Inhibition of WAVE Regulatory Complex Activation by a Bacterial Virulence Effector Counteracts Pathogen Phagocytosis

Graphical Abstract

Highlights

- WAVE regulatory complex (WRC) and Arf and Rac1 direct phagocytosis of EPEC and EHEC
- Virulence effector EspG inhibits the WRC to counteract pathogen phagocytosis
- EspG blocks Arf6 signaling to ARNO that activates Arf1
- EspG blocks Arf1 collaboration with Rac1 and WRC activation

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In Brief
Humphreys et al. find that the virulence effector EspG of enteropathogenic and enterohaemorrhagic Escherichia coli inhibits pathogen phagocytosis by inhibiting activation of the WAVE regulatory complex (WRC). EspG uncouples the small GTPases Arf1 and Arf6 from Rac1, thus initiating WRC-dependent actin polymerization.
Inhibition of WAVE Regulatory Complex Activation by a Bacterial Virulence Effector Counteracts Pathogen Phagocytosis

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SUMMARY
To establish pathogenicity, bacteria must evade phagocytosis directed by remodeling of the actin cytoskeleton. We show that macrophages facilitate pathogen phagocytosis through actin polymerization mediated by the WAVE regulatory complex (WRC), small GTPases Arf and Rac1, and the Arf1 activator ARNO. To establish extracellular infections, enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) Escherichia coli hijack the actin cytoskeleton by injecting virulence effectors into the host cell. Here, we find that the virulence effector EspG counteracts WRC-dependent phagocytosis, enabling EPEC and EHEC to remain extracellular. By reconstituting membrane-associated actin polymerization, we find that EspG disabled WRC activation through two mechanisms: EspG interaction with Arf6 blocked signaling to ARNO while EspG binding of Arf1 impeded collaboration with Rac1, thereby inhibiting WRC recruitment and activation. Investigating the mode of EspG interference revealed sites in Arf1 required for WRC activation and a mechanism facilitating pathogen evasion of innate host defenses.

INTRODUCTION
Professional phagocytes cells represent the first line of host defense against bacterial pathogens. To eradicate pathogenic bacteria, professional phagocytes employ myriad host cell-surface receptors that bind the target bacterium directly (e.g., bacterial surface sugars) or indirectly through host-derived opsonins (e.g., antibodies, complement) (Celli and Finlay, 2002; Sarantis and Grinstein, 2012). Receptor binding triggers polymerization of actin filaments that guide the plasma membrane around the pathogen to facilitate bacterial uptake and destruction within an intracellular microbicidal phagolysosome compartment. The actin polymerization requires Rho GTPases Rac1 and Cdc42 that anchor by lipid prenylation to the membrane where they recruit and activate myriad cellular effectors responsible for directing cytoskeleton remodeling via the Arp2/3 complex (Caron and Hall, 1998; May et al., 2000).

Counteracting phagocytosis is a central paradigm in bacterial pathogenicity. For example, to inhibit opsonin-dependent trans-phagocytosis Staphylococcus aureus secretes protein A, which sequesters antibodies, while several pathogens use cell-surface capsule polysaccharides to inhibit deposition of complement (Celli and Finlay, 2002; Sarantis and Grinstein, 2012). However, phagocytes offset this strategy through myriad non-opsonic phagocyte receptors that directly bind bacteria and mediate cis-phagocytosis independent of opsonins. Nevertheless, uniting the diverse uptake mechanisms is the role of the actin cytoskeleton whose remodeling is required for phagocytosis (May et al., 2000). Consequently, pathogens have evolved sophisticated measures to interfere with the actin cytoskeleton and antagonize a spectrum of phagocytic mechanisms at the molecular level.

Enteropathogenic and enterohaemorrhagic Escherichia coli (EPEC and EHEC) are major global human health threats causing gastroenteritis and bloody diarrhea, respectively (Hartland and Leong, 2013). To cause disease, they inject a cocktail of virulence effectors into host cells via a type 3 secretion system (T3SS) to enable cell-surface colonization on intestinal epithelia where the pathogen forms lesions characterized by the destruction of brush border microvilli. Here, the bacteria encounter macrophages that infiltrate sites of infection yet EPEC and EHEC are able to block their own phagocytosis through the injected virulence effectors (Santos and Finlay, 2015). Indeed, mutants of type 3 secretion are phagocytosed by macrophages (Goosney et al., 1999; Marchès et al., 2008). Four virulence effectors are known to contribute to anti-phagocytosis (Santos and Finlay, 2015); EspB interacts with the actin binding motor protein myosin-1c (Iizumi et al., 2007), EspF inhibits PI3 kinase signaling (Celli et al., 2001), EspH inhibits the Dbl subfamily of Rho guanine nucleotide exchange factors (GEFs) (Dong et al., 2010) and EspJ impedes phagocytosis through inhibition of Src kinase activity (Young et al., 2014).

EPEC and EHEC employ multiple mechanisms to disable phagocytosis. While it is clear that the pathogens target the actin cytoskeleton, we do not yet understand the identity of the cellular actin nucleation machinery governing pathogen phagocytosis.
and therefore the mechanisms of bacterial interference. Consequently, we first sought to identify the players underlying the actin filament polymerization that are targeted by virulence effectors.

RESULTS

EPEC Opposes Phagocytosis Directed by the WAVE Regulatory Complex

To address how pathogenic Escherichia coli resist engulfment by macrophages, we infected differentiated human THP1 macrophage cells with wild-type EPEC (EPECWT) or T3SS-deficient mutant EPEC (EPECΔT3SS) labeled with pHrodo, a pH-sensitive dye that fluoresces red in the low pH of phagosomes and signified pathogen uptake (Figure 1A). Intracellular bacteria were inaccessible to antibodies against EPEC that marked extracellular bacteria. Only ~27% of EPECWT were found intracellular within phagosomes of host cells (actin) and labeling of extracellular bacteria with antibodies demonstrated that the majority of bacteria had counteracted phagocytosis (Figures 1A and 1B). In contrast, very few extracellular EPECΔT3SS were observed as ~92% of bacteria had been phagocytosed. Equivalent results were observed in RAW267.4 mouse macrophage cells (Figure S1A), confirming that EPEC fights phagocytosis using T3SS effectors.

Given the central role of Arp2/3-dependent actin polymerization in phagocytosis (May et al., 2000), it seemed likely that EPEC targeted activators of Arp2/3. The WAVE regulatory complex (WRC) is one such activator that is known to drive pathogen macropinocytosis in epithelial cells (Humphreys et al., 2012b, 2013) and has been implicated in phagocytosis by Dictyostelium (Seastone et al., 2001), mammalian granulocytes (Plis et al., 2012), and neutrophils and macrophages (Park et al., 2008). The WRC is a heteropentameric complex comprising Cyfip, Nap/Hem, Abi, and HSPC300 or their homologs (Gautreau et al., 2004), which must be activated directly by Rac1 effector p21 activated kinase (PAK) and GTP-bound Arf, thereby maintaining the GTP-bound form of Arf, and therefore likely uses an alternative mechanism. One possibility included the EPEC effector EspG that was previously shown to act as a molecular scaffold by simultaneously binding the Rac1 effector p21 activated kinase (PAK) and GTP-bound Arf GTPases (e.g., Arf1, Arf6) (Selyunin et al., 2011). EspG directly activates PAK, while EspG interaction with Arf sterically hinders Arf GAPs, thereby maintaining the GTP-bound form of Arf, which still has portions of its switch 1 and 2 domains exposed to permit interactions with some, but not all, of its cellular effectors (Selyunin et al., 2011, 2014).

First, we examined whether EspG antagonizes WRC-dependent cytoskeleton remodeling by infecting THP1 macrophages with pHrodo-labeled EPECWT or an isogenic strain with null mutations in espG and its close homolog espG2, henceforth EPECΔespG (Figure 1D). In contrast to wild-type bacteria, EPECΔespG bacteria were incapable of resisting phagocytosis, and ~85% were observed intracellularly mirroring the phagocytosis of EPECΔT3SS (Figures 1B and 1D). Furthermore, this EspG role appears conserved as ΔespG strain of the related pathogen EHEC was also susceptible to phagocytic uptake while wild-type EHEC were resistant (Figure S1D).

We next investigated the mechanism of WRC interference by EspG. The phosphoinositide PIP3 is known to activate the WRC through Rac1 and Arf GTPases (Lebensohn and Kirschner, 2009; Koronakis et al., 2011), and PIP3 is a major regulator of pathogen phagocytosis (Cox et al., 1999; Celli et al., 2001; Quittard et al., 2006), but how EPEC counteracts PIP3-driven pathways is unclear. We first reconstituted WRC-dependent actin polymerization driven by PIP3 using a motility assay in cell-free extracts as previously described (Hume et al., 2014). Silica microspheres coated with a phospholipid bilayer containing equal amounts of phosphatidylcholine (PC) and phosphatidylserine (PS) plus 2% PIP3 (PIP3) were added to cell-free extract containing fluorescent rhodamine-labeled actin and non-hydrolysable GTPγS to activate GTPases. In the control, PIP3 microspheres triggered actin polymerization and generated actin-comet tails (of ~14 μm) on the membrane surface that propelled the beads through the extract (Figure 1E). When PIP3-driven motility was examined in extract containing purified recombinant EspG, actin comet tail formation was abrogated and there was no actin assembly on the membrane surface (+EspG). This mirrored the phenotype observed with a Rac1 inhibitor (EHT1864) (Figure 1E) indicating EspG inhibition of the WRC.

To demonstrate that EspG was blocking WRC activation by Arf1 and Rac1, PC:PI microspheres (i.e., without PIP3) were anchored with purified constitutively active GTP-bound myristoylated Arf1-Q71L (Arf1QL) and prenylated Rac1-Q61L (Rac1QL) and then added to cell-free extract in the presence or absence of
EspG (Figure 1F). WRC-dependent actin comet tail formation (~15 μm) was observed in extracts containing PC:PI microspheres co-anchored with Arf1QL and Rac1QL, but actin polymerization was abolished in extract containing EspG.

To further examine how EspG disables WRC-dependent actin polymerization, we scaled up the motility assays to isolate the components recruited to the membrane co-anchored with Arf1QL and Rac1QL in the presence or absence of EspG (Figures 1G–1H).
EspG Targeting of Arf1 Antagonizes WRC-Mediated Phagocytosis

EspG is a multifunctional virulence effector and may interfere with WRC-mediated cytoskeleton remodeling in several ways: EspG binds active Arf GTPases and deactivates Rab GTPases with WRC-mediated cytoskeleton remodeling in several ways: EspG binds active Arf GTPases and deactivates Rab GTPases (Selyunin et al., 2011; Dong et al., 2012), and both Arf and Rab GTPases are known to promote Rac1-dependent actin filament polymerization (Palamidessi et al., 2008; Koronakis et al., 2011). EspG also activates PAK (Selyunin et al., 2011) that modulates actin filament dynamics, e.g., by deactivating cofilin (Edwards et al., 1999). Consistent with a possible role for PAK1, the presence of EspG at membranes co-anchored with Arf1QL and Rac1QL was co-incident with enhanced PAK1 recruitment (Figure 1H). To resolve how EspG disables WAVE complex activation, we first purified recombinant EspG mutants (Figure 2A) incapable of deactivating Rabs (EspGΔR; mutation Q293A) (Dong et al., 2012), binding PAK (EspGΔP; D205A, R208A) (Germane and Spiller, 2011), or binding Arf and PAK (EspGΔPA; I152S, P351A, P355A) (Selyunin et al., 2011) before assessing interference with WRC-dependent actin-based motility directed by Arf1QL and Rac1QL (Figure 2B). Like EspGWT, mutant derivatives EspGΔR and EspGΔP both abrogated actin-comet tail formation showing that EspG interaction with PAK and its Rab GAP activity were dispensable for WRC inhibition. This was not the case for EspGΔPA, which had no effect on WRC activity as actin comet tails (of ~14 μm as control) were formed and the microspheres moved through the extract. Thus, EspG targeting of Arf1 and not PAK or RabS blocked WRC activation.

Consistent with this view, only EspGΔPA was deficient in binding Arf1QL-anchored membranes in buffer (Figure 2C). Moreover, when EspGWT was pre-incubated with membranes co-anchored with Arf1QL and Rac1QL to form an Arf1-EspG
complex before incubation in extract, WRC-dependent actin comet tail formation was not observed (data not shown). Furthermore, chemical inhibitors of PAK (i.e., IPA3) have been shown to inhibit activation by EspG (Selyunin et al., 2011), yet WRC-dependent actin-based motility was observed in PAK-inhibited extract (Figure S2B), reaffirming that EspG inhibits the WRC independently of PAK.

To further examine EspG inhibition of the WRC, we assessed WRC recruitment by Arf1<sup>DL</sup> and Rac1<sup>DL</sup> from cell extract in the presence of the EspG derivatives (Figures 2D and 2E). WRC recruitment to the membrane was obstructed by EspG<sup>WT</sup>, EspG<sup>ΔN</sup>, and EspG<sup>ΔA</sup> but not EspG<sup>ΔAP</sup> as exemplified by the SDS-PAGE (Figure 2D, green arrows) and immunoblotting of Hem (Figure 2E). All EspG variants except EspG<sup>ΔAP</sup> were recruited to Arf1<sup>DL</sup> and Rac1<sup>DL</sup> co-anchored membranes. Interestingly, EspG<sup>ΔA</sup> but not EspG<sup>ΔAP</sup> recruited PAK1 indicating that PAK recruitment was dependent upon the Arf1-EspG interaction and localization of the virulence effector at the membrane.

In parallel, we assessed phagocytic uptake of EPECΔ<sup>espG</sup> expressing either EspG<sup>WT</sup>, EspG<sup>ΔN</sup>, EspG<sup>ΔA</sup>, EspG<sup>ΔAP</sup>, or the empty vector as a control (Figures 2F and S2A). THP1 macrophages phagocytosed ~82% of EPECΔ<sup>espG</sup> encoding the vector alone, while bacteria expressing EspG<sup>WT</sup> resisted WRC-dependent uptake that was reduced to ~45% (Figures 2F and S2A), mirroring the resistance imposed by wild-type EPEC (Figure 1).

Similarly, EPECΔ<sup>espG</sup> expressing EspG<sup>ΔN</sup> or EspG<sup>ΔA</sup> also antagonized phagocytosis. In contrast, ~79% of bacteria expressing EspG<sup>ΔAP</sup> were incapable of resisting the WRC and were phagocytosed to the same extent as the EPECΔ<sup>espG</sup> vector control strain. Thus, EspG-mediated interaction with Arf GTTPases, and not PAK or Rabs, combats WRC-directed pathogen phagocytosis.

Next, we examined whether EspG could counteract the activities of other pathogens dependent upon the WRC. In contrast to extracellular pathogens EPEC and EHEC, Salmonella Typhimurium is an intracellular pathogen that invades host epithelial cells by activating the WRC-Rac1-Arf1 axis (Humphreys et al., 2012b, 2013). When we examined *Salmonella* invasion in HeLa cells expressing HA-tagged EspG, pathogen uptake was reduced by ~64% relative to control, which was not observed in cells expressing EspG<sup>ΔAP</sup> (Figure 3A), thus providing further evidence of EspG interference of WRC-dependent cytoskeleton remodeling.

**EspG Incapacitates Arf6 and ARNO Upstream of WRC Activation**

EspG is known to bind Arf6 in an analogous fashion to Arf1 (Selyunin et al., 2011), though no function has been ascribed for this host-pathogen interaction. To trigger WRC-dependent invasion, *Salmonella* Typhimurium hijacks Arf6 to recruit and activate the Arf1 GEF ARNO of the cytohesin family (Humphreys et al., 2012b, 2013; Stalder et al., 2011). We therefore speculated that EspG might also incapacitate WRC activation by inhibiting Arf6 upstream of ARNO-mediated activation of Arf1.

First, we examined whether the cytohesin family (i.e., ARNO) facilitated phagocytosis. THP1 cells treated with the cytohesin inhibitor secinh3 impeded the relative uptake of EPECΔ<sup>espG</sup> from ~82% to ~30% (Figures 3B and 3C). ARNO is known to facilitate macropinocytosis (Humphreys et al., 2012b, 2013), but the uptake of EPECΔ<sup>espG</sup> was not affected by the macropinocytosis inhibitor eipa (Figure 3B). Secinh3 but not eipa also inhibited the phagocytosis of EPECΔ<sup>espG</sup> opsonized with human serum (Figure S2C). Thus, ARNO directs phagocytosis of EPEC, which was counteracted by EspG.

We next examined the mechanism by which EspG antagonized ARNO by reconstituting Arf6-driven activation of WRC as previously reported (Humphreys et al., 2013). PC:PI microspheres anchored with recombinant myristoylated Arf6 activated with GTP<sub>γ</sub>S were incubated in extract with or without (−) recombinant ARNO (Figure 3D). Arf6 only triggered actin assembly in the presence of ARNO, but this was abrogated in extract containing EspG<sup>WT</sup> but not EspG<sup>ΔAP</sup>. To determine how EspG impeded Arf6-dependent actin polymerization, PC:PI microspheres were isolated from extract then analyzed by immunoblotting (Figure 3E). To trigger WRC activation, Arf6 must recruit ARNO (Humphreys et al., 2013). Indeed, Arf6 alone (− ARNO) recruited very little Arf1 and Hem, which was enhanced upon addition of recombinant ARNO (+ ARNO). However, in the presence of EspG, Arf6 was incapacitated as the recruitment of ARNO as well as downstream players Arf1 and Hem were impeded. This was not the case with the Arf binding mutant EspG<sup>ΔAP</sup> that had no effect on the Arf6 cascade.

Finally, to investigate whether EspG directly inhibits ARNO via binding to Arf6, we examined interactions in buffer with purified components and PC-coated microspheres (Figures 3F and 3G) that minimize known ionic interactions between the ARNO pleckstrin-homology domain and acidic phospholipids such as PI (Macao et al., 2000). ARNO weakly bound PC microspheres alone, but its recruitment was potentiated by Arf6 (Figures 3F and 3G). In the presence of EspG<sup>WT</sup>, the virulence effector was recruited through Arf6 that blocked interaction with ARNO (Figures 3F and 3G). In contrast, the Arf binding mutant EspG<sup>ΔAP</sup> was not recruited by Arf6 and was incapable of impeding ARNO (Figures 3F and 3G). Thus, EspG directly disables Arf6-dependent actin polymerization by blocking signaling to its cellular effector ARNO.

**The Molecular Basis of WRC Interference by EspG**

Activated Arf1 mediates interaction with cellular effectors via its switch 1 (residues 40–51) and 2 (68–81) domains (Nie et al., 2003). EspG exhibits an unusual Arf binding interface that is rotated away from the switch 2 site (Figure 4A) where it interacts with the switch 1 and the alpha-1 helix (29–37) positioned outside of the canonical switch regions (Selyunin et al., 2011). Consequently, Arf1 bound to EspG can still bind cellular effectors that interact with its switch 2 domain such as the Arf binding GAT domain of GGA vesicle adaptors (Selyunin et al., 2014; Kuai et al., 2000) as depicted in Figure 4A and confirmed experimentally in Figure S3A.

We took advantage of the distinct binding modes of EspG and GAT3 to investigate the mechanism of Arf1-mediated WRC activation and EspG interference. First, we examined actin-based motility in extracts containing equivalent concentrations of either EspG or GAT3 (Figure 4B). EspG was more potent at inhibiting WRC than GAT3, which impeded robust comet tail formation but still permitted initiation of actin assembly and small comet...
EspG interaction with Arf1 is key to inhibiting WRC activation (Figure 2). Thus, we set out to resolve the molecular basis of EspG interference further by purifying an array of Arf1QL derivatives incorporating mutations within the alpha-1 helix (Y35Q), switch 1 (T45I, I49T), or switch 2 (I74T, Y81H) domain (Figure 4A), which have been implicated in interactions with EspG or its cellular effectors (Selyunin et al., 2011; Kuai et al., 2000). We examined EspG and GAT3 interactions with PC:PI microspheres anchored with each Arf1QL mutant derivative in buffer (Figures 3D and 3E, and comprehensively shown in Figures S3B and S3C). EspG bound control Arf1QL but interaction was evidently weaker (Figure 3D). The remaining Arf1 mutations had no effect. In contrast, GAT3 bound each Arf1QL variant except the switch 2 mutant Arf1QL-I74T (Figure 3E).

EspG interaction with Arf1 activates the WRC but the results suggested that EspG inhibits recruitment of Arf1 effectors that bind switch 1. This hypothesis was further substantiated by immunoblotting of the switch 1-binding protein AP-1 (Austin et al., 2002; Ren et al., 2013) that was recruited in the presence of GAT3 but not EspG (Figure 4C, AP-1).

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EspG Targets Arf1 Residues Essential to Cooperation with Rac1 and WRC Activation

As the Arf1 residue Y35, and to a lesser extent I49, likely underlie EspG inhibition of the Arf1-Rac1-WRC axis, we next examined whether these sites in Arf1 were key for collaboration with Rac1 in WRC recruitment and activation at the membrane. In contrast to Arf1QL or Rac1QL alone, only membranes co-anchored with both GTPases (ctrl) triggered recruitment of the WRC (Figures 5A and 5B) and robust actin comet tail formation (Figures 5C and S4A) demonstrating that small GTPase cooperation was required for WRC activation. Interestingly, Arf1QL alone recruited AP-1 (Figure 5B), a marker for classical Arf1 effectors that binds the alpha-1 helix and switch 1 domain of Arf1 (Austin et al., 2002; Ren et al., 2013). However, when Arf1QL combined with Rac1QL (ctrl) the presence of AP-1 was diminished relative to Arf1QL alone, while WRC recruitment was enhanced uncovering a remarkable switch in effector interplay by Arf1 when collaborating with Rac1 (Figure 5B). Thus, when working in synergy with Rac1 the results indicate that Arf1 recruits and activates the WRC via its alpha-1 helix and switch 1 domain in place of classical effectors such as AP-1.

When we examined the Arf1QL derivatives mutated in the alpha-1 helix, switch-1 or switch-2 domain, they all collaborated with Rac1 by recruiting the WRC (Figures 5A and 5B and comprehensively shown in Figures S4C and S4D). Given this observation, we were surprised to find that certain Arf1 mutations had a substantial impairment in WRC activation (Figures 5C and S4B). Like Arf1QL, mutants Arf1QL-T45I, -I74T, and -Y81H formed robust actin comet tails (exemplified by I74T in Figure 5C). In contrast, the motility of membranes anchored with Arf1QL-Y35Q or -I49T was markedly impaired (Figure 5C). We noticed that a small proportion of actin shells (~10%) surrounding the Arf1QL-Y35Q and Arf1QL-I49T membranes broke symmetry to form stumpy comet tails of ~2 μm (exemplified by green arrows in Figure 5C), indicating weak activation of the WRC. We speculated that the weak activation of the WRC by Arf1QL-Y35Q or -I49T was markedly impaired (Figure 5C). We noticed that a small proportion of actin shells (~10%) surrounding the Arf1QL-Y35Q and Arf1QL-I49T membranes broke symmetry to form stumpy comet tails of ~2 μm (exemplified by green arrows in Figure 5C), indicating weak activation of the WRC. We speculated that the weak activation of the WRC by Arf1QL-Y35Q would be resistant to interference by EspG, which binds Y35 (Figure 4D). Sure enough, while EspG blocked the formation of comet tails generated by the switch 2 mutant Arf1QL-I49T, stumpy comets were still formed by Arf1QL-Y35Q even in the presence of EspG (Figure 5C).

Finally, as both Arf1 residues Y35 and I49 mediated interaction with EspG (Figure 4D), we examined WRC recruitment and actin-based motility at membranes anchored with a double mutant (Figures 5A–5C). Arf1QL-Y35Q/I49T was incapable of collaborating with Rac1QL as the WRC was neither recruited (Figures 5A and 5B) nor activated (Figure 5C). Thus, EspG targets specific residues in the alpha-1 helix and switch 1 domain of Arf1 that control +GAT3

Rac1QL

Arf1QL

+EspG

Sw

itch1 Switch

2

EspG

GAT3

Arf1

Y35Q

α

1-helix

GAT3EspGctrl

Rac1QL

Arf1QL

GAT3

EspG

Hem

AP-1

GST

Arf1

Rac1

T45I

I49T

I74T

E

ctrl Y35Q I49T-

PCPI

EspG

Arf1

GAT3

ctrl Y35Q I49T-

PCPI

EspG

Arf1QL

I74T

I74T

GAT3

Arf1

PCPI

Figure 4. Molecular Basis of WRC Interference by EspG

(A) Cartoon depicting Arf1 interaction with EspG or GAT3 of GGA3 with key residues and domains in Arf1 shown.

(B) WRC-dependent actin-based motility directed by Arf1QL and Rac1QL in extract alone (control), or with GAT3 or EspG. Insets magnify actin-comet tails. Scale bars 5 μm.

(C) Immunoblotting of proteins recruited by membrane-anchored Arf1QL and Rac1QL from extract alone (ctrl), or from extract containing EspG or GAT3, or both in combination. Anti-GST antibodies detected GAT3 and EspG.

(D) Interaction of EspG with PCPI membranes alone (–) or with membranes anchored with Arf1QL (ctrl) or Arf1QL derivatives containing indicated mutations.

(E) Experiment performed as (B) with GAT3.

See also Figure S3.
facilitate small GTPase co-operation and actin filament polymerization by the WRC.

**DISCUSSION**

To avoid phagocytosis bacterial pathogens employ a wide range of strategies. For example, many pathogens secrete immunoglobulin proteases to cleave antibodies and impede FcR-mediated uptake (Sarantis and Grinstein, 2012). However, not all phagocytic mechanisms are driven by opsonization emphasizing the need for other inventive virulence strategies. We show that EPEC and EHEC circumnavigate this problem by inhibiting WRC signaling to the actin cytoskeleton whose remodeling is at the very center of phagocytosis. The role of Rac1 and Arf6 in phagocytosis is well established (Niedergang et al., 2003; Zhang et al., 1998; Caron and Hall, 1998), yet the contribution of the WRC (Park et al., 2008) and Arf1 is less clear (Bee-miller et al., 2006; Sendide et al., 2005). WRC activation by Arf1 and Rac1 is known to mediate lamellipodia formation and *Salmonella* macropinocytosis into host cells (Humphreys et al., 2012a, 2013). Here, we show a crucial role for the WRC in pathogen phagocytosis and establish that collabo-

![Figure 5. EspG Targets Arf1 Residues Essential to Synergy with Rac1 and WRC Activation](image)

- **A** WRC-dependent actin-based motility directed by Rac1Q26 alone (−) or in combination with Arf1Q26 (ctrl) or in combination with Arf1Q26 containing indicated mutations. Insets magnify actin-comet tails. Scale bars, 5 μm.
- **B** Proteins recruited by membrane-anchored Arf1Q26 or Rac1Q26 alone or Rac1Q26 in combination with Arf1Q26 containing indicated mutations as (A). Green arrows indicate cyfip and Hem.
- **C** Immunoblotting of samples from (B) with indicated antibodies (right).
- **D** WRC-dependent actin-based motility directed by Rac1Q26 in combination with indicated Arf1Q26 mutants in extract containing EspG. Insets magnify actin-comet tails. Scale bars, 5 μm.

See also Figure S4.
cytohesin-1 in opsonin-dependent phagocytosis (Beemiller et al., 2006; Sendide et al., 2005). ARNO is activated at the plasma membrane by Arf6 (Stalder et al., 2011; Cohen et al., 2007), a long established regulator of phagocytosis (Niedergang et al., 2003; Zhang et al., 1998). EspG directly abrogated Arf6 recruitment and activation of ARNO thereby impeding Arf1 activation and describing a role for the Arf6-EspG interaction.

In summary, by targeting both Arf6 and Arf1 our work establishes a dual mechanism by which a single virulence effector uncouples two arms of the WRC regulatory pathway and ultimately inhibits phagocytic uptake to evade innate host defenses (depicted in our model in Figure 6).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**

EPEC E2348/69 and EHEC EDL933 (TUV93-0 Shiga toxin deficient derivative) strains were used. Isogenic mutant EPEC ΔespG1/ΔespG2 (Prof. Feng Shao) and EHEC ΔespG (Dr. Ken Campellone) were kind gifts. For infections, bacteria were cultured as previously described (Smith et al., 2010).

**Plasmids**

The following plasmids were generated by Invitrogen Gateway methodology: pET15b-espG, pGEX2T-espG, pcDNA-HA-espG (encoding effector domain residues 48–398) and pTrc99FA-espG (full length). Plasmids pET20b-Arf1, pET20b-Arf6, pET15b-Rac1, pGEX2T-ARNO-2G, and pGEX2T-GGA3-GAT1–313 were described previously (Humphreys et al., 2013). Point mutations were introduced by site-directed mutagenesis into pET15b-espG, pGEX2T-espG (EspGΔ residues D205A, R208A; EspGΔN Q293A; EspGΔA R Q293A) and pET20b-Arf1 or pET20b-Arf6 (mutations indicated in the text), GST- and His-tagged proteins were expressed in E. coli Rosetta (Novagen) at 18 °C before affinity purification (Humphreys et al., 2012b).

**Antibodies**

Antibodies were purchased from Abcam (Rac1, ab33186; Arf1, ab58578; Arf6, ab16650; ARNO, ab56510; Actin, A2066; Cyfip, P0092; Nap1, N3788; AP1, A4200), GE Healthcare Life Sciences (GST, 27457701), Qiagen (Abi1, A5106; Actin, A2066; Cyfip, P0092; Nap1, N3788; AP1, A4200), GE Healthcare Life Sciences (GST, 27457701), QIAGEN (His, 34660) or were raised against recombinant proteins D205A, R208A; EspGΔN Q293A; EspGΔA R Q293A and pET20b-espG (Prof. Feng Shao) (Genta) at 18 °C before affinity purification (Humphreys et al., 2012b).

**Mammalian Cell Culture and Transfection**

The human monocyte-like cell line THP1s (kind gift from Prof. Gordon Dougan) and mouse macrophage-like RAW264.7 cells were cultured in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), 200 μg/mL streptomycin, and 100 U/mL penicillin (Thermo Fisher Scientific) before washing with Tris (pH 7.4)-buffered saline.

**Phagocytosis Assays**

Prior to infection EPEC and EHEC strains were harvested by centrifugation, washed in phosphate-buffered saline (PBS) then incubated with pH-Rodo (Thermo Fisher Scientific) before washing with Tris (pH 7.4)-buffered saline. Approximately 2 × 10^7 mammalian cells seeded onto glass coverslips were

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Figure 6. Model for EspG Incapacitation of the WRC

(A) Arf6 recruits and activates ARNO that activates Arf1 which consequently anchors via its exposed myristoylation moiety to the plasma membrane (brown lines). While the Arf1 binding partner remains speculative (e.g., Hem), nevertheless, membrane anchored active Arf1 and Rac1 work in synergy to recruit and activate the WRC (i.e., release of the WAVE veroprolin homology cofilin homology acidic region [VCA] domain) that induces Arp2/3-dependent polymerization of actin filaments (red) and pathogen phagocytosis. Empty (GDP) and filled blue (GTP) circles.

(B) EspG interaction with Arf GTPases blocks actin polymerization via a dual mechanism: EspG impedes Arf6-activation of ARNO and Arf1-activation of the WRC (highlighted by the red cross).

Given the central role of Rho GTPases in phagocytosis, it is not surprising that many pathogens employ GAPs to deactivate GTPases (e.g., Yersinia YopE, Pseudomonas ExoS) (Black and Bliska, 2000; Goehring et al., 1999). Yet, EPEC and EHEC encode no known GAPs. Instead, the pathogens interfere with Rho GTPase cooperation with Rac1 in driving cytoskeleton remodeling. Thus, EPEC and EHEC likely inhibit WRC-dependent phagocytosis by nullifying both Rho activation via EspH and Arf signaling via EspG.

The study also shows that ARNO, a plasma membrane GEF of Arf1, operates in this phagocytic pathway, which is consistent with a previously reported role for Arf1 and the ARNO homolog...
infected with pH-Rodo-labeled bacteria (1 hr, 37°C, 5% CO₂) before washing with PBS and fixation using 4% paraformaldehyde. Fixed cells were incubated with rabbit anti-intrin (EPEC/EHEC outer membrane protein) antibodies, washed with PBS, and then incubated with anti-rabbit Alexa Flour 350 antibodies and bodies visualized phalloidin-FITC-488 (Thermo Fisher Scientific) in PBS supplemented with Tx100. Phagocytosis was quantified by counting the number of extracellular bacteria labeled with intrin antibodies relative to intracellular bacteria showing pH-Rodo fluorescence using automated Velocity software (Improvision). When appropriate, cells were incubated with 25 μM Secinh3 (Merck), Immunofluorescence microscopy and images assembled as described (Humphreys et al., 2012b). All experiments were performed at least three times.

Salmonella Invasion of Non-Phagocytic Host Cells
Wild-type Salmonella enterica serovar Typhimurium SL1344 were used to assay invasion into non-phagocytic cells as previously described (Humphreys et al., 2013). Salmonella encoding pM975 that expresses GFP via the SPI2 promoter when bacteria are within Salmonellas containing vacuoles (SCVs) (Schlumberger et al., 2007) were used to infect HeLa cells (10 min), and the number of fluorescent bacteria was counted per cell microscopically.

In Vitro WRC-Dependent Actin-Based Motility
Preparation of porcine brain extract, actin-based motility by phospholipid-coated beads, and isolation of bead membrane-associated proteins have been described in detail (Hume et al., 2014). When indicated, extract or buffer containing recombinant EspG derivatives, GAT3, or ARNO was used. Quantification of comet tail length was performed on 50 comet tails per experiment using Velocity measurement software (Improvision). All experiments were performed at least three times.

Statistics
All experiments were performed at least three times. Geometric means were calculated, and significance was determined by Student’s t test or one-way ANOVA followed by a post hoc Dunnett’s comparison. *p < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.039.

AUTHOR CONTRIBUTIONS
D.H., V.S., and V.K. performed experiments and wrote the paper.

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