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Identification of interactions among host and bacterial proteins and evaluation of their role early during *Shigella flexneri* infection

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Abstract

Shigella species cause diarrhoea by invading and spreading through the epithelial layer of the human colon. The infection triggers innate immune responses in the host that the bacterium combats by translocating into the host cell cytosol via a type 3 secretion system bacterial effector proteins that interfere with host processes. We previously demonstrated that interaction of the *Shigella* type 3 secreted effector protein IcsB with the host protein Toca-1 inhibits the innate immune response microtubule-associated protein light-chain 3 (LC3)-associated phagocytosis, and that IcsB interaction with Toca-1 is required for inhibition of this host response. Here, we show that Toca-1 *in vitro* precipitated not only IcsB, but also the type 3 secreted proteins OspC3, IpgD and IpaB. OspC3 and IpgD precipitation with Toca-1 was dependent on IcsB. Early during infection, most of these proteins localized near intracellular *Shigella*. We examined whether interactions among these proteins restrict innate host cell responses other than LC3-associated phagocytosis. In infected cells, OspC3 blocks production and secretion of the mature pro-inflammatory cytokine IL-18; however, we found that interaction of OspC3 with IcsB, either directly or indirectly via Toca-1, was not required for OspC3-mediated restriction of IL-18 production. These results indicate that interactions of the host protein Toca-1 with a subset of type 3 effector proteins contribute to the established function of some, but not all involved, effector proteins.

INTRODUCTION

Shigella spp. are the most common cause of diarrhoea in children worldwide [1]. *Shigella* spp. are facultative intracellular pathogens that cause disease by entering intestinal epithelial and immune cells, altering cellular processes and spreading through tissues of the intestinal mucosa. *Shigella* entry into non-phagocytic cells occurs by bacterium-induced macropinocytosis. Once internalized, the bacteria rapidly escape from the uptake vacuole into the host cytosol, whereupon they use the host actin polymerization-promoting factor N-WASP to polymerize host actin into a propulsive tail that moves them to the cell periphery and into adjacent cells [2, 3]. Infection and spread through the intestinal mucosa is accompanied by acute inflammation [4].

The process of bacterial uptake into cells and many of the subsequent alterations in host cell signaling are mediated by effector proteins delivered into cells by the *Shigella* type 3 secretion system. Upon initial contact with host cells, the type 3 secretion system inserts a translocon pore into the plasma membrane. The bacteria dock onto host cells in a manner that depends on the translocon pore [5], and docking triggers secretion and delivery of bacterial effectors into the host cytosol [6]. Among the first wave of effector proteins delivered into the cell are those that trigger macropinocytosis of the bacterium and those that inhibit early innate immune responses.

A critical early innate immune response to *Shigella* and other intracellular pathogens is autophagy, a process that promotes cellular homeostasis by engulfing undesirable cargo in membranes and delivering the membrane-bound cargo to the late endosome/lysosome compartments for destruction. Microtubule-associated protein light-chain 3 (LC3) is a key cellular regulator of autophagosome formation. LC3 participates in all autophagy pathways, including xenoautophagy, in which

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Abbreviations: LC3, microtubule-associated protein light chain 3; PMA, phorbol 12-myristate 13-acetate.

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One supplementary figure is available with the online version of this article.

foreign cytoplasmic particles (including pathogens) are engulfed in double-membrane vacuoles, and a non-canonical autophagy pathway called LC3-associated phagocytosis, in which single-membrane phagosomes that contain undesirable material are marked and delivered to lysosomes [7, 8]. During LC3-associated phagocytosis, enrichment of the phagosomal membrane that forms during pathogen uptake with LC3 accelerates phagosome maturation and killing of the pathogen [8].

Upon entry of *S. flexneri* into cells, the secreted *Shigella* type 3 effector protein IcsB, in conjunction with the host protein Toca-1, restricts LC3-associated phagocytosis [9]. Toca-1 is an effector of the small GTPase CDC42 that has been implicated in autophagy, actin assembly, membrane invagination and endocytosis [10–15]. During *S. flexneri* infection, Toca-1 is both associated with the uptake vacuole and required for activation of N-WASP-mediated polymerization of actin tails [9, 12]. Recruitment of Toca-1 to the uptake vacuole depends on IcsB [9], and the presence of IcsB and Toca-1 is associated with protection from LC3 recruitment around entering bacteria, indicating that IcsB and Toca-1 inhibit LC3-associated phagocytosis triggered by bacterial entry.

The presence of Toca-1 and IcsB around early-uptake vacuoles and the dependence on IcsB of Toca-1 recruitment to these sites [9] raised the possibility that Toca-1 and/or IcsB might also interact with and modulate the function of other *Shigella* effector proteins. To test this, we identified bacterial proteins that interact with Toca-1 *in vitro* and tested the role of interacting proteins identified *in vitro* in the disruption of host cell signalling early during infection.

METHODS

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. The wild-type *S. flexneri* strain used in this study is serotype 2a strain 2457T, and all other strains are isogenic to it. A strain carrying an unmarked non-polar deletion of *ospC3* was constructed by removing the kanamycin cassette in Δ*ospC3*::FRT-km^r-FRT using FLP recombinase [16]. This non-polar deletion of *ospC3* was generated in the same way that the *ospC3* deletion used in the published description of OspC3 inhibition of caspase 4 [17]. *ospC3*::FRT-km^r-FRT was introduced into 2457T Δ*icsB* by P1 phage transduction to generate 2457T Δ*icsB* Δ*ospC3*::FRT-km^r-FRT. *S. flexneri* was grown overnight in tryptic soy broth from individual colonies that were red on agar containing Congo red. Expression of IPTG-inducible effector constructs was essentially as described [18]. Alignment of OspC2 and OspC3 was performed using SerialCloner version 2-6-1.

To increase bacterial adherence to cells, *S. flexneri* strains used for A431 infections produced the uropathogenic *E. coli* pilus AFA-I, cloned into pBAD33 [19, 20]. The coding sequence of the AFA-I pilus and its native promoter from pIL22 [20] were sub-cloned as an NruI and EcoRI fragment that was blunt ended and ligated into pBAD33 lacking the arabinose promoter. pCS2-ZZ-TEV was generated by removal of the Toca-1 coding sequence from pCS2-ZZ-TEV-Toca-1 (gift of Marc Kirschner) by digestion with FseI and AscI, and ligation of the resulting plasmid backbone with double-stranded oligonucleotides encoding a stop codon and possessing FseI- and AscI-compatible overhangs.

Table 1. Strains and plasmids used in this study

Bacterial strain	Genotype or description	Reference
Wild-type	Wild-type serotype 2a *S. flexneri* strain 2457T	[32]
Δ*icsB*	2457T *icsB*::FRT	[9]
Δ*ospC3* (km)	2457T *ospC3*::FRT-km^r-FRT	Gift of Cammie Lesser
Δ*ospC3*	2457T *ospC3*::FRT	This study
Δ*icsB* Δ*ospC3* (km)	2457T *icsB*::FRT *ospC3*::FRT-km^r-FRT	This study
Plasmids		
pDSW206	IPTG-inducible expression plasmid containing 3xFLAG, Amp^r	[9]
pDSW206-IcsB-FLAG	IPTG-inducible expression of IcsB-3xFLAG, Amp^r	[9]
pDSW206-OspC3-FLAG	IPTG-inducible expression of OspC3-3xFLAG, Amp^r	Gift of Cammie Lesser
pDSW206-VirA-FLAG	IPTG-inducible expression of VirA-3xFLAG, Amp^r	Gift of Cammie Lesser
pCS2-protein A-TEV	Constitutive expression of protein A-TEV tag in mammalian cells, Amp^r	This study
pCS2-protein A-TEV-Toca-1	Constitutive expression of protein A-TEV-Toca-1 in mammalian cells, Amp^r	[11]
pABB1	pBAD33 encoding *E. coli* afimbrial adhesin AFA-I under the native promoter, Cm^r	This study
psPAX2 (pCMV-dR8.74)	Lentiviral packaging plasmid, Amp^r	Gift of Didier Trono (Addgene plasmid #12260)
pMD2.G	Lentiviral VSV-G envelope plasmid, Amp^r	Gift of Didier Trono (Addgene plasmid #12259)
pLKO.1	Lentiviral vector, Amp^r	[21]
pLKO.1-Toca.shRNA lenti C12	Lentiviral vector carrying shRNA against human Toca-1, Amp^r	[21]

Mammalian cell lines and culture conditions

THP-1 cells (gift of Jonathan Kagan) were maintained in RPMI medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 2 mM L-glutamine and 55 μM beta-mercaptoethanol at 37 °C in 5 % CO_2. A431 [21], HEK293T (ATCC) and HeLa cells (ATCC) were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated FBS. Where appropriate to maintain the stably integrated lentiviral vector encoding an shRNA against Toca-1 or the stably integrated empty lentiviral vector [21], 0.5 μg ml^{-1} (THP-1 cells) or 1 μg ml^{-1} (A431 cells) puromycin was included in the medium. A431 cell lines carrying control shRNA or A431 Toca-1 shRNA were a gift of Andrew Craig [21].

THP-1 shRNA knock-down

Lentivirus carrying empty lentiviral vector or lentiviral vector containing Toca-1 shRNA sequences was generated by transfecting HEK293T cells with psPAX2, pMD2.G and lentiviral control or Toca-1 shRNA vector using Fugene6, as we have previously [22] and per the manufacturer's instructions. Twenty-four hours following transfection, the medium was replaced with DMEM containing 30 % FBS. Twenty-four hours after medium exchange, supernatants containing lentivirus were collected and filtered through a 0.45 μm filter. THP-1 cells were transduced with lentivirus by spinfection as follows. HEK293T supernatants containing lentivirus and 8 μg ml^{-1} polybrene were added to 0.5×10^6 THP-1 cells maintained in RPMI in a 12-well plate at a 1 : 1 ratio, yielding a final concentration of 4 μg ml^{-1} polybrene. Cells were spun for 2 h at 1000 ***g*** at 32 °C, incubated for 2 h at 37 °C with 5 % CO_2, washed with RPMI containing 10 % FBS and resuspended at 0.2×10^6 cells per ml. After 24 h, selection for stable integration of the vectors was performed by the addition of puromycin to 1 μg ml^{-1} for 7 days.

Toca-1 precipitation assays

HEK293T cells were transfected with 20 μg pCS2-protein A-TEV-Toca-1 (encoding a protein A-Toca-1 fusion) or pCS2-protein A-TEV (encoding protein A) per 10 cm^2 tissue culture dish using the calcium phosphate method. The next day, transfected HEK293T cells were washed and provided with fresh medium. Twenty-four hours later, the cells were gently washed with PBS, knocked off the plate by vigorous pipetting and recovered by centrifugation. Recovered cells were lysed in ice-cold lysis buffer (30 mM HEPES pH 7.8, 300 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 % Triton X-100) supplemented with EDTA-free protease inhibitor (Roche, 1 tablet per 10 ml) on ice for 30 min. Lysates were clarified by centrifugation at 21 130 ***g*** for 15 min at 4 °C. Cleared lysates were aliquotted and stored at −20 °C until use. Protein A or protein A-Toca-1 was bound to lysis buffer-washed IgG sepharose beads (GE) by incubation of the beads with cleared lysates containing these proteins for 4 h at 4 °C with gentle rotation. Unbound protein was removed by washing once each with ice-cold Wash A (30 mM HEPES pH 7.8, 500 mM NaCl, 1 mM DTT, 0.5 % Triton X-100), Wash B (30 mM HEPES pH 7.8, 300 mM NaCl, 1 mM DTT, 0.5 % Triton X-100) and Wash C (30 mM HEPES pH 7.8, 300 mM NaCl, 1 mM DTT, 0.01 % Triton X-100). Prior to performing precipitation experiments, the amount of cleared 293T lysate containing protein A-Toca-1 or protein A needed to saturate IgG sepharose beads was determined experimentally. To ensure that equivalent amounts of protein A-Toca-1 or protein A were present in subsequent precipitation experiments, this amount was consistently applied to the beads.

S. flexneri strains carrying pDSW206 expression plasmids containing FLAG-tagged *S. flexneri* effector proteins were grown overnight in medium containing 0.1 % glucose, to repress expression of the effector protein. Strains were sub-cultured at 37 °C to an OD_{600} of 0.5–0.8. To induce expression of the effector protein, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 100 μM for the last 75 min of sub-culture, as described [18]. To induce secretion of bacterial effector proteins, Congo red was added to the cultures at 10 μM, and the cultures were incubated for 30 min at 37 °C in a water bath. Bacteria were pelleted by centrifugation at 2000 ***g***, and culture supernatants were collected. The supernatants were supplemented to a final concentration of 25 μg ml^{-1} gentamicin, supplemented with EDTA-free protease inhibitor (Roche, 1 tablet for each 10 ml of bacterial supernatant) and pre-cleared by incubation with IgG sepharose beads for 1 h at room temperature. Pre-cleared supernatants were incubated with protein A-bound or protein A-Toca-1-bound IgG sepharose beads at 37 °C for 3.5 h with gentle rotation. The beads were recovered by centrifugation at 1000 ***g***, washed once each with Wash D (30 mM HEPES pH 7.8, 500 mM NaCl, 0.5 % Triton X-100), Wash E (30 mM HEPES pH 7.8, 300 mM NaCl, 0.1 % Triton X-100) and Wash F (30 mM HEPES pH 7.8, 300 mM NaCl) and resuspended with equal volume 2.5x SDS-PAGE sample buffer.

Antibody cross-reactivity occurred between the IpaB antibody and the protein A tag on protein A-Toca-1, which obscured IpaB visualization. To eliminate cross-reactivity, following Wash F, beads were resuspended in TEV protease buffer, incubated with TEV protease for 1 h at room temperature with gentle agitation and resuspended in 2.5x sample buffer to achieve a final volume equal to other precipitation samples.

Primary antibodies used for Western blot were anti-Flag M2 (mouse monoclonal, Sigma F1804) and anti-IpaB clone H16 ([23], mouse monoclonal, gift of Philippe Sansonetti), each used at a dilution of 1 : 10 000, and anti-IpgD (mouse monoclonal, gift of Philippe Sansonetti and Armelle Phalipon), used at a dilution of 1 : 8000. The secondary antibody was HRP-conjugated Affinipure F(ab')$_2$ fragment donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc. 715-036-150), used at a dilution of 1 : 5000. To detect protein A and protein A-Toca-1, non-immune rabbit serum was used as a primary antibody at a dilution of 1 : 40 000,

and HRP-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories Inc. 111-035-003) was used at a dilution of 1 : 10 000.

Bacterial infection of mammalian cells

THP-1 cells, seeded at 1×10^6 cells per well in a six-well tissue culture plate, were differentiated by incubation with 50 μM PMA (Sigma) for 48 h before infection. A431 cells were seeded at 6×10^5 cells per well of a six well plate 24 h before infection. *S. flexneri* infection was performed essentially as described [24, 25]. Specifically, *S. flexneri* were grown to an OD_{600} of 0.3–0.5, recovered by centrifugation, resuspended in cell culture media (lacking FBS), and added to cell monolayers at a multiplicity of infection (MOI, bacteria:cell) of 10–20 for THP-1 cells and 25–35 for A431 cells. Bacterial entry was synchronized by centrifugation at 800 ***g*** for 10 min at 25 °C. Cells were incubated for 30 min (THP-1 cells) or 50 min (A431 cells) at 37 °C with 5 % CO_2, washed twice with warm PBS and incubated in cell culture media (lacking FBS) containing 25 μg ml^{-1} gentamicin for an additional 1, 2 or 3 h. Co-culture supernatants were collected, TCA precipitated (final concentration 10 % TCA) and resuspended in SDS-PAGE sample buffer. After removal of co-culture supernatant, the cell monolayers were lysed in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1 % SDS, 1 % nonidet-P40, 0.5 % sodium deoxycholate), supplemented with EDTA-free protease inhibitor (1 tablet per 10 ml, Roche) on ice, after which SDS-PAGE sample buffer was added. Primary antibodies used for Western blot were anti-IL-18 (rabbit polyclonal, MBL PM014), used at a dilution of 1 : 1000, anti-Toca-1 (mouse monoclonal, gift of Giorgio Scita), used at a dilution of 1 : 100 and anti-β-actin-HRP (mouse monoclonal, Sigma A3854), used at a dilution of 1 : 25 000. Secondary antibodies were HRP-conjugated goat anti-rabbit or goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories Inc. 111-035-003 and 115-035-003), each used at a dilution of 1 : 5000.

For immunofluorescence microscopy, HeLa cells were seeded at 3×10^5 cells in six-well plates on acetone-washed coverslips. The following day, *S. flexneri* Δ*ospC3* pDSW206-OpsC3-FLAG was grown as described above with IPTG added at a final concentration of 100 μM for the last 1 h of sub-culture, and was added to HeLa cells at an MOI of approximately 200 in the presence of IPTG. Infected monolayers were centrifuged at 800 ***g*** for 10 min and incubated at 37 °C for 15 min, washed with culture medium and incubated for an additional 15 min in fresh culture medium containing 100 μM IPTG (40 min total infection). Monolayers were washed with culture medium, fixed in 3.7 % paraformaldehyde in F-buffer for 20 min and permeabilized with 1 % Triton X-100. Immunofluorescence labelling was performed using the Flag M2 antibody (mouse monoclonal, Sigma F1804) at a dilution of 1 : 100 as the primary antibody. The secondary antibody used was anti-mouse IgG (H+L) Alexa Fluor 488 (Thermo Fisher Scientific A-11001) at a dilution of 1 : 100. DAPI (100 μM, Thermo Fisher Scientific 62247) was used to stain DNA.

Mass spectrometry

Precipitates from pull-downs of *S. flexneri* culture supernatants with protein A-Toca-1- or protein A-bound beads, generated as described above, were loaded onto an SDS-PAGE gel and allowed to migrate to the top of the separating gel, whereupon for each sample, a band containing all of the protein was cut out of the gel. Protein containing gel fragments was processed by tryptic digest and electrospray ionization tandem mass spectrometry at the Harvard Taplin Mass Spectrometry facility. Peptides were matched to the *Shigella* protein database, and secreted *Shigella* proteins were identified.

Data and statistical analysis

Unless otherwise noted, all experiments were performed at least three times on independent days. For densitometry analysis of bands on Western blots, autoradiography films were scanned and band intensity was determined using ImageJ software (NIH). All statistical analysis was performed using Graphpad Prism 6 software (Graphpad Software). For data sets containing two experimental groups, statistical significance was determined using a Student's *t*-test. For data sets containing three or more groups, statistical significance was determined using a one-way ANOVA followed by a Dunnett's or a Tukey's *post hoc* test, or two-way ANOVA followed by a Dunnett's or a Sidak's *post hoc* test.

RESULTS

A subset of secreted *S. flexneri* proteins precipitate with the host protein Toca-1 *in vitro*

We previously showed that early during infection, the host protein Toca-1 forms rings around ~60 % of intracellular *S. flexneri* and is required for inhibition of LC3-associated phagocytosis [9, 12]. The specific and early recruitment and role of Toca-1 suggested that it might serve as a scaffold that promotes the localization and/or function of *S. flexneri* type 3 secreted effector proteins that play a role early in infection. To test this, proteins secreted via the type 3 secretion system were precipitated with Toca-1. Secretion of type 3 secretion system proteins into the culture supernatant was induced with Congo red [26, 27], and the secreted proteins were precipitated by beads bound with protein A-Toca-1 or by control protein A beads. Precipitated proteins were identified by mass spectrometry.

Among the more than 30 type 3 secretion system proteins secreted by *S. flexneri*, four, OspC2/3, IpaB, IpgD and IcsB, were markedly enriched by protein A-Toca-1 precipitation as compared to the control precipitation (Table 2). For each of these four proteins, multiple peptides were detected (11 for OspC2/3, 12 for IpaB, 11 for IpgD and 7 for IcsB). By mass spectrometry, it was not possible to distinguish whether OspC2, OspC3 or both were present in Toca-1 precipitates, since in this strain (2457T) the two proteins are 92.4 % identical (Fig. S1, available with the online version of this article) [28], and the reads did not include a peptide

Table 2. Spectral counts of *S. flexneri* proteins precipitated by Protein A-Toca-1, as detected by mass spectrometry

Secreted *Shigella* protein	Spectral counts		*P* value
	Protein A-Toca-1	Protein A	
OspC2/3	16	0	0.004
IpaB	15	0	0.004
IpgD	12	0	0.004
IcsB	7	0	0.007
OspD2	1	0	0.045

that differs between the two. A fifth *S. flexneri* type 3 secreted protein, OspD2, was detected in the Toca-1-precipitated proteins at such low levels (one spectral count and one peptide) that the significance of its presence is uncertain. IpaB is one of two *S. flexneri* type 3 secretion system proteins that form a plasma membrane-embedded translocon pore in the host cell. OspC2, OspC3, IpgD, IcsB and OspD2 are each type 3 effector proteins that are delivered through the translocon pore into the host cell cytosol.

To validate the mass spectrometry results of the four proteins that displayed significantly higher spectral counts in the Toca-1 precipitate than in the control precipitate (OspC2/C3, IpaB, IpgD and IcsB), the levels of these proteins in Toca-1 and control precipitates were compared using Western blot analysis. Where antibodies to the native proteins were unavailable, FLAG-tagged derivatives were introduced into strains lacking the native protein. IcsB-FLAG, IpgD, OspC3-FLAG and IpaB each precipitated with Toca-1 (Fig. 1a–f).

Precipitation of each of these effectors was dependent on Toca-1, since minimal precipitation occurred with control beads. Moreover, these interactions were not due to non-specific binding of Toca-1 to type 3 effector proteins, since Toca-1 did not precipitate the type 3 effector VirA (Fig. 1g, h). This specificity is consistent with the detection by mass spectrometry of only the indicated subset of type 3 secreted proteins in Toca-1 precipitates, whereas a much larger number of type 3 secreted proteins are present in the Congo red-induced culture supernatants used as input for these studies [27]. For reasons that are unclear, effector proteins migrate more rapidly in the pull-down sample than in the input sample (Fig. 1a, d and g); we observe this consistently for all effector proteins (unpublished). Together, these pull-down data indicate that the *S. flexneri* type 3 effector proteins IcsB, OspC3 and IpgD, and the *S. flexneri* translocon pore protein IpaB, interact with Toca-1 *in vitro*.

Early during infection, type 3 effector protein OspC3 largely localizes near intracellular bacteria

Following bacterial internalization, host Toca-1 and type 3 secreted effector protein IcsB form rings around bacterial vacuoles, and Toca-1 and IcsB co-localize around the same intracellular bacteria [9, 12]. IcsB and the translocon pore protein IpaB are detected as puncta surrounding intracellular bacteria ([9, 29] and Fig. 2a). Using immunofluorescence microscopy, we tested whether the type 3 secreted effector OspC3 localizes to intracellular bacteria. At 40 min of infection, more OspC3 puncta were present near intracellular bacteria than in other parts of infected cells (Fig. 2b), whereas few puncta were present in uninfected cells within the same microscopic field. Even though OspC3 staining is weak, it was not dissimilar to that observed for IcsB ([29] and Fig. 2a), which we previously showed both localizes to intracellular bacteria and is required for recruitment of Toca-1 around intracellular bacteria [9]. The presence of a few scattered puncta elsewhere in the cytosol could have resulted from a number of events, including that intracellular *S. flexneri* had been present at these sites but had moved via actin-based motility to another location, leaving behind OspC3 and possibly other proteins. These observations suggest that in infected cells, Toca-1, IpaB, IcsB and OspC3 may be in close proximity to one another, consistent with the hypothesis that during *Shigella* infection, Toca-1 that surrounds intracellular bacteria interacts with a small subset of type 3 secreted proteins.

Type 3 effector OspC3 restricts IL-18 secretion from macrophage-like cells

OspC3 inhibits innate immune responses in human epithelial cells by binding the 19 kD processed fragment of caspase-4 and blocking caspase-4 activity [17]. In the absence of OspC3, *S. flexneri* infection induces activation of caspase-4, leading to processing of inactive pro-IL-18, secretion of mature IL-18 and inflammatory cell death. OspC3 disruption of caspase-4 activation results in decreased IL-18 processing and secretion.

We previously showed that IcsB restriction of LC3-associated phagocytosis depends on IcsB interactions with the host protein Toca-1 at early times during bacterial infection [9]. We therefore postulated that, like IcsB, interaction of OspC3 with Toca-1 early during infection might be important to OspC3 function inside cells.

We tested whether, as observed for human epithelial cells, OspC3 represses IL-18 cleavage and secretion in the macrophage-like cell line THP-1. We used THP-1 cells because macrophages are an important immune cell type infected by *Shigella* during human infection [30]. THP-1 monocytes were differentiated into adherent macrophage-like cells by incubation with phorbol 12-myristate 13-acetate (PMA). By 100–160 min of infection, PMA-differentiated THP-1 cells co-cultured with *S. flexneri* secreted mature IL-18 into the supernatant (Fig. 3a). At 220 min of infection, the level of mature IL-18 in the supernatant of cells infected with *S. flexneri* Δ*ospC3* was significantly increased compared to that in the supernatant of cells infected with WT *S. flexneri* (Fig. 3, $P<0.05$). The levels of pro-IL-18 in the supernatant, presumably released from dying cells, followed a similar general pattern (Fig. 3a). These results indicate that, as in epithelial cells, OspC3 represses IL-18 secretion from THP-1 cells.

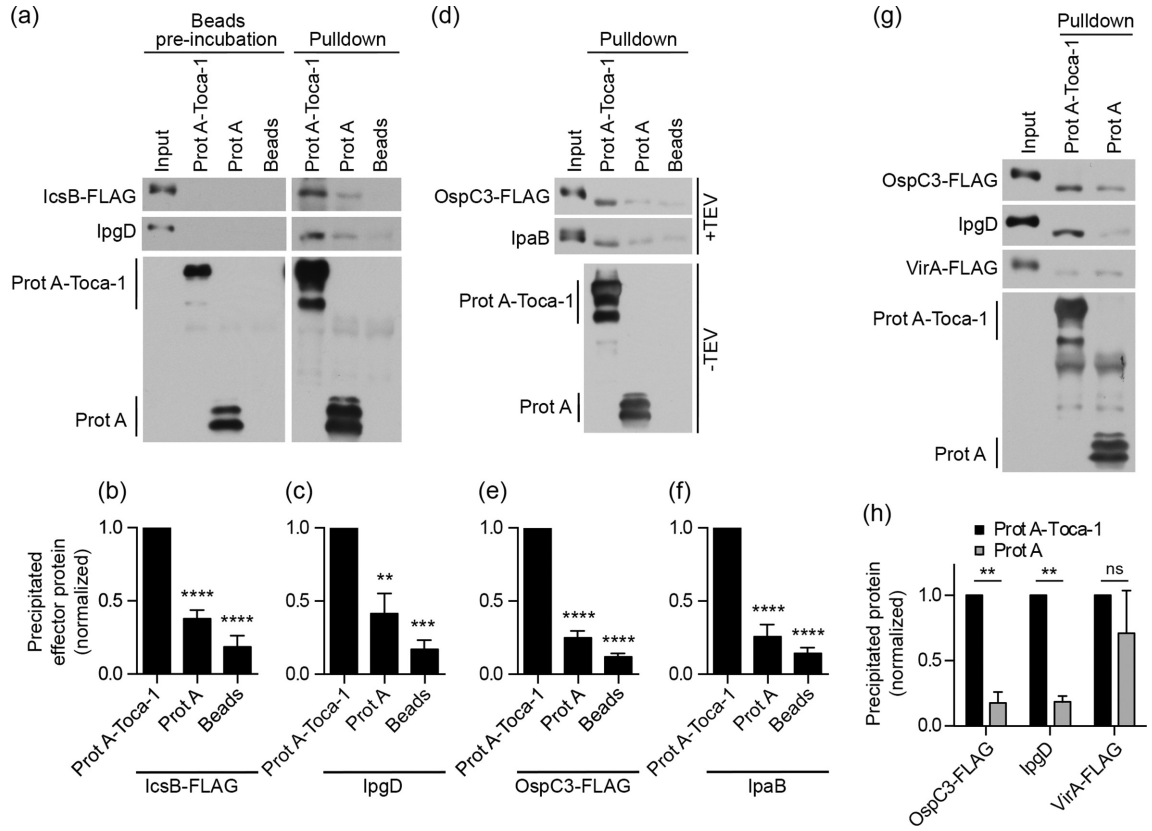

Fig. 1. A subset of secreted *S. flexneri* proteins specifically precipitate with the host protein Toca-1. *S. flexneri* proteins precipitated from culture supernatants by beads loaded with an equivalent amount of protein A-Toca-1 or protein A, or by beads alone. (a–c) Protein A-Toca-1 precipitation of IcsB-FLAG and IpgD. Representative Western blots (a) and band densitometry of precipitated protein (b, c). For each antibody used, both parts of the blot are from the same blot. (d–f) Protein A-Toca-1 precipitation of OspC3-FLAG and IpaB. Representative Western blots (d) and band densitometry (e, f). Following pull-down, to discriminate IpaB from a cross-reactive protein A-Toca-1 band at the same molecular weight, the protein A tag was removed with TEV protease in all samples (+TEV). To visualize the amounts of protein A-Toca-1 and protein A in the samples, samples obtained in parallel that were not treated with TEV protease are shown in the lower panel (-TEV). (g) and (h) Protein A-Toca-1 beads do not precipitate the secreted *S. flexneri* effector protein VirA. OspC3-FLAG and VirA-FLAG were co-expressed in *S. flexneri* prior to induction of type 3 secretion, and were differentiated on Western blots by molecular weight. Representative Western blots (g) and band densitometry (h). In each panel, the densitometry was normalized to that of protein precipitated by protein A-Toca-1 beads. Within each panel of the figure, all blots are from the same experiment; gels having distinct acrylamide concentrations, used to optimize separation of bands, were run in parallel. Data represent the mean±S.E.M. of four independent experiments. Input, secreted *S. flexneri* proteins in culture supernatant. **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$; ns, not significant; one-way ANOVA with Dunnett *post hoc* test (c) and (e) or two-way ANOVA with Sidak *post hoc* test (g).

Precipitation of *S. flexneri* effector IpgD with Toca-1 is potentiated by IcsB

We previously showed that Toca-1 recruitment around intracellular *S. flexneri* is dependent on the presence of the effector IcsB [9]. IcsB and Toca-1 reciprocally co-precipitate [9], consistent with our findings here (Table 2, Fig. 1a, b). The observed interactions between IcsB and Toca-1 suggested that IcsB might be required for the interactions between IpgD or OspC3 and Toca-1. We tested whether the interaction of IpgD with Toca-1 depended on IcsB by performing Toca-1 pull-down assays of culture supernatants that contained or lacked IcsB. In the absence of IcsB, there was a significant decrease in IpgD precipitated by Toca-1 beads (Fig. 4a, b, $P<0.05$). In the absence of IcsB, IpgD was precipitated by Toca-1 more than by control beads alone (Fig. 4a, b, $P<0.05$), indicating that IpgD interacts with Toca-1 in the absence of IcsB and that this interaction is strengthened by the presence of IcsB. Thus, IcsB potentiates IpgD binding to Toca-1.

Precipitation of *S. flexneri* effector OspC3 with Toca-1 depends on IcsB

We similarly tested whether the interaction of OspC3 with Toca-1 depended on IcsB, by performing Toca-1 pull-down assays of culture supernatants that contained or lacked IcsB. In the absence of IcsB, there was a significant decrease in OspC3-FLAG precipitated by Toca-1 beads (Fig. 4c, d, $P<0.01$), indicating that the interaction of OspC3 with

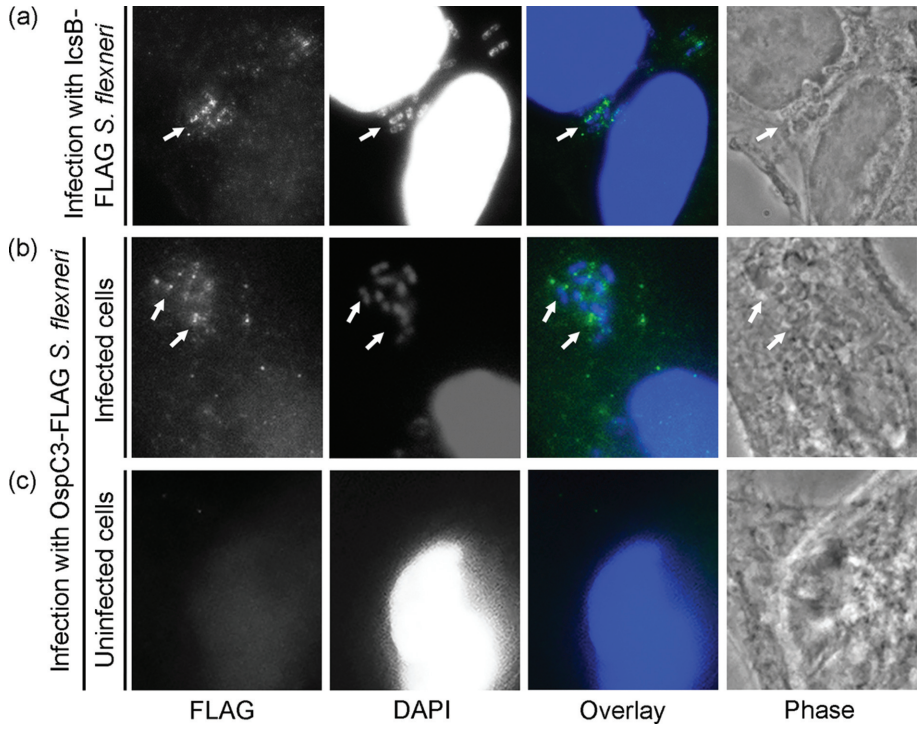

Fig. 2. Localization of OspC3-FLAG in *S. flexneri*-infected cells. Localization of IcsB-FLAG (a) or OspC3-FLAG (b) around *S. flexneri* at 40 min of infection of HeLa cells. (c) Uninfected cells in the same microscopic field as those imaged in panel (b), displaying minimal signal with FLAG antibody. Immunofluorescence to FLAG (left panels and green in overlay), staining of bacterial and cellular DNA with DAPI (second from left, and blue in overlay) and phase. Images are representative and are from three independent experiments.

Toca-1 is dependent on IcsB. Although the amount of OspC3-FLAG precipitated in the absence of IcsB appeared slightly greater for Toca-1 beads than for beads alone, this difference was not statistically significant. We did not test whether IpaB precipitation with Toca-1 is dependent on IcsB. These results indicate that, *in vitro*, the host protein Toca-1 interacts, either directly or indirectly, with the *S. flexneri* type 3 proteins IcsB, OspC3, IpgD and IpaB.

In macrophage-like cells, IcsB does not contribute to OspC3-mediated repression of IL-18 secretion

Interactions between Toca-1 and *S. flexneri* type 3 proteins *in vitro* raised the possibility that similar interactions occur during *S. flexneri* infection and function in type 3 effector modulation of host cell processes. We tested whether interactions of Toca-1 with *S. flexneri* effectors promote caspase-4 inhibition by OspC3, thereby restricting IL-18 maturation and secretion. Since IcsB is required for precipitation of OspC3 with Toca-1 *in vitro* (Fig. 4a, b), we tested whether the absence of IcsB reproduces the phenotype of the *ospC3* mutant, namely increased IL-18 cleavage and secretion (Fig. 3 and [17]). The levels of mature IL-18 secreted from THP-1 cells infected with *S. flexneri* Δ*icsB* were comparable to those from cells infected with WT *S. flexneri* (Fig. 3a, b), indicating that IcsB does not restrict IL-18 maturation.

In macrophage-like cells, the absence of Toca-1 is associated with reduced levels of IL-18

We postulated that by localizing OspC3 to a particular site within the cell, the interaction of Toca-1 with *S. flexneri* effectors *per se* might promote OspC3 activity, thereby restricting IL-18 cleavage and secretion. We attempted to test this by generating THP-1 cells that lack Toca-1 by virtue of stable integration of Toca-1 shRNA (Fig. 3c). Unexpectedly, pro-IL-18 was 2-fold less abundant in whole cell lysates of the THP-1 Toca-1 knockdown cells than in cell lysates of the control cells (Fig. 3d). Consequently, the importance of Toca-1 in IL-18 processing and secretion in THP-1 cells could not be assessed.

In keratinocytes, neither IcsB nor Toca-1 contributes to OspC3 restriction of IL-18 secretion

Because THP-1 cells could not be used to address the role of Toca-1 in IL-18 processing and secretion, we instead tested this question in keratinocyte cells, in which the functional relationship of Toca-1 and IcsB and the function of OspC3 had both been previously established [9, 17]. In contrast to THP-1 cells, in the keratinocyte cell line A431, the level of pro-IL-18 was similar upon shRNA knockdown of Toca-1 as compared to the shRNA control (Fig. 5a). We therefore tested the role of Toca-1 on IL-18 processing using A431 cells. Since OspC3 precipitation by

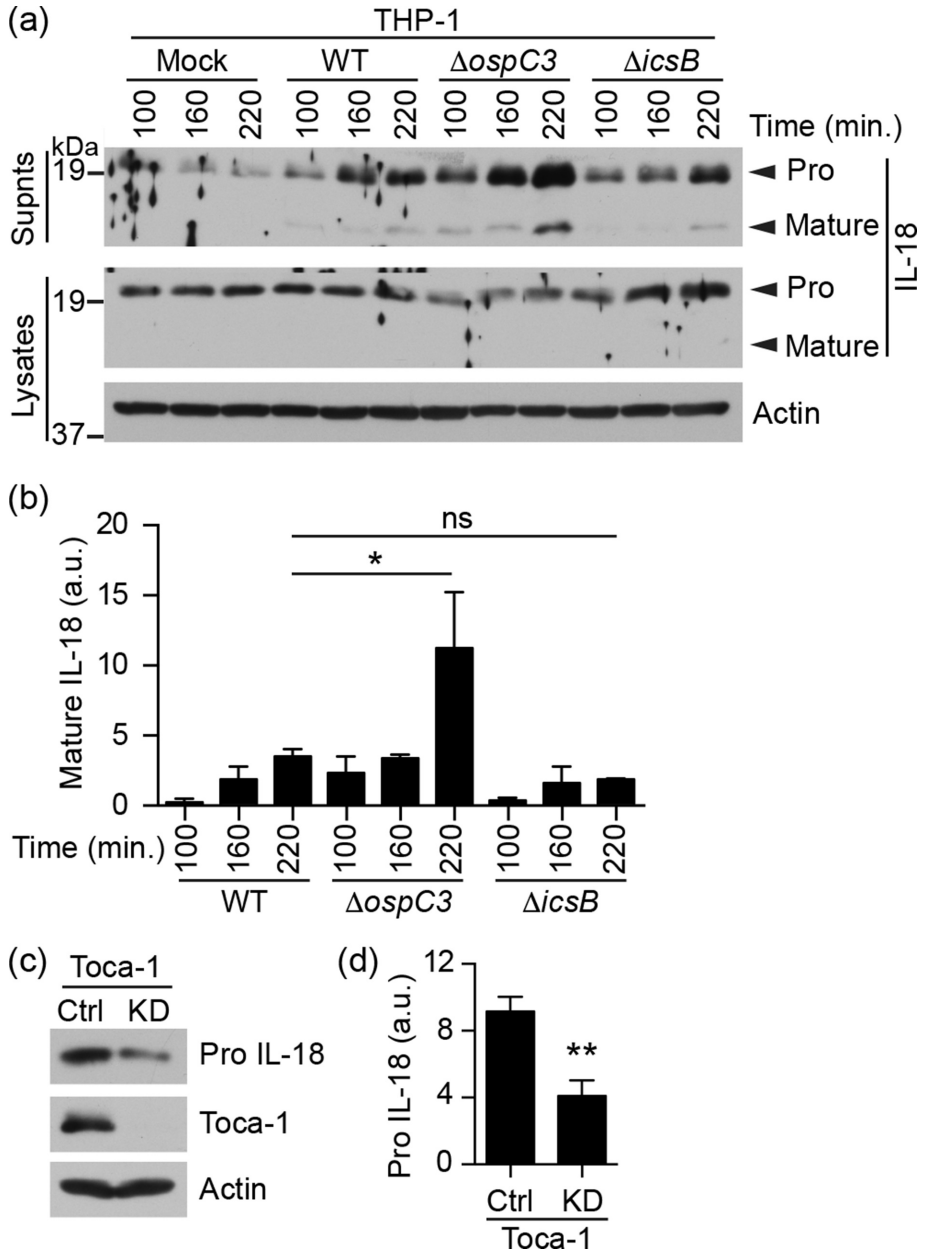

Fig. 3. IL-18 in infected or uninfected THP-1 macrophage-like cells. (a) and (b) Time course of IL-18 cleavage and release into the supernatants of macrophage-like THP-1 cells infected with *S. flexneri* wild-type (WT), Δ*ospC3* or Δ*icsB*, or mock infected. Representative Western blots (a) and band densitometry of mature IL-18 levels in the supernatant, normalized to actin in the corresponding lysates (b). (c) and (d) Pro-IL-18 levels in lysates from uninfected THP-1 Toca-1 knockdown (KD) or control (Ctrl) cells. Representative Western blot (c) and band densitometry of pro-IL-18 in lysates normalized to actin (d). Data represent the mean±S.E.M. of two (b) or three (d) independent experiments. *, $P<0.05$; **, $P<0.01$; ns, not significant; two-way ANOVA with Dunnett *post hoc* test (b) or unpaired two-tailed *t*-test (d).

Toca-1 depends on IcsB, we tested whether IcsB was required for restricting IL-18 secretion in these cells. In agreement with previous studies [17], levels of mature IL-18 were increased in the co-culture supernatant of keratinocytes infected with the *S. flexneri* Δ*ospC3* mutant as compared to that of cells infected with WT *S. flexneri* (Fig. 5, Toca-1 WT). In contrast, the level of mature IL-18 in the co-culture supernatant of A431 cells infected with the *S. flexneri* Δ*icsB* mutant was no different from that of cells infected with WT *S. flexneri* (Fig. 5, Toca-1 WT), indicating that IcsB does not play a role in restricting IL-18 processing and secretion.

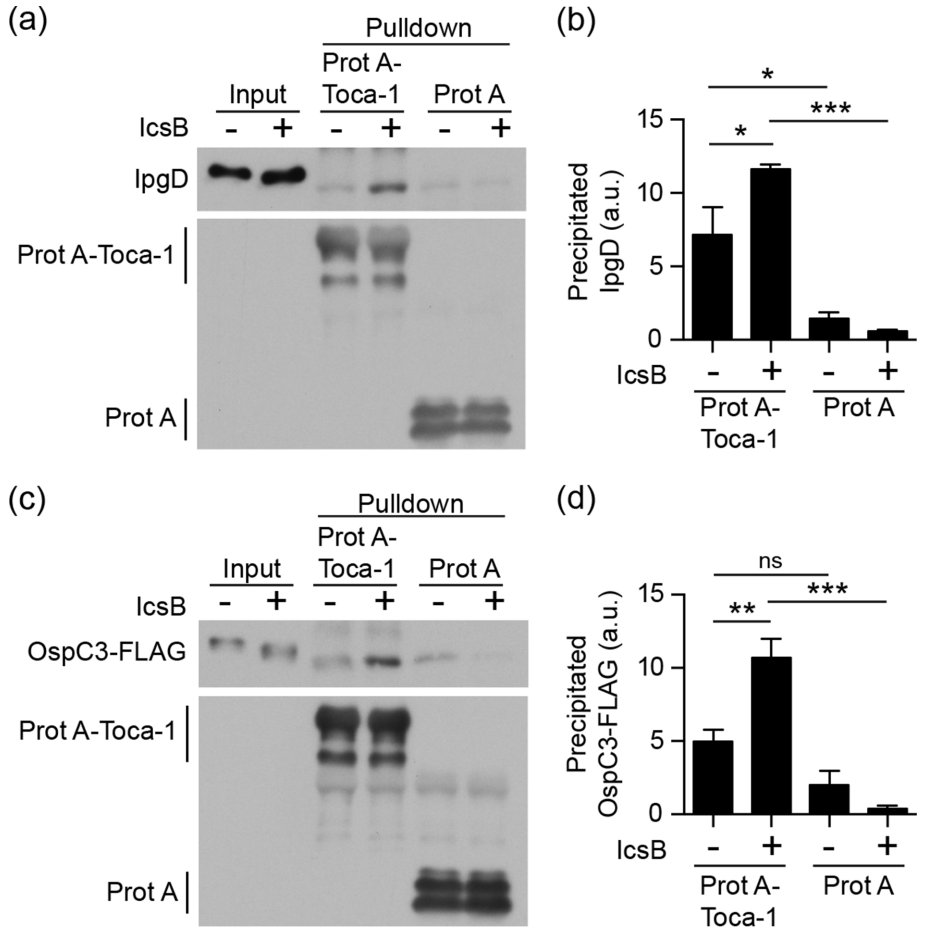

Fig. 4. IpgD precipitation with Toca-1 is potentiated by IcsB, and OspC3 precipitation with Toca-1 depends on IcsB. Protein A-Toca-1 precipitation of IpgD and OspC3-FLAG from culture supernatants of *S. flexneri* producing or lacking IcsB. (a) and (b) Precipitation of IpgD. Representative Western blots (a) and band densitometry (b). (c) and (d) Precipitation of OspC3-FLAG. Representative Western blot (c) and band densitometry (d). Input, *S. flexneri* culture supernatant. Data represent the mean±S.E.M. of three independent experiments. a.u., arbitrary units. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, ns, not significant; one-way ANOVA with Tukey *post hoc* test.

To test whether, in A431 cells, Toca-1 is required for restricting IL-18 maturation and secretion, we compared levels of mature, secreted IL-18 in cells that contained or lacked Toca-1. Upon infection with WT *S. flexneri*, levels of mature IL-18 in the co-culture supernatant of A431 cells in which Toca-1 was depleted were indistinguishable from those in the co-culture supernatant of control shRNA cells (Fig. 5). These data indicate that neither IcsB nor Toca-1 facilitates OspC3-mediated restriction of IL-18 maturation and secretion, and suggest that incorporation of OspC3 into a complex with Toca-1 and IcsB does not modulate the anti-caspase activity of OspC3 in infected cells.

DISCUSSION

Early during infection by *S. flexneri*, the type 3 effector IcsB restricts LC3-associated phagocytosis of the invading pathogen [9]. Restriction of LC3-associated phagocytosis depends on IcsB interactions with the host protein Toca-1 during and at early times after bacterial uptake [9]. IcsB and Toca-1 co-localize around intracellular *S. flexneri* coincident with bacterial containment within uptake vacuoles and with bacterial lysis of these vacuoles [9]. Here, we demonstrate that Toca-1 interacts *in vitro* not only with IcsB, but also with the type 3 translocon protein IpaB and the type 3 effector proteins OspC3 and IpgD. In contrast, the type 3 effector VirA did not specifically interact with Toca-1, suggesting that the interaction of Toca-1 with IpaB, OspC3 and IpgD is not due to non-specific aggregation of effectors *in vitro*.

The presence of IpaB among these interacting proteins raised the possibility that the proteins assemble on the cytoplasmic side of vacuolar membranes. Consistent with this are our observations, together with those of other investigators, that IcsB, OspC3, IpaB and Toca-1 are each detected near intracellular bacteria early during infection (Fig. 2; [9, 29]). The methodology used to perform the localization of these proteins detects proteins accessible in the host cell, but

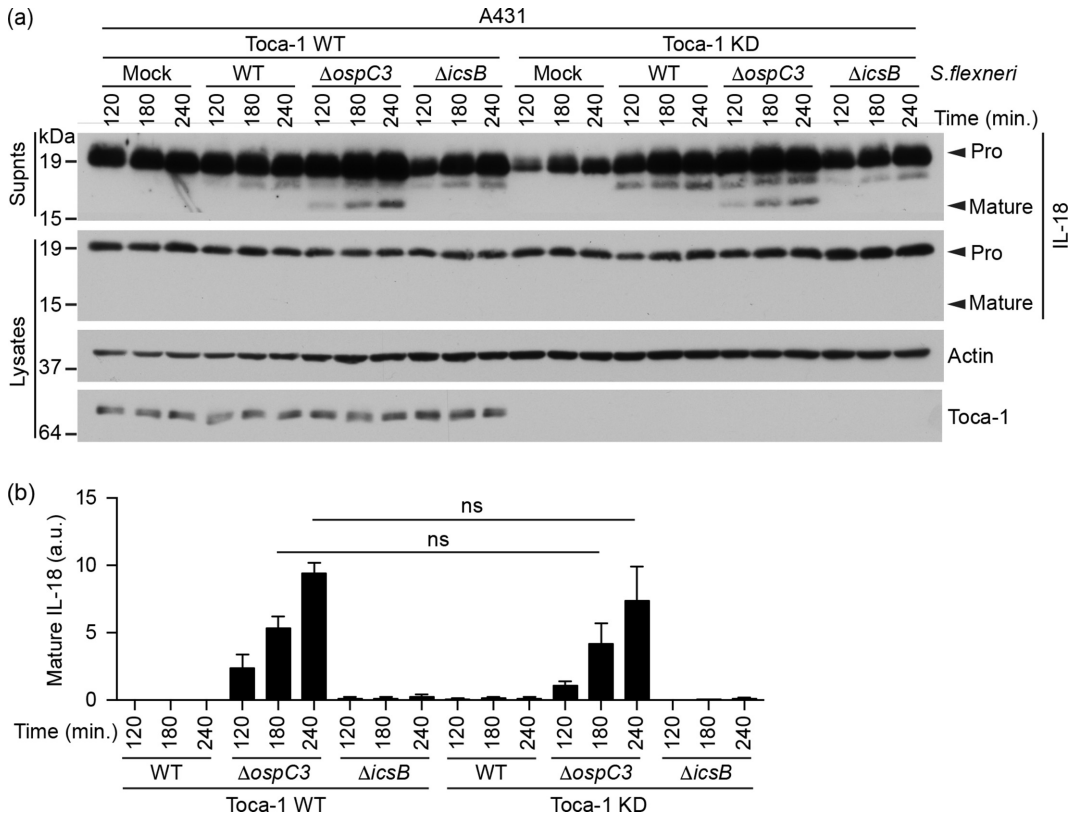

Fig. 5. In keratinocytes, neither Toca-1 nor IcsB contributes to OspC3 restriction of IL-18 secretion. Time course of IL-18 cleavage and release into the supernatants of A431 Toca-1 knockdown (Toca-1 KD) or control (Toca-1 WT) cells infected with *S. flexneri* wild-type (WT), Δ*ospC3* or Δ*icsB*, or mock infected. (a) Representative Western blots. (b) Band densitometry of mature IL-18 in the supernatants, normalized to actin in the corresponding lysates. Data represent the mean±S.E.M. of three independent experiments. a.u., arbitrary units; ns, not significant; two-way ANOVA with Sidak *post hoc* test.

not those retained within the bacteria, indicating that the visualized proteins have been secreted into the host cell. Whereas the localization of these proteins is consistent with interactions occurring among them at these sites early during infection, further studies are necessary to test this hypothesis. We did not examine the sub-cellular localization of IpgD.

Since IpaB localizes near intracellular bacteria [29], its interaction *in vitro* with Toca-1 and other effectors raises the possibility that, after vacuole lysis, IpaB could serve as an anchor for effector proteins on vacuolar membrane remnants. Since membrane remnants are sites of active host innate immune responses [31], concentration of these proteins at these sites would position them well for combatting these responses.

The interaction between Toca-1 and IcsB is functionally significant during infection, as it restricts LC3-associated phagocytosis [9]. In the absence of Toca-1, IcsB or a functional interaction between the two, recruitment of LC3 around *S. flexneri* at early times during infection is significantly increased. In contrast, we found OspC3 interaction with IcsB was not important for OspC3-mediated immune evasion. Precipitation of OspC3 by Toca-1 *in vitro* depended on IcsB, yet in the absence of IcsB, OspC3 inhibition of IL-18 maturation and secretion was unchanged (Figs 3a, b and 5). In addition, at least in keratinocytes, the absence of Toca-1 had no impact on IL-18 processing. We were unable to assess the importance of Toca-1 to OspC3 function in macrophage-like cells, since the absence of Toca-1 was associated with decreased levels of IL-18 (Fig. 3c, d). Whether the association of OspC3 with Toca-1 and the other interacting *S. flexneri* effectors *in vitro* might be relevant under distinct experimental conditions is currently unclear. Determining whether other interactions among these proteins are important in *S. flexneri* pathogenesis may provide additional insights into mechanisms of bacterial manipulation of host responses.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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