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Ubiquitination of Innate Immune Regulator TRAF3 Orchestrates Expulsion of Intracellular Bacteria by Exocyst Complex

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SUMMARY

Although the intracellular trafficking system is integral to most physiologic activities, its role in mediating immune responses to infection has remained elusive. Here, we report that infected bladder epithelial cells (BECs) mobilized the exocyst complex, a powerful exporter of subcellular vesicles, to rapidly expel intracellular bacteria back for clearance. Toll-like Receptor (TLR) 4 signals emanating from bacteria containing vesicles (BCVs) were found to trigger K33-linked polyubiquitination of TRAF3 at Lys168 which was then detected by RalGDS, a guanine nucleotide exchange factor (GEF) that precipitated the assembly of the exocyst complex. While this distinct modification of TRAF3 served to connect innate immune signaling to the cellular trafficking apparatus, it crucially ensured temporal and spatial accuracy in determining which among the many subcellular vesicles was recognized and selected for expulsion in response to innate immune signaling.

Graphical abstract

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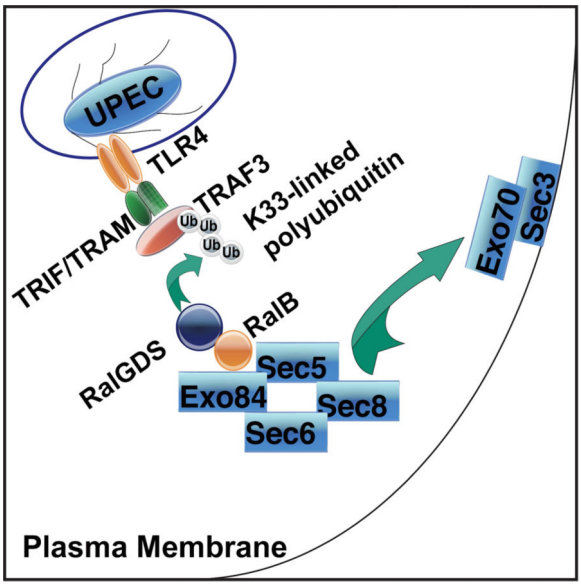
AUTHOR CONTRIBUTIONS

Studies were designed by Y.M. and S.N.A.. Y. M. carried out experiments, with J.W. providing key assistance. Data was analyzed by Y.M., with advice from S.N.A. The manuscript was primarily written by Y.M. and S.N.A. All authors contributed to discussions and manuscript review.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online



INTRODUCTION

In view of their highly compartmentalized nature, the membrane trafficking system of eukaryotic cells has emerged as a pivotal component of the immune defense network. Several excellent examples illustrating this role include cooption of multiple trafficking machineries such Rab5-mediated endocytosis (Kagan et al., 2008) and Rab11a-mediated trafficking (Husebye et al., 2010) to coordinate the accurate assembly of Toll-like receptor-4 (TLR4) signaling complexes on the endosomes, evoking robust Type I interferon responses as well as the utilization of an unconventional trafficking pathway to transport Toll-like receptor-9 (TLR9) from the endoplasmic reticulum (ER) into endolysosomes to achieve activation (Latz et al., 2004). However, unlike these examples of the involvement of the trafficking machinery in promoting immune recognition, almost nothing is known about whether these transporters can be directly mobilized for combating intracellular pathogens.

It was recently reported that infected epithelial cells exhibit a capacity to expel most of the intracellular bacteria suggesting an active role for the intracellular trafficking machinery in host defense (Bishop et al., 2007) (Miao et al., 2015). Since upon expulsion, the bacteria are either rapidly cleared by immune cells in the vicinity or eliminated by the flow of body fluids, such as urine, this activity represents a powerful host defense mechanism to reduce intracellular bacterial burden (Abraham and Miao, 2015). Using urinary tract infections (UTIs) as a model, these studies also revealed that following invasion of bladder epithelial cells (BECs) and entering Rab27b^{+} subcellular vesicles, uropathogenic *E.coli* (UPEC) within these bacteria-containing vesicles (BCVs) are promptly detected and expelled (Bishop et al., 2007) (Song et al., 2009). Since during this exocytosis-like process, bacteria appeared to be transported within cellular membranes and expelled without any loss of cell viability, the underlying mechanisms conceivably involve active participation of the subcellular trafficking machinery as well as innate immune recognition systems that activate them. Although the TLR4 molecules have been implicated in this activity (Song et al.,

2009), it remains unclear which set of cellular trafficking machinery is mobilized by TLR4 signaling. It is also unclear how this putative apparatus accurately distinguishes and specifically selects BCVs for expulsion from other subcellular compartments.

Much is already known regarding the TLR4 signaling circuitry activated following exposure to lipopolysaccharide (LPS) (Akira and Takeda, 2004). The classical view of the functional role of TLR4 signaling is that it serves as a “yellow alert” which initiates the production of a network of cytokines. Depending on its subcellular location, TLR4 activates two distinct signaling pathways resulting in different cytokine responses. Upon binding to LPS, TLR4 molecules in the plasma membrane will engage with MyD88 and TIRAP adaptor proteins resulting in the classical NF-κB responses and culminating in proinflammatory reactions (Kawai and Akira, 2011), whereas if the activated TLR4 is transported into subcellular compartments, it will interact with a different set of adaptor proteins, TRIF and TRAM which primarily initiate production of type I interferon (Barton and Kagan, 2009). Upon TLR4 activation, these distinct pathways appear to be coordinated by various ubiquitination modifications of TRAF3, a key downstream E3 ligase. For example, K48-linked polyubiquitination of TRAF3 results in its early degradation causing enhancement of cellular NF-κB responses, whereas when a K63-linked ubiquitin chain is added onto TRAF3, interferon production results (Tseng et al., 2010). Since TLR4 signaling results in the production of various cytokines, many of which serve to recruit professional immune cells to the site of infection to control and clear pathogens, the ultimate targets of this signaling pathway have been assumed to be extracellular. However, recent studies have revealed several outcomes of TLR4 signaling that are intracellular. For example, upon initiation of TLR4 signaling via MyD88 and TIRAP, TRAF6, has been reported to interact with mitochondrial respiratory proteins to directly augment bactericidal activity within infected cells (West et al., 2011). TLR4 signaling is also observed to activate autophagy, the key machinery involved in controlling intracellular infection (Xu et al., 2007). Seemingly TLR4 signaling is also an integral part of the cell autonomous immune system, mediating a myriad of intracellular responses with the ultimate goal of protecting the infected cell.

Here, we report that exocyst complex is directly mobilized by TRIF and TRAM mediated TLR4 signaling circuitry which emanates from intracellular compartments housing bacteria. We also demonstrate that a unique modification of TRAF3, the E3 ligase downstream of TRIF and TRAM, orchestrates the communication between TLR4 signaling and exocyst complex to achieve precise recognition and selection of BCVs for expulsion. This communication crucially ensured temporal and spatial accuracy in determining which among the many subcellular vesicles was recognized and selected for expulsion in response to innate immune signaling.

RESULTS

Compartmentalized TLR4 signaling triggers bacterial exocytosis

We started our quest by demonstrating the relevance of TLR4 signaling in bacterial export in a murine model of urinary tract infection (UTI), as a previous report on the involvement of TLR4 was obtained exclusively from *in vitro* studies using cultured BECs (Song et al., 2009). Since C3H/HeJ mice carry a mutation in their *Tlr4* gene, resulting in their inability to

recognize LPS, we employed these mice for our studies and C3H/HeN mice as wild type controls. After infection of both groups of mouse bladders employing standard procedures (See Method), we assessed bacterial expulsion after eliminating all of the residual extracellular bacteria with gentamycin treatment. As expected, the bacterial expulsion in the C3H/HeJ mice was minimal, whereas robust bacteria expulsion was observed in bladders of the control C3H/HeN mice (**Figure 1A**). In order to exclude the possibility that the repeated bladder expansion associated with washing of the bladder contributed to bacterial expulsion, we collected infected bladders from C3H/HeN or C3H/HeJ mice and incubated them in aerated medium *ex vivo* and assessed bacterial expulsion. It is known that the bladder epithelial tissue remain viable and functional for at least 4 hours under these experimental conditions (Wieser et al., 2011). As expected, the bacteria expelled from C3H/HeJ mice remained minimal in contrast to their C3H/HeN counterparts (**Figure 1A**). Based on these *in vivo* and ex vivo studies, we conclude that the TLR4 signaling-mediated expulsion is a critical defense mechanism in the bladder to reduce intracellular bacterial burden immediately after their entry into $Rab27b^{+}$ BCVs.

Having established the relevance of TLR4 for bacterial expulsion, we sought to uncover its intracellular signaling components. Predictably, by uncovering the signaling circuitry initiated by TLR4 in infected human BECs, we should be able to identify key intracellular trafficking mediators involved in the export of bacteria. First, we investigated which of the two known TLR4 signaling pathways was responsible for initiating bacterial exocytosis by selectively silencing the expression of the key adaptor proteins involved in each of the two pathways in the human 5637 BEC line. Compared to control BECs, TRIF silencing, but not MyD88 silencing led to significant reduction in bacterial exocytosis activity, a phenotype which was comparable to what we observed in TLR4 silencing BECs (**Figure 1B**). The importance of TRIF, in contrast to MyD88 was further demonstrated by using gene deficient mice. When we compared *in vivo* bacterial expulsion between *Tlr4*$^{-/-}$, *Ticam1*$^{-/-}$, *Myd88*$^{-/-}$ and WT mice, we observed that *Ticam1*$^{-/-}$, but not *Myd88*$^{-/-}$, exhibited reduced bacterial expulsion to a similar degree as that observed in *Tlr4*$^{-/-}$ mice (**Figure 1C**).

This observation led us to assume initially that an interferon-activated gene was responsible for mediating bacterial exocytosis, as during infection, the TRIF and TRAM pathway invariably elicits type I interferon responses (Kagan et al., 2008). However, several independent observations indicate that this is not the case. When we silenced IRF3, the key transcription factor downstream of TRIF and TRAM for activating the production of type 1 interferon (Doyle et al., 2002), we didn't detect any significant difference in bacterial exocytosis (**Figure 1B**). Blocking IRF3 function by overexpressing a dominant negative mutant (IRF3Δ55) (**Figure S1A**) (Doyle et al., 2002) also failed to show a phenotype with regard to bacterial exocytosis (**Figure S1B**). To more definitely demonstrate that the TLR4 initiated, TRIF and TRAM mediated exocytic pathway was distinct from the well-characterized interferon response, we investigated the exocytosis of a Δ*msbB* mutant *E.coli* strain from infected BEC. This mutant *E.coli* has a mutation in the *msbB* gene and so lacks the myristic acid moiety of lipidA, therefore, its LPS is poorly recognized by TLR4 (Somerville et al., 1996). Consequently, the exocytosis of Δ*msbB* mutant *E.coli* from infected BECs was markedly impaired compared to the wild type (WT) *E. coli* strain

(**Figure 1D**). Importantly, this defect could not be rescued by treatment of infected BECs with recombinant type I interferon (**Figure 1D**), despite strong interferon responses being evoked (**Figure S1C**). Thus, the bacterial exocytosis observed in infected BECs triggered by TLR4 and mediated by TRIF and TRAM is a distinct and interferon-independent pathway. What is currently known of the early signaling events following activation of TLR4 by LPS within subcellular vesicles is that TRAM shuttles to the cytosolic side of these vesicles where it recruits TRIF which in turn recruits TRAF3 molecules, forming a stable signaling complex that eventually leads to the early activation of the transcription factor IRF3 via TBK1 kinase (Barton and Kagan, 2009) or late mobilization of interferon responses by IKKε kinase (Solis et al., 2007). When we silenced each of these components and examined their relevance to bacterial export we found that TRAM and TRAF3 were critical, but not TBK1 or IKKε (**Figure 1B**). The apparent noninvolvement of TBK1 or IKKε in bacterial exocytosis could be relevant as it may represent the precise juncture where this signaling pathway deviates from the known interferon response.

Since the TLR4 initiated and TRIF and TRAM mediated signaling originate from intracellular vesicles rather than the plasma membrane of the cell (Barton and Kagan, 2009) (Kagan et al., 2008), we investigated if these molecules or their downstream signaling components were also physically associated with BCVs in WT or Δ*msbB* mutant *E.coli* infected BECs employing a recently developed technique to specifically purify BCVs (Lonnbro et al., 2008; Miao et al., 2015). Here we pre-tagged bacteria with magnetic nanoparticles (see Methods) before they were employed for BECs infection. One hour post infection (h.p.i), we mechanically disrupted the plasma membrane of BECs and the BCVs were isolated using a magnet. The purity of these vesicle preparations and the rigor of the isolation methods were initially assessed by immunofluorescence staining and western blot probing various subcellular compartment markers in BCVs fractions isolated from WT bacteria infected BECs (**Figure S2**). We verified the enrichment of critical TLR4 signaling molecules, such as TRIF or TRAF3 in the BCVs fractions purified from WT *E.coli* infected BECs but remarkably, not in any of the vesicles containing Δ*msbB* mutants (**Figure 2A**), despite the fact that comparable amount of BCVs were examined as evidenced using the BCV marker, Rab27b. What is noteworthy is that TLR4 molecules could be found in both types of vesicles. However, the downstream signaling molecules in the TLR4 pathway were only recruited onto the BCVs encasing WT bacteria which were able to activate the TLR4 receptor (**Figure 2A**). This determination was further made by immunofluorescence staining where we observed that TRAM was found co-localized with an overwhelming majority of BCVs of WT *E.coli* and minimally with BCVs of Δ*msbB* mutant *E.coli* infected BECs (**Figure 2B**). Notably, neither TBK1, IKKε nor IRF3 could be found on the BCVs of the wild type bacteria, further demonstrating the irrelevance of type I interferon responses (**Figure 2A**).

Since the physical presence of the TLR4 signaling components on BCVs of WT infected BECs are not necessarily indicative that signals initiating expulsion were specifically emanating from these vesicles and not from other intracellular compartments or from the plasma membrane of the cell, we designed additional studies to address this question. We undertook a coinfection assay where BECs were simultaneously infected with WT and

Δ*msbB* mutant *E.coli* so that each BECs would harbor both bacteria (See Methods). Since each of the bacteria bear a different antibiotic resistance marker, it was possible to distinguish and enumerate how many of each strain were expelled. We reasoned that if the specific cues for bacterial exocytosis originate exclusively from BCVs, then only WT *E.coli* would be expelled, however, if the exocytic signals emanated from neighboring vesicles or from the plasma membrane then Δ*msbB* mutant *E.coli,* which are encased in compartments that intrinsically fail to signal, would also be expelled due to the presence of WT bacteria in the same cell. We found that whereas WT *E.coli* continued to be exocytosed from the coinfected BECs, the exocytosis of Δ*msbB* mutant *E.coli* from these cells remained minimal (**Figure 2C**). Therefore, this assay revealed that the regulated bacterial exocytosis is a highly selective activity, and only BCVs that are capable of emanating TLR4 signaling are selected for export.

The exocyst complex is recruited onto signaling BCVs for bacterial expulsion.

Having identified TRAM, TRIF and TRAF3 as critical mediators of compartmentalized TLR4 signals, we reasoned that one or more of these signaling moieties would be involved in the direct recruitment of the cellular exocytic machinery. We hypothesized that by employing these signaling molecules as bait, we could identify the corresponding transporters of BCVs. Each of these signaling proteins were immunoprecipitated from cell lysates of naive or infected BECs and proteins showing strong binding to the bait proteins after infection were selected and subjected to mass spectrometry for identification (see method). A pull-down protein of particular interest associated with TRAF3 was the guanine nucleotide exchange factor RalGDS (Figure S3A), and western blots confirmed the interaction between TRAF3 and RalGDS (**Figure 3 A, B and C**). RalGDS functions as a guanine nucleotide exchange factor (GEF) activating either RalA or RalB (Neel et al., 2011), a pair of small GTPases which regulate the assembly of a protein complex called exocyst (Moskalenko et al., 2002) which is composed of eight subunits implicated in intracellular transport. Whereas subunits such as Sec5, Exo84, and Sec8 are recruited to cytosolic surfaces of intracellular vesicles, other subunits of this complex including Sec3 are specifically located on the cell surface (Guo and Novick, 2004; He and Guo, 2009). Upon receipt of the activation signals, the exocyst complex promotes the movement of intracellular vesicles towards the cell surface, and its subsequent tethering to plasma membrane (Inoue et al., 2003). This action is driven by the components of the exocyst complex on the vesicle and their complementary counterparts on the plasma membrane which are drawn to assemble into an eight-component complex (Inoue et al., 2003). The functional contribution of the exocyst was demonstrated by silencing key exocyst components in BECs such as Sec5, Exo84 or Sec8 mRNAs which resulted in defective bacterial exocytosis following infection (**Figure 3D**). In view of its proposed role in activating small GTPases implicated in vesicular transport, we also examined the effect of silencing RalGDS mRNA and in addition, sought to identify which Ral GTPase it was specifically activating. Silencing RalGDS and also RalB, but not RalA mRNAs exhibited a defective bacterial exocytosis phenotype that was comparable to that observed in TLR4- or TRAF3 mRNA silenced BECs (**Figure 3D**). Further evidence implicating the exocyst complex in bacterial export came from assays probing for the activation of RalB and the assembly of the exocyst complex during this process. We show that compared to naïve BECs and Δ*msbB* mutant *E.coli*

infected BECs, WT *E.coli* infection of BECs caused a marked increase in GTP binding on RalB (**Figure 3E**). Consistent with this result, WT but not Δ*msbB* mutant *E.coli* infections induced the assembly of the exocyst complex in infected BECs (**Figure 3F**).

By examining the BCVs isolated from WT or Δ*msbB* mutant *E.coli* infected BECs, we found that the exocyst complex components mentioned above were selectively deposited only on vesicles containing wild type bacteria (**Figure 3G**). Immunofluorescence microscopy confirmed the localization of RalB on BCVs containing WT bacteria (**Figure 3H**), but not Δ*msbB E.coli*-containing vesicles. Therefore, the exocyst complex appears to be the critical exocytic machinery mobilized by the compartmental TLR4 signaling to mediate exocytosis of bacteria contained within their vacuole in the infected BECs.

We then sought to establish the contribution of components of exocyst complex to the host defense network. We first showed that silencing various exocyst complex proteins led to significant increase in the bacteria load in cultured BECs (**Figure 4A**). Next, we utilized our murine model of UTIs to demonstrate the physiological significance of this defense pathway. Due to nonavailability of gene-ablated mice deficient in various exocyst components, because such deficiency leads to a lethal phenotype (Friedrich et al., 1997), we opted to employ *in vivo* silencing of exocyst complex components selectively in the bladder epithelium. This was achieved by injecting into the mouse bladder, modified forms of siRNA oligos that are able to readily penetrate the plasma membrane upon binding to BECs (see method). This reagent has been successfully employed by multiple studies to silence genes in mice, resulting in both systemic and local alteration of various signaling pathways (Cheng et al., 2012; Difeo et al., 2009). In view of the impregnability of the tight epithelial barrier of the bladder, only superficial epithelial cells received the instilled siRNA. The siRNAs which comprised of either scrambled control sequence or targeted to either RalGDS or Sec5 mRNAs were instilled into the bladders of female mice and 72hours later, the same organs were challenged with UPEC. Approximately 6 hours post infection, the bacterial burden in various mouse bladders were compared. We chose this early time point in order to minimize the impact of recruited immune cells on bacterial numbers. As expected, bladders exposed to siRNA against RalGDS or Sec5 mRNAs exhibited a significantly enhanced bacterial burden compared to controls (**Figure 4B**). Thus, cumulatively the components of exocyst complex play a critical role in reducing intracellular infection load following infection.

K33-linked polyubiquitination of TRAF3 is necessary for RalGDS binding

So far, we have shown that the exocyst complex mediates bacterial expulsion following compartmentalized TLR4 signaling from BCVs. It is striking that the exocyst complex is able to precisely target BCVs emanating TLR4 signals from amongst the large pool of nondescript cellular vesicles, as depicted in **Figure 3G**. Therefore, we sought to investigate the basis for this cargo selection process. Initially, we reasoned that selectivity was achieved when RalGDS, the exocyst activator, came in contact with TRAF3 proteins anchored on BCVs. However, very limited binding interactions were observed between TRAF3 and RalGDS when immunoprecipitating endogenous proteins in naive cells (**Figure 3B**), or even when both proteins were overexpressed in naïve human BECs (**Figure 3A**). When we

examined the interactions between TRAF3 and RalGDS in BECs infected with *ΔmsbB* mutant *E.coli*, we again noticed a limited interaction which was in sharp contrast to the strong interaction seen between the same two signaling molecules in BECs infected with WT bacteria (**Figure 3C**). Seemingly, physical proximity between RalGDS and TRAF3 is not sufficient to mediate a binding interaction. Thus, TRAF3 only appears to efficiently bind RalDGS upon receipt of an activation signal. One possible explanation for this conditional binding interaction is a post-translational modification on either TRAF3 or RalGDS following TLR4 activation that permits consequential interactions. In this regard, TRAF3 is known to undergo various forms of ubiquitination upon activation by TLR4 resulting in marked changes in its activities leading to distinct cellular responses (Tseng et al., 2010). To examine if any such ubiquitination of TRAF3 was involved in the binding between RalGDS and TRAF3 which preceded bacterial exocytosis, we first overexpressed in BECs an Ubiquitin b mutant (Ub null) in which all the lysine residues of the Ubiquitin protein were replaced by arginine so that it would be able to function as a dominant negative mutant (Bloom and Pagano, 2005). Indeed, in BECs expressing this mutant, strong inhibition of interactions between TRAF3 and RalGDS, as well as bacterial exocytosis were observed (**Figure 5 A, B**). Thus, we speculate that the exocyst complex-mediated selection of BCVs for export is not simply through the recognition of bound TRAF3 molecules, but rather, through the detection of an ubiquitin-modified form of TRAF3. To test this hypothesis, we sought to map the ubiquitination site that was critical for bridging TRAF3 and RalGDS, and to identify the type of polyubiquitin chain that was added onto TRAF3. Our plan was to introduce specific mutations to either TRAF3 or to ubiquitin, and then examine the functional impact of ubiquitination on the selective exocytosis of BCVs. TRAF3 contains an N-terminal RING finger domain (a signature motif of E3 ubiquitin ligase), followed by several zinc finger motifs, and by a C-terminal TRAF-C domain (**Figure S3B**). After careful deletion analysis of the various fragments of TRAF3, we found that only the deletion in the beginning portion of the zinc finger motif could abolish the interaction between TRAF3 and RalGDS (**Figure S3C**), indicating that the putative modification site was within this region. We then substituted every lysine residue within this region with arginine, and in some cases mutated two or more lysine residues, and then co-transfected the resulting mutants with RalGDS, and examined their interactions in BECs following infection. We found that a single amino acid substitution at Lys168 of TRAF3 was sufficient to abolish its strong interaction with RalGDS in infected BECs (**Figure 5C**). We next proceeded to characterize the type of ubiquitination introduced to this site upon infection. During a typical polyubiquitin modification, an ubiquitin molecule is first added to a specific lysine residue on the substrate protein, and then a series of additional ubiquitin molecules become linked to a specific lysine residue on the previously added ubiquitin molecule. Structurally different polyubiquitin chains mediating distinct functional responses will result depending on the specific lysine residue (K6, K11, K27, K29, K33, K48, or K63) on the previous ubiquitin that the next ubiquitin molecule is linked to. To probe which ubiqutination linkage on TRAF3 is critical for bacterial expulsion, we first overexpressed a series of ubiquitin mutants in BECs. In each of these mutants, only one lysine was mutated into arginine, so it can potentially form all the other types of polyubiquitin chains, but not the one that was mutated (e.g. for K33R: the lysine on K6, K11, K27, K29, K48 and K63 were kept, only K33 was mutated into arginine). We found that, in contrast to the BECs transduced with

empty vector or with other ubiquitin mutants, only overexpression of K33R in BECs abrogated bacterial exocytosis activity (**Figure 5B**) to a similar degree as overexpressing ubiquitin null mutants. To further confirm that the ubiquitination modification for bacterial expulsion is K33-linked, we then co-transfected WT or the K168R mutant form of TRAF3 together with the K33 only ubiquitin mutant. (for K33 only: the lysine on K6, K11, K27, K29, K48 and K63 were mutated into arginine, only K33 remained unchanged), so this ubiquitin mutants could only form K33-linked polyubiquitin chain. As controls, we also transfected the BECs with K48-only, or K63-only ubiquitin mutants, two types of ubiquitination that are known to be added on TRAF3 during activation of TLR4 signaling. Importantly, when we undertook immunoprecipitation reactions, both WT and K168R TRAF3 mutants could be co-immunoprecipitated with K48, or K63 ubiquitin mutants, suggesting that these other types of polyubiquitin modifications of TRAF3 are not added onto Lys168 (**Figure 5D**). However, when we co-transfected WT or K168R TRAF3 with K33-only ubiquitin mutant, whereas WT TRAF3 efficiently pull-down the K33-only ubiquitin chain, we failed to detect a strong ubiquitination signal in IP fractions from K168R TRAF3 transfected BECs (**Figure 5D**). In addition, we further confirmed that only K33R overexpression was able to blunt the interaction between TRAF3 and RalGDS (**Figure 5E**).

Ubiquitination of TRAF3 is the BCV selection signal for the exocyst complex

Our studies imply that both the physical presence of TRAF3 on BCVs and its ubiquitin modification are necessary for the exocyst complex to recognize and transport BCV in response to TLR4 signaling. To establish this point, we sought to demonstrate that the anchoring of TRAF3 on BCVs was necessary but not sufficient to recruit the exocyst complex and then that TRAF3 ubiquitination was needed before any BCV was selected for export. We specifically blocked K33-linked ubiquitination of TRAF3 by mutating Lys168, and then compared the deposition of the export machinery on BCVs in BECs expressing WT or this mutant TRAF3. For this experiment, we first created a $TRAF3^{-/-}$ BEC line where the endogenous expression of TRAF3 was completely abolished employing CRISPR technology (**Figure S4 and Figure 6A**). We then reconstituted these $TRAF3^{-/-}$ cells with either WT or the K168R mutant form of TRAF3. When we isolated BCVs from these cells following bacterial infection, we observed that both WT and the K168R mutant form of TRAF3 were anchored in comparable amounts on BCVs indicating that as a result of TLR4 signaling from each of the BCVs, TRAF3 were recruited and further, that its binding to BCVs was unaffected even when bearing a K33-linked ubiquitination deficient mutation (**Figure 6A**). Since the export of the BCVs was critically dependent on its recognition by the export machinery, we next examined the presence of exocyst complex components on BCVs. We found that whereas exocyst complex components failed to deposit on BCVs isolated from $TRAF3^{-/-}$ cells, BCVs from WT TRAF3-reconstituted BECs were amply enriched with these components (**Figure 6A**), indicating that TRAF3 was necessary for recognition by the export machinery. Importantly, we found that transduced BECs expressing the K168R mutant form of TRAF3 were also incapable of recruiting exocyst components indicating that while the presence of TRAF3 on BCVs was necessary for recruiting export machinery it was not sufficient and that its ubiquitination at position 168 was also required (**Figure 6A**). When we examined cellular fractions of the various BEC constructs described above following infection, for the presence of GTP-bound RalB

(**Figure 6B**) and assembled exocyst complex (**Figure 6C**), we found these indicators of an activated export machinery were detectable in ample amounts only in cells where ubiquitinated TRAF3 was associated with BCVs (**Figure 6B, C**). To establish that only BCVs tagged by K33-linked polyubiquitination on TRAF3 were selected for exocytosis we assessed bacterial exocytosis in various BECs following infection with WT *E.coli*. We found that bacterial exocytosis was evident only in BECs where BCVs could be tagged by K33-linked polyubiquitinated TRAF3 (**Figure 6D**).

These findings reveal that following receipt of an activation signal, there is a specific requirement for polyubiquitination of a signaling molecule installed on intracellular vesicles before these compartments were selected for exocytosis.

DISCUSSION

In systematically elucidating the signaling circuitry associated with TLR4-mediated expulsion of UPEC from infected BECs, we have discovered that the subcellular trafficking machinery is a powerful weapon employed by the cell autonomous immune system to eradicate intracellular bacteria. This mechanism is distinct from the recently described expulsion activity described in UPEC infected BECs, where bacteria encapsulated in lysosomes were spontaneously discharged when the bacteria attempts to neutralize the pH of this organelle (Miao et al., 2015). Whereas lysosome-mediated bacterial expulsion seem a byproduct of the cell's attempt to maintain homeostasis and occurs as a reaction to eliminate the bacteria that have escaped from their initial Rab27b^{+} vesicles (Miao et al., 2015), the expulsion mechanism described here is triggered specifically by intracellular immune surveillance molecules immediately upon bacterial entry into Rab27^{+} vesicles and involves the active participation of the cell's export machinery. It is noteworthy that whereas trafficking proteins such as Rab11a and Rab5 have recently been reported to regulate TRIF and TRAM mediated TLR4 signaling (Husebye et al., 2010) (Kagan et al., 2008), our studies reveal that the converse is also true. Namely, that the cellular trafficking system are subject to regulation by immune surveillance molecules for direct bacterial clearance.

Although we have shown that TLR4 orchestrated bacterial expulsion is independent of TLR4 mediated type I interferon responses, they seem to share several signaling intermediaries such as exocyst complex components(Chien et al., 2006). Since type 1 interferon responses are typically a delayed reaction to LPS exposure (≥ 2 hours post infection)(Doyle et al., 2002) whereas bacterial expulsion is activated almost immediately after bacterial entry, it is conceivable that these responses are initiated by the same TLR4 signaling pathway, with the type 1 interferon response being activated only if bacteria still persist in the host cell. Thus, mechanisms could exist to switch one TLR4 response to another as required. A candidate molecule to play this putative regulatory role is TRAF3, as this E3 ligase was prominent in both bacterial expulsion and type I interferon responses, especially in light of our observation that the mobilization of the exocyst complex for bacterial expulsion was initiated by a previously unrecognized mode of ubiquitination modification on TRAF3. We observed that when a K33-linked polyubiquitination on TRAF3 occurred, this modification directed the exocyst complex to export bacteria. It is already known that the K63-linked polyubiquitination of TRAF3 is important for initiating type I

Interferon production (Tseng et al., 2010). Thus, it appears that TRAF3 can function as a molecular rheostat, coordinating distinct subsets of innate signaling events and dictating their outcome based on the different ubiquitin modifications that it experiences.

In this study, we have also demonstrated a “two factor authentication” mechanism that could be in play to ensure the accurate onset of bacterial expulsion by anchoring an ubiquitinated TRAF3 on the bacteria-loading vesicle, providing spatial as well as temporal accuracy in the selection of the correct cargo. Presumably, installment of TRAF3 onto BCVs provides spatial accuracy in export, as it distinguishes these compartments from numerous otherwise nondescript intracellular vesicles. The K33-linked polyubiquitin chain that is added on TRAF3 following activation ensures temporal specificity for the export activity as this modification is evoked only by the activation of TLR4 signaling on BCVs. Both the physical presence of TRAF3 and its post-translational modification are required for recognition by exocyst complex, and for the onset of bacterial expulsion. This two-factor authentication requirement could be an intrinsic cellular mechanism to avoid inappropriate or untimely release of unrelated subcellular compartments which could be harmful. Additionally, the ubituitination activity described here also seems to mirror the uptake of membrane receptor and ligand complexes by the endocytic machinery following engagement by extracellular ligands. When receptors destined for internalization are bound by ligands, these molecules or other down-stream signaling proteins promptly become ubiquitinated which is the critical signal for recruitment of the cellular endocytic machinery (Hicke and Dunn, 2003) (Mukhopadhyay and Riezman, 2007). Thus, the cargo selection process for both endocytosis and exocytosis in cells involve ubiquitin modifications on crucial molecules in the signaling pathway. Whereas the polyubiquitin chain for endocytosis is mainly K63-linked(Geetha et al., 2005), the post-translational modification for exocytosis involves K33- linked ubiquitination of the substrate protein. This reversible form of post-translational modification appears to be a common mechanism employed by the cell to determine which and when cargo are internalized or exported, and this mechanism is utilized by the innate immune system to ensure accurate onset of its responses. Thus, the bacterial expulsion appears to be a powerful cellular exocytic activity whose spatial and temporal accuracy is orchestrated by the intimate communication between innate immune signaling and intracellular trafficking machineries.

EXPERIMENTAL PROCEDURES

Bacterial exocytosis assay

2×10^5 human 5637 BECs cells were seeded 12h before infection. One hour after exposure of BECs to bacteria, the un-invaded bacteria were washed and killed with 100 g/mL gentamicin for one hour. This time point was considered as 0 hour post infection (h.p.i). At 0 h.p.i, 0.1% Triton X-100 was added into 6 wells of infected BECs to release intracellular bacteria and 50 L cell lysates were plated on MacConkey agar plates to determine the initial bacteria load at 0 h. To the remaining wells, 500 l expulsion medium (culture medium containing 100 mM methyl -D-mannopyranoside for preventing re-attachment of expelled bacteria and 25 g/mL bacteriostatic agent trimethoprim to prevent bacterial proliferation) was added. After two hours, 50 l of culture supernatant was plated on MacConkey agar plates to quantify the

exocytosed bacteria, and this number was normalized against initial intracellular bacterial load at 0 h and expressed as percentage of bacterial expulsion. What worth noticing is that the bacterial exocytosis appears to be initiated as soon as bacterial invasion, which results in higher initial bacterial load when various key components in TLR4 pathway or Exocyst Complex were silenced. To compensate this difference in initial bacterial load and to prevent the possibility that the difference in expelled bacterial number is a result of different initial load, the control KD BECs was usually infected with MOI at 300:1, while BECs in which key bacterial exocytosis components (e.g. TLR4, TRIF, TRAF3, RalGDS, RalB, or Sec5) were silenced, was infected with MOI at 100:1. The initial bacterial load was then compared to ensure the intracellular bacteria numbers in control or KD BECs were comparable.

Isolation of intracellular BCVs

To isolate intracellular BCVs, UPEC cell were conjugated with BioMag carboxyl magnetite nanoparticles (BM570, Bangs Laboratories) to generate “magnetic bacteria”. For this purpose, the particles were first washed twice in 0.1 M MES buffer (2-(N-morpholino) ethanesulphonic acid, pH 5.2), followed by activation with 4 mg EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). The activated and washed particles were mixed with 0.5×10^9 live E.coli, and incubated for 30 min at 37°C. After blocking with 1% BSA, the magnetic bacteria were added to 2×10^7 BECs at different MOI (WT: 300:1; ΔmsbB mutant: 100:1 to ensure initial bacterial load in different strain infected BECs were comparable). One hour later, the infected cells were scrapped and the plasma membrane was disrupted by repeated passage of the cell suspension through Gauge30 needles. The BCVs in the supernatant was then placed on a magnetic cell separation rack (BD IMagnet™, BD Biosciences, 552311) for washing and purification at 4°C. The presence and absence of specific interested molecules on purified BCVs was analyzed after SDS-PAGE and western blotting. The amount of Rab27b, the marker for BCVs was used to confirm similar amount of BCVs were isolated from different BECs or BECs infected with distinct strains.

*In vivo*UPEC infection and *in vivo or ex vivo* bacterial expulsion assay

Mice underwent deep anesthesia with nembutal, followed by transurethral inoculation with 2×10^8 UPEC using polyethylene catheters. For in vivo bacterial expulsion assay, the mice were infected by UPEC strain CI5 to prevent bacteria-initiated cell death. One hour after inoculation of the bacteria, the bladder was emptied and 30 l of PBS containing 100 g/ml gentamicin was administered for one additional hour to kill all the extracellular bacteria. After all of the gentamicin was washed away, 50 l of fresh HBSS buffer was injected into bladder. Two hours later, the fluid in the bladder which contained exocytosed bacteria and urine was collected, and the bacteria in the fluid was plated and quantified. For ex vivo expulsion assay, after the one hour gentamicin treatment, the mice were sacrificed, and the infected bladder was collected. After extensive washing, the bladder was cut open, and incubated in the oxygenated chamber filled with HBSS buffer for two hours. Any expelled bacteria into the extracellular buffer was plated on MacConkey agar plates and quantified.

In vivo RNA silencing

To silence various exocyst complex components in mouse bladder, Accel siRNA with non-targeting control sequence or simultaneously targeting four different regions of mouse

Ralgds (SMARTpool, Dharmacon Inc.E-062150-00-0020) or Sec5 (SMARTpool, Dharmacon Inc. E-042601-00-0020) were inoculated transurethrally 96 hours before infection. 24 hours later, the treatment was repeated, and 72 hours after the second treatment, the bladder was infected with clinical UPEC strain J96. After one hour infection, the gentamicin was inoculated to kill any un-invaded bacteria. After 4 hours, before the recruitment of neutrophils, the mice were sacrificed, the bladder tissue was collected, extensively washed, and homogenized to quantify bacterial burden in the bladder tissue

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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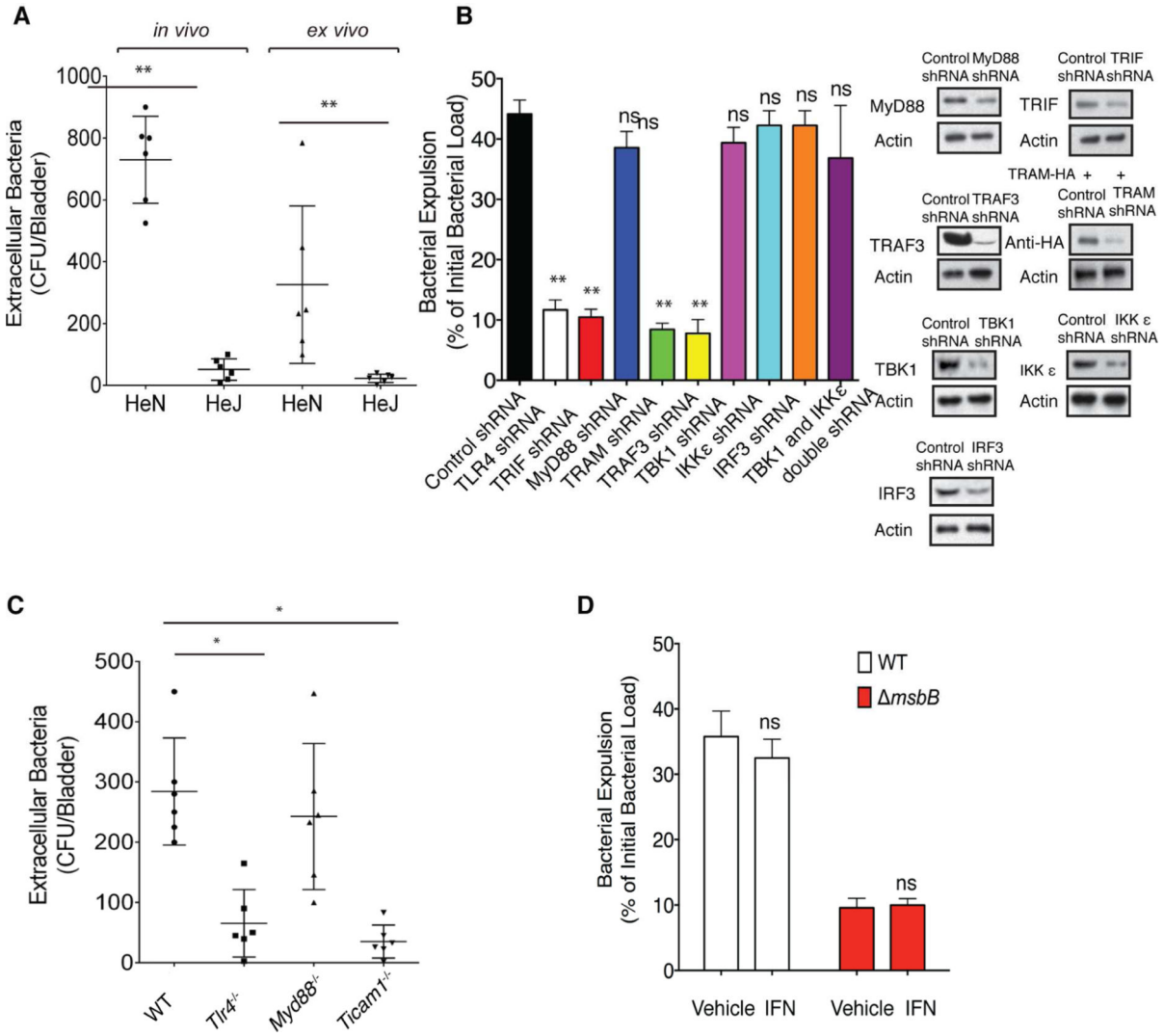

Figure 1. TRIF and TRAM mediated, but interferon-independent TLR4 signaling triggers bacterial expulsion

(**A**) *Tlr4* competent (HeN) or inactivated (HeJ) mice were infected with UPEC for 1h, followed by exposure to gentamicin for 1 h to kill extracellular bacteria. Upon extensive washing, the bacterial expulsion from the infected bladder epithelium was measured *in vivo* or *ex vivo* after 2 h incubation in buffer. Error bars represent SEM. The experiments were repeated three times.

(**B**) Bacterial expulsion from infected BECs transfected with control shRNA or shRNA plasmids targeting TLR4, TRIF, MyD88, TRAM, TRAF3, TBK1, IKKε, IRF3 or both TBK1 and IKKε respectively. Silencing efficiency is indicated by western blots. Error bars represent SEM. The experiments were repeated three times, where each experiment employed n=6 wells.

(**C**) Extracellular bacteria expelled from the infected bladder epithelium *in vivo* from WT C57/BL6 or *Tlr4*$^{-/-}$, *Ticam1*$^{-/-}$, *Myd88*$^{-/-}$ mice during 2 hours of incubation post infection. Error bars represent SEM.

(**D**) Bacterial expulsion from BECs infected with WT or Δ*msbB* mutant *E.coli* following exposure to vehicle or 1000U recombinant type I interferon. See also Figure S1.

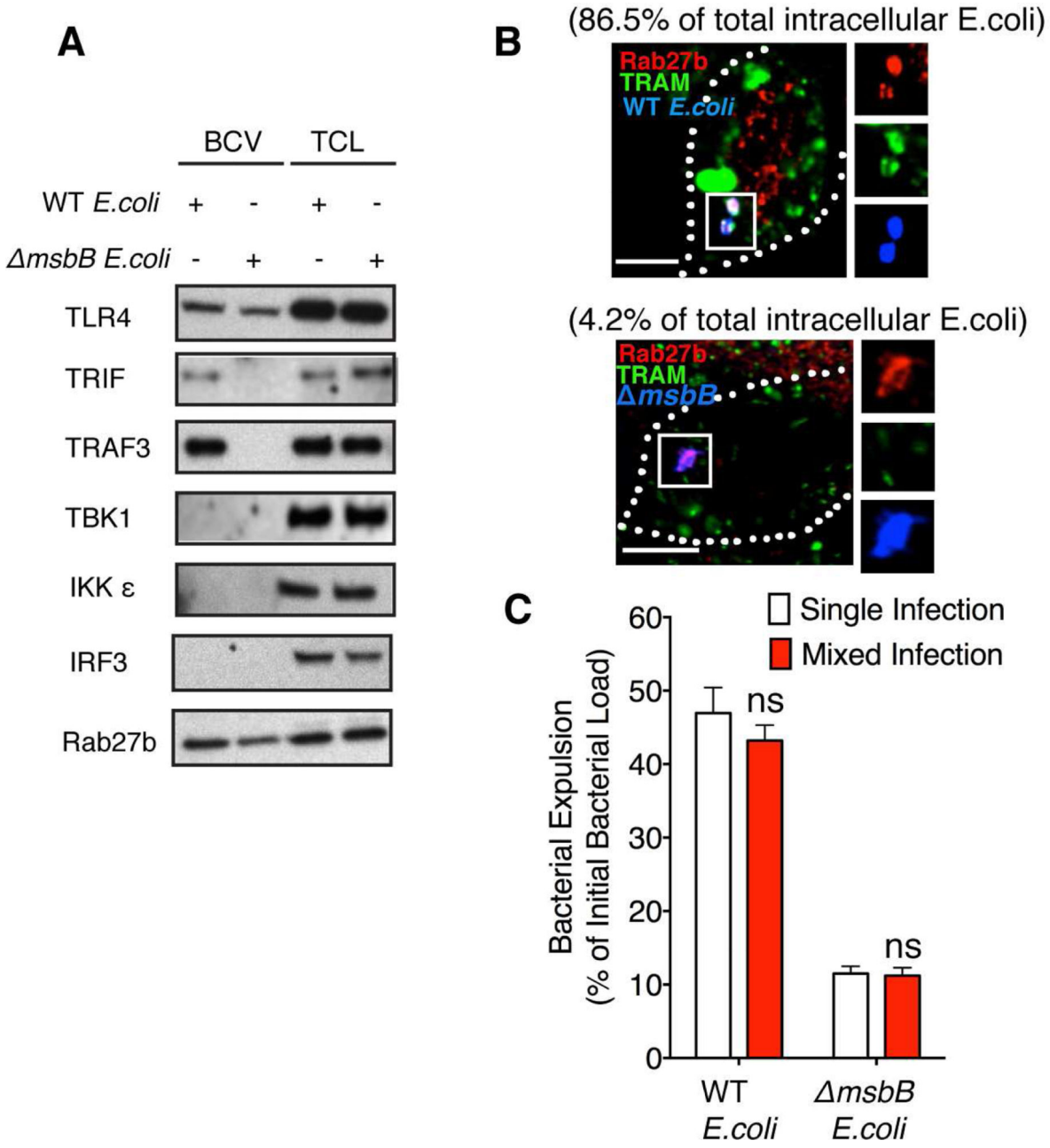

Figure 2. Signaling components regulating bacterial expulsion are compartmentalized on BCVs

(**A**) Protein immunoblot analysis of various TLR4 signaling molecules in bacteria-containing vesicle (BCV) fractions isolated from BECs infected with either WT or Δ*msbB* mutant *E.coli*. The BCV marker, Rab27b, was used to show that equal amounts of BCVs were employed for the various comparisons. Total cell lysates (TCLs) were used as controls to show that similar number of infected cells was employed.

(**B**) Immunofluorescence staining of infected BECs revealing the co-association of TRAM (green) with Rab27b^{+} vesicle (red) containing WT *E.coli* (blue) (upper), but not with vesicles containing the Δ*msbB* mutant (lower). Single channel images with high magnification of the area in the rectangle are shown alongside on the right. Quantification of

TRAM$^+$ BCV: This is indicated as percentage of total Rab27$^+$ BCVs in WT or Δ*msbB* mutant infected BECs and this is parenthesis. Dashed lines indicate the border of the cell. n=3slides Scale bar: 5 μm.

(**C**) Bacterial expulsion from BECs infected with a single bacterial strain (white bar) or with a mixture of WT and Δ*msbB* mutant *E.coli* (red bar). Error bars represent SEM. The experiments were repeated three times, where each experiment employed n=6 wells. See also Figure S2.

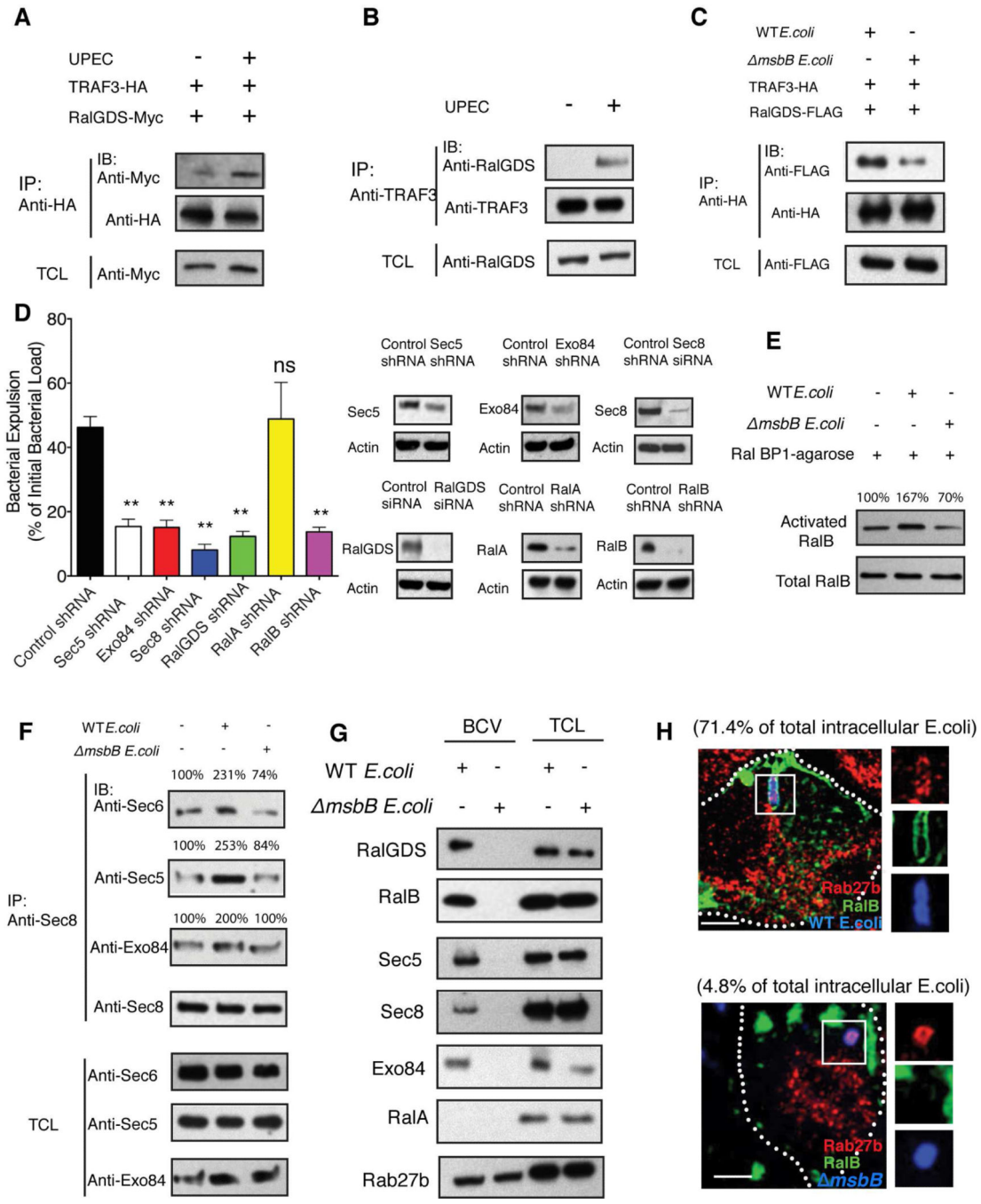

Figure 3. Exocyst complex is mobilized for bacterial expulsion

(**A**) TRAF3-FLAG was immunoprecipitated from naïve or infected BECs. The association between RalGDS and TARF3 in different conditions was examined by western blotting of the IP fractions. The protein of interests in the total cell lysate (TCL) was depicted to indicate that similar amount of RalGDS was present in each fraction.

(**B**) Endogenous TRAF3 was immunoprecipitated from naive or infected BECs, the presence of RalGDS in each IP lysate was detected with antibody recognizing endogenous RalGDS.

(**C**) The interaction between RalGDS and TRAF3 was examined by probing for RalGDS in TRAF3 IP fractions from BECs infected with either WT or ΔmsbB mutant *E.coli.*
(**D**) Bacterial expulsion in infected BECs transfected with control shRNA or shRNA plasmids targeting Sec5, Exo84, Sec8, RalGDS, RalA, or RalB mRNAs respectively. Silencing efficiency is indicated by the western blot. Error bars represent SEM. The experiments were repeated three times, where each experiment employed n=6 wells.
(**E**) Activation of RalB was examined by pulling down GTP-bound RalB using recombinant Ral BP1, the RalB effector conjugated on agarose beads, and the amount of activated RalB in naïve or BECs infected with WT or Δ*msbB* mutant *E.coli* was determined by immunoblotting.
(**F**) Assembly of exocyst complex was examined by blotting various exocyst complex components in the IP fractions using Sec8 antibody as probe. The amount of Sec6, Sec5, or Exo84 interacting with Sec8 was determined in naïve or BECs infected with WT or Δ*msbB* mutant *E.coli.*
(**G**) Protein immunoblot analysis of various exocyst complex components in bacteria-containing vesicles (BCV) fractions purified from BECs infected with either WT or *ΔmsbB* mutant *E.coli*. The BCV marker, Rab27b was used to show that equal amounts of BCVs were isolated for the various comparisons. Total cell lysates (TCLs) were used as controls to show that similar number of infected cells were examined.
(**H**) Immunofluorescence staining of infected BECs revealing the co-association of RalB (green) with $Rab27b^+$ vesicle (red) containing WT *E.coli* (blue), but not with vesicles containing Δ*msbB* mutant. Single channel images with high magnification of the area in the rectangle are shown alongside on the right. Quantification of $RalB^+$ BCV: This is presented as the percentage of total $Rab27^+$ BCVs in WT or Δ*msbB* mutant-infected BECs is indicated in the parenthesis. Dashed lines indicate the border of the cell. n=3 slides. Scale bar: 5 m. See also Figure S3.

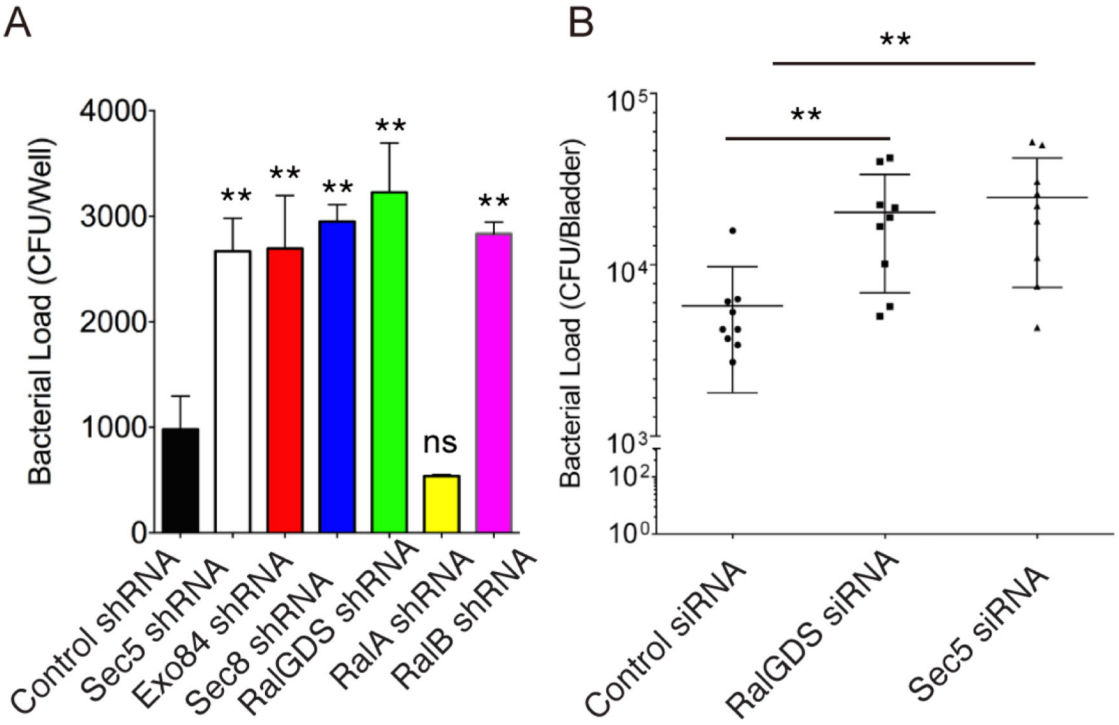

Figure 4. Critical role of exocyst complex in host defense against UPEC infections of the bladder

(**A**) Intracellular bacterial load measured in vitro cultured BEC line receiving control siRNA or siRNA targeting Sec5, Exo84, Sec8, RalGDS, RalA, or RalB, mRNAs at 2 h.p.i.. Error bars represent SEM. The experiments were repeated three times, where each experiment employed n=6 wells.

(**B**) Bacterial burden in the infected bladder tissue was measured in mice receiving modified siRNA targeting a scrambled control, *Ralgds* or *Sec5* which were intravesicularly injected into the mice bladder 72 hours before infection. Error bars represent SEM. The experiments were repeated three times, where each experiment employed n=3 mice, and the data were pooled.

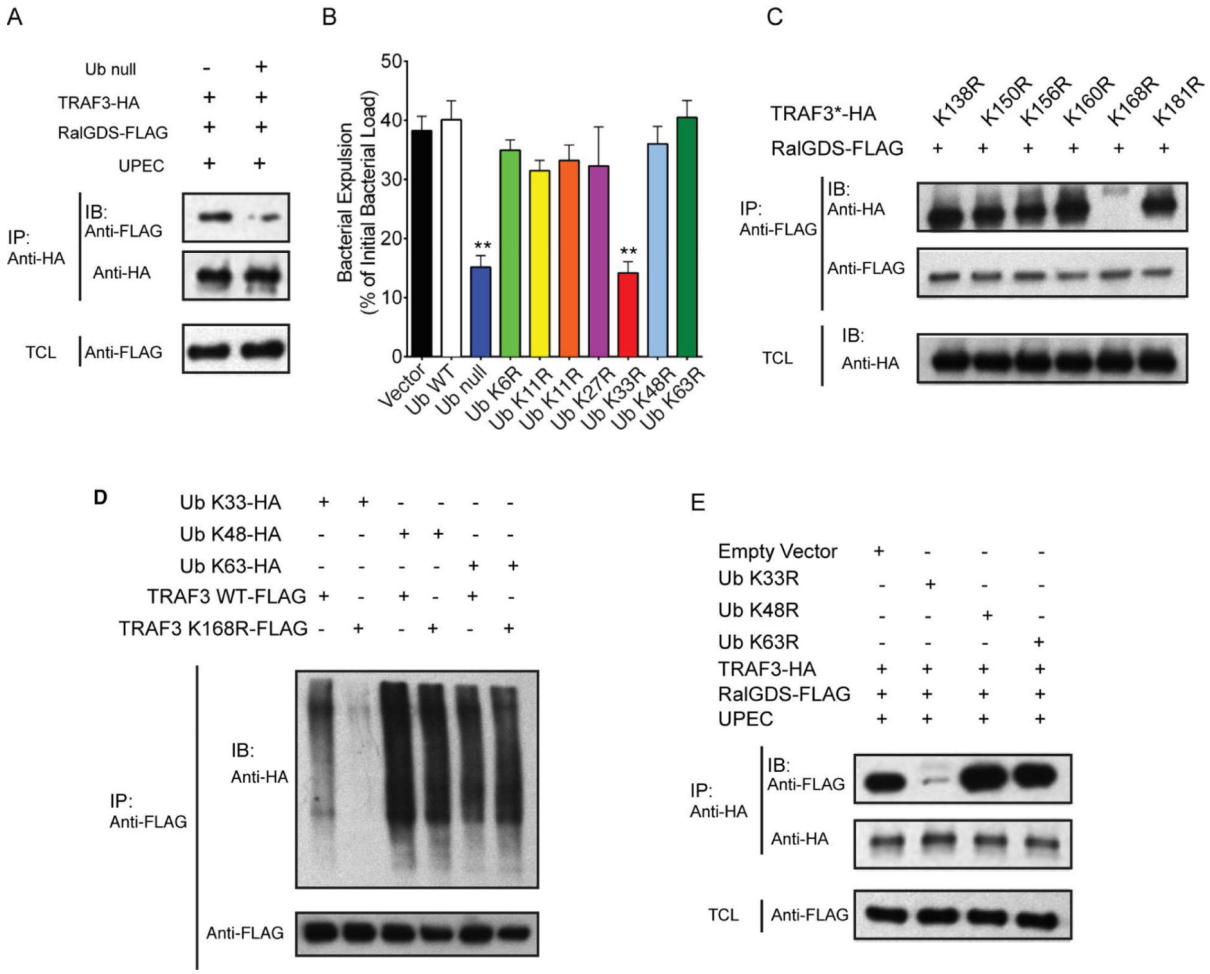

Figure 5. K33-linked polyubiquitination of TRAF3 is necessary for RalGDS binding

(**A**) The association between RalGDS and TARF3 was examined by immunoblotting RalGDS in TRAF3 IP fractions from infected BECs overexpressing dominant negative Ub null mutants, which cannot form a polyubiquitin chain. The empty vector was used as control here.

(**B**) Bacterial expulsion in infected BECs transduced with control vector or overexpressing Ub null, Ub K6R, Ub K11R, Ub K27R, Ub K29R, Ub K33R, Ub K48R, or Ub K63R mutants. Error bars represent SEM. The experiments were repeated three times, where each experiment employed n=6 wells.

(**C**) The association between RalGDS and a series of TARF3 mutants was examined by immunoblotting TRAF3 in RalGDS IP fractions from infected BECs. In each of these mutants, one lysine residue was changed to arginine. Notice that only the mutation at Lys168 abolished interaction with RalGDS.

(**D**) The WT or K168R TRAF3 mutants was co-transfected with an ubiquitin mutant, each of which can only undergo one single type of ubiquitination (including K33, K48, or K63-only ubiquitin mutants). The ubiquitination of either WT or K168R mutants was examined by immunoblotting of different ubiquitin mutants in the IP fractions of TRAF3.

(**E**) Different ubiquitin mutants were overexpressed in BECs to block each type of polyubiquitination. The association between RalGDS and TRAF3 was examined by immunoblotting RalGDS in TRAF3 IP fractions from BECs expressing each of these ubiquitin mutants. Representative blots were shown from three repeats of the experiments.

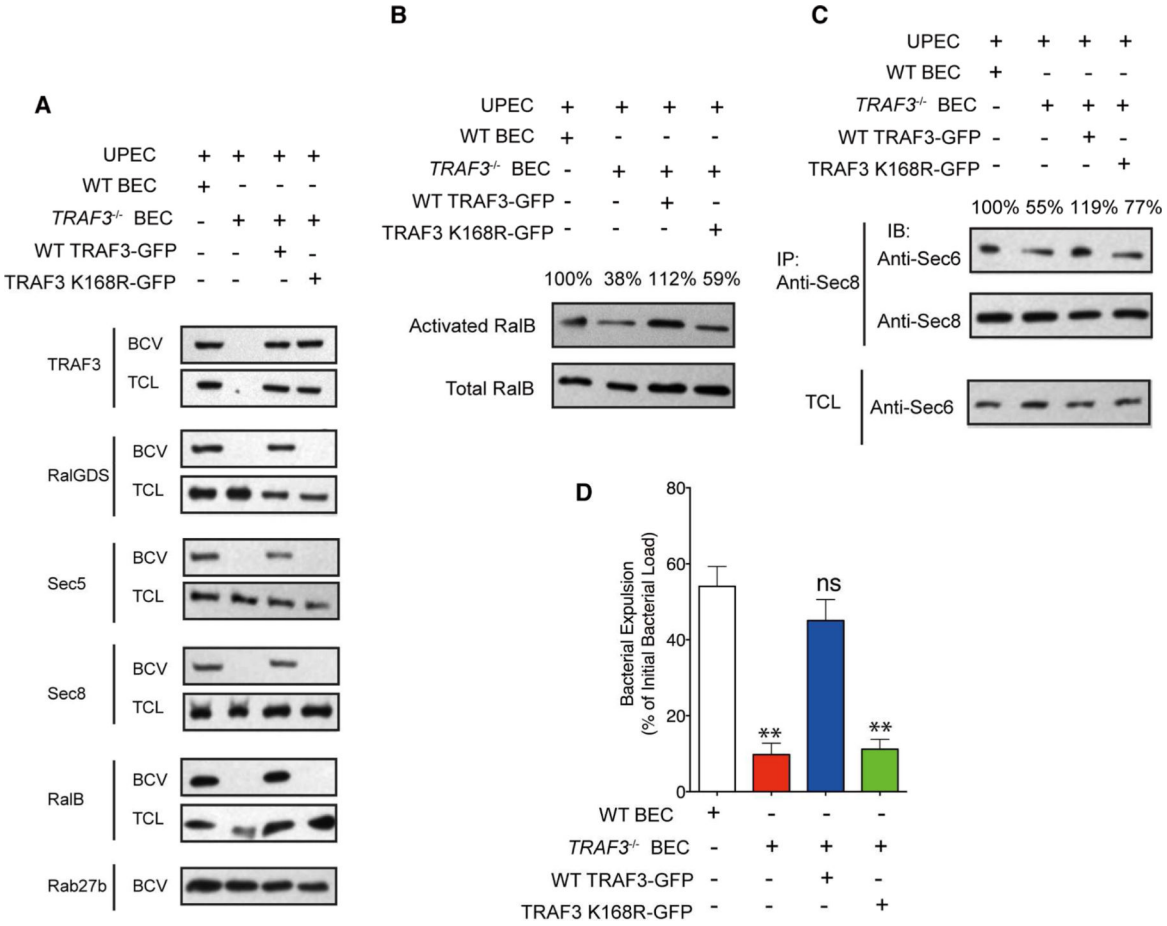

Figure 6. Ubiquitination of TRAF3 is the selection signal for the docking of the exocyst complex on BCVs

(**A**) Protein immunoblot analysis of TRAF3 or various exocyst complex components in bacteria-containing vesicles (BCV) fractions purified from UPEC infected WT BECs, *TRAF3*$^{-/-}$ BECs, or *TRAF3*$^{-/-}$ BECs reconstituted with WT TRAF3 or K168R mutant. Assessment of Rab27b amount was used to show that equal amounts of BCVs were utilized for the various comparisons. Total cell lysates (TCLs) were used as controls to show that similar number of infected cells were used.

(**B**) Relative amounts of activated RalB in infected WT BECs, *TRAF3*$^{-/-}$ BECs, or *TRAF3-*$^{-/-}$ BECs reconstituted with WT TRAF3 or K168R mutant determined by immunoblotting. Activated RalB was observed by pulling down GTP-bound RalB using recombinant Ral BP1 conjugated on agarose beads.

(**C**) Assembly of the exocyst complex assessed by blotting Sec6 in the IP fractions using Sec8 antibody as probe. The amount of Sec6 interacting with Sec8 was determined in infected WT BECs, *TRAF3*$^{-/-}$ BECs, or *TRAF3*$^{-/-}$ BECs reconstituted with WT TRAF3 or K168R mutant.

(**D**) Bacterial expulsion in infected WT BECs, *TRAF3*$^{-/-}$ BECs, or *TRAF3*$^{-/-}$ BECs reconstituted with WT TRAF3 or K168R mutant. Error bars represent SEM. The

experiments were repeated three times, where each experiment employed n=6 wells. See also Figure S4.