amoxcl) all cells were included, but in infected samples only infected cells (based on the presence of IpaH1.4 and IpaH2.5) were counted. In experiments with pEGFP−C2-IpaH1.4/2.5, 1×/10^5 (0.35 µg per well) of each sample was included. A p65 signal lower in the nucleus than in the cytoplasm was scored as no translocation, and a p65 signal higher in the nucleus than in the cytoplasm was scored as translocation. The outcome of cells in a monolayer was determined by visualizing the cytoketol of cells using Phalloidin stain. Fluorescence intensity in cross-sections of cells was determined using the plot profile tool in Image J.

Reagents. The antibodies were anti-LexA (06-719, Millipore), anti-Myc 9E10 (MMS-150P, Covance), anti-Flag M2 (A8592, Sigma Aldrich), anti-GFP (632592, Clontech), anti-Actin (A2066, Sigma Aldrich), anti-HOIP (MAB8039, R&D systems) and anti-RBCK1/HOIL-1L (HPA024185, Sigma Aldrich). Anti-Phospho-TCIβ (2859, anti-IgCβ (4814), anti-Phospho-p65 (3033), anti-p65 (8242), anti-TRADD (3684), anti-TRA2 (4712), anti-IKBKG/NEMO (4505) were purchased from Cell Signaling. Anti-IKBKG/NEMO antibodies were purchased from Abcam. Anti-β-actin was purchased from Sigma-Aldrich or with the protocol (3.0 µl) of 0.4 to 0.6 β-actin. The cell lines used in this study, HEK293T (CRL-3126), HeLa (CCL-2), CaCo-2 (HTB-37) and UOS (HTB-96), were purchased from ATCC. Parental cell lines were tested and determined to be mycoplasma free.

Statistical analysis. A two-tailed unpaired Student’s t-test was used to calculate statistical differences. Experiments were performed at least three times independently. A P value of less than 0.05 was considered significant.

Construction of Shigella mutants. S. flexneri MT90, IpaH1.4, IpaH2.5 and Ipa301 single and double mutants were constructed by λ red recombinase mediated replacement by homologous recombination of respective genes with a kanamycin resistance cassette followed by flippase (FLP) recombinase catalysed removal of the cassette4. Primers used were IpaH1.4−F (CGGTGCCCTGAGATTCGATCAATATAAAA CATTGAACCTCCTTGGATACCCCTGTAGACGCTGAGCTGACGCTTCTC) and IpaH1.4−R (CTTCCATGAGAAACAAATATGCGGAGCAGTATATTTT GCTCGGTTTCAGGCTATGATATCTCCTTATTG) for deletion of IpaH1.4. For
deletion of ipaH2.5 primers ipaH2.5-F (GATTTATCAGGGAAAGTTGCGCGAGATTTCCATAGAG) and ipaH2.5-R (GGCGACATTTCCGCTGATCACTCCTGAGAACATGCATGCGTGGCCTGCTCTC) were used. For the ∆ipaH1.4/2.5 double mutant this procedure was repeated for the ipaH1.4 gene in ∆ipaH2.5 background using primers ipaH1.4-F2 (GATTTATCAGGGAAAGTTGCGCGAGATTTCCATAGAG) and ipaH1.4-R2 (GGCGACATTTCCGCTGATCACTCCTGAGAACATGCATGCGTGGCCTGCTCTC) and ipaH1.4-R2 and ipaH1.4-F2 primers flanked with actA (underlined) and simultaneously cured of pKD46 by growth at 42 °C overnight. The kanamycin resistance gene was eliminated through the introduction of the pCP20 helper plasmid that contains the FLP recombinase. Subsequent curing of pCP20 was carried out by growing strains at 42 °C for 5 h. Deletions were confirmed by PCR using primers flanking the deletion site and sequencing of the PCR product.

Construction of l. monocytogenes strains. Shigella effectors IpaH1.5 (WT and C638S) were cloned into pActAN100-pPL2 (ref. 44), in which effectors were Gibson assembled (New England Biolabs) together with a 3xFlag-tag and a 529 nt l. monocytogenes actA fragment (247 nt of the actA promoter region and 282 nt of the actA ORF). Thus, the resulting construct ActA(1-94a)-3xFlag-IpaH1.5aa(1-163) contains the ActA Sec secretion signal from Listeria and is under transcriptional control of the actA promoter. The resulting plasmids were introduced into the (attenuated) Listeria 10403S ∆luxA strain through conjugation with E. coli SM10.

CRISPR/Cas9 genome editing. For CRISPR/Cas9 mediated knockout of MAP3K7/TAK1 on E. coli/HOPS or RNF31/HOP from U2OS cells, a previously described method was used46. Sequences TGTGCGGTCGGCAACGGCTCAT in the first exon of the RNF31 gene and GTGTGACCTAAGAGAGTGTCG in the first exon of the MAP3K7 gene were cloned into lentiviral vector and introduced into U2OS cells through lentiviral infection, to facilitate genomically targeting of these sites by Cas9. After obtaining a culture derived from a single cell, knockout of RNF31/HOP or MAP3K7/TAK1 was confirmed by western blotting and sequencing of the affected site.

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Author contributions
M.F.d.J. and N.M.A. were responsible for study design, the analysis and interpretation of data and for writing the manuscript. M.F.d.J. performed and analysed the experiments. Z.L. and D.C. developed essential assays systems.

Additional information
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Competing interests
The authors declare no competing financial interests.