Distribution of CD147 During Enteropathogenic *Escherichia coli* and *Salmonella enterica* Serovar Typhimurium Infections

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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) and *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) are highly infectious gastrointestinal human pathogens. These microbes inject bacterial-derived effector proteins directly into the host cell cytosol as part of their disease processes. A common host subcellular target of these pathogens is the actin cytoskeleton, which is commandeered by the bacteria and is used during their attachment onto (EPEC) or invasion into (*S*. Typhimurium) the host cells. We previously demonstrated that the host enzyme cyclophilin A (CypA) is recruited to the actin-rich regions of EPEC pedestals and *S*. Typhimurium membrane ruffles. To further expand the growing catalogue of host proteins usurped by actin-hijacking bacteria, we examined the host plasma membrane protein and cognate receptor of CypA, CD147, during EPEC and *S*. Typhimurium infections. Here, we show that CD147 is enriched at the basolateral regions of pedestals but, unlike CypA, it is absent from their actin-rich core. We show that the CD147 recruitment to these areas requires EPEC pedestal formation and not solely bacteria-host cell contact. Additionally, we demonstrate that the depletion of CD147 by siRNA does not alter the formation of pedestals. Finally, we show that CD147 is also a component of actin-rich membrane ruffles generated during *S*. Typhimurium invasion of host cells. Collectively, our findings establish CD147 as another host component present at dynamic actin-rich structures formed during bacterial infections. Anat Rec, 00:000–000, 2019. © 2019 American Association for Anatomy

Key words: membrane ruffles; basigin; EMMPRIN

The host actin cytoskeleton is a key target of many enteric bacterial pathogens (Goley and Welch, 2006; Lambrechts et al., 2008; Campellone, 2010; Ruetz et al., 2011; Law et al., 2012; Dhanda et al., 2018a). Several medically relevant actin-hijacking microbes include members of the *Enterobacteriaceae* family of bacteria such as enteropathogenic *Escherichia coli* (EPEC) and *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium). These pathogens commandeer the host Arp2/3-mediated actin polymerization machinery during critical steps of their infectious processes such as during their entry into as well as for their movement atop host cells (Kalman et al., 1999; Stender et al., 2000; Criss and Casanova, 2003; Velle and Campellone, 2017). In order to exploit intracellular host

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proteins, the microbes utilize a multisubunit proteinaceous needle-like apparatus (commonly referred to as the type three secretion system [T3SS]) to deliver specialized effector proteins directly into the host cytoplasm (Jarvis et al., 1995). These injected effectors hijack a multitude of cellular processes and organelles within the host cell, ultimately leading to disease of the infected individual.

EPEC is a noninvasive human intestinal pathogen and is a major cause of diarrheal disease worldwide (Kotloff et al., 2013). During these infections, these bacteria cause the loss (effacement) of microvilli in the localized area where the bacteria attach to the host enterocytes (Iizumi et al., 2007). EPEC use over 20 different multifunctional effector proteins to target an array of host cell components (Dean and Kenny, 2009). One of these effectors is the translocated intimin receptor (Tir), which embeds itself within the host plasma membrane once delivered through the T3SS (Kenny et al., 1997). Mechanistically, the extracellular domain of Tir binds to the EPEC surface protein intimin, which anchors the bacteria to the host cell surface (Kenny et al., 1997). The cytoplasmic domains of Tir then trigger the recruitment of the host actin polymerization machinery. This occurs through an assortment of phosphorylation events on Tir, the most prominent being at Y474 by host tyrosine kinases that elicit the recruitment of Nck, which in turn recruits N-WASp and the Arp2/3 complex within the host cells, beneath the regions of EPEC attachment (Rosenshine et al., 1992; Kalman et al., 1999; Kenny, 1999; Gruenheid et al., 2001). The activation of the Arp2/3 complex generates an extensive branched meshwork of filamentous actin (F-actin) that distends the plasma membrane, raising the bacteria off the natural surface of the host cell resulting in actin-rich protrusions (commonly referred to as pedestals). Other crucial host cell actin-associated proteins at pedestals include the Arp2/3 activating protein cortactin, as well as the F-actin stabilizing proteins SM22, calponin 1, and calponin 2 (Cantarelli et al., 2002; Chua et al., 2018a, 2018b). Recent evidence also points toward a role for the formin family actin nucleator protein, mDia, as a potential generator of actin filament seeds for the Arp2/3 complex during pedestal biogenesis (Velle and Campellone, 2018). Pedestal biogenesis is further complicated by the crucial yet noncanonical role of clathrin endocytic machinery proteins such as dynamin 2, CD2AP, epsin 1, Eps15, and Hsc70 at the structures (Unsworth et al., 2007; Veiga et al., 2007; Guttman et al., 2010; Lin et al., 2011; Walker et al., 2018). Once generated, EPEC pedestals become motile and permit the bacteria to "surf" atop the infected cells and spread onto neighboring ones (Sanger et al., 1996; Velle and Campellone, 2017).

In contrast to EPEC, *S*. Typhimurium uses effectors injected through one of its T3SSs to actively invade host cells. These disease-causing proteins usurp the host actin polymerization machinery by: (1) directly influencing F-actin organization (SipA) or (2) activating host cell signaling pathways which govern Arp2/3-based actin polymerization (SopB, SopE, and SopE2) (Galyov et al., 1997; Hardt et al., 1998; Zhou et al., 1999a; Bakshi et al., 2000; Stender et al., 2000). Activation of the Arp2/3 complex at sites of bacteria-host contact results in the formation of large actin-rich dynamic ruffles of the plasma membrane that capture nearby *S*. Typhimurium bacteria to enable their entry into the cell (Francis et al., 1993). While actin constitutes a major cytoskeletal component of pedestals and membrane ruffles, this cytoskeletal element does not work alone as an assortment of publications have demonstrated that other host cytoskeletal systems such as the spectrin cytoskeleton as well as intermediate filament proteins are hijacked during both pedestal generation and bacterial invasion (Batchelor et al., 2004; Ruetz et al., 2011, 2012).

We have previously demonstrated that the eukaryotic enzyme/chaperone, cyclophilin A (CypA), is hijacked by EPEC and *S*. Typhimurium at pedestals and membrane ruffles, respectively (Law et al., 2015; Dhanda et al., 2018b). CypA localizes specifically within the actin-rich regions of these structures. CypA commonly interacts with its cognate receptor CD147 (Yurchenko et al., 2002), which is also known as extracellular matrix metalloproteinase inducer and basigin (Miyauchi et al., 1990; Biswas et al., 1995). This plasma membrane glycoprotein is crucial for several cellular processes that involve the actin cytoskeleton (Curtin et al., 2005; Qian et al., 2008; Zhao et al., 2011; Grass et al., 2012; Liang et al., 2014). CD147 promotes the proper formation and functioning of focal adhesions as well as lamellipodia in cultured cancer cells and regulates actin-rich invadopodia (Curtin et al., 2005; Zhao et al., 2011; Grass et al., 2012; Liang et al., 2014). CD147 also functions extracellularly, where it mediates leukocyte migration (chemotaxis) via its interaction with extracellularly derived CypA (Yurchenko et al., 2002). Here, we examine the distribution and role of CD147 during *in vitro* EPEC and *S*. Typhimurium infections. Because paired host receptor–ligand systems are present at an assortment of bacterially induced actin-rich structures (Finlay et al., 1992; Goosney et al., 2001; Pust et al., 2005), we tested the hypothesis that the cognate receptor of CypA, CD147, would also be present at actin-rich structures generated by EPEC and *S*. Typhimurium.

MATERIALS AND METHODS

Cell Culture

Human cervical epithelial cells (HeLa; American Type Culture Collection (ATCC) [number CCL-2]) were cultured using Dulbecco's modified Eagle's medium (DMEM) containing high glucose (Hyclone, GE Healthcare) supplemented with 10% FBS. Cells were maintained in a cell culture incubator at 37°C and 5% CO_2. Cells were trypsinized and seeded into clear polystyrene 6- or 24-well format plates (Corning) containing glass coverslips.

Bacterial Strains and Growth Conditions

The bacterial strains used in this study included wild type *S*. Typhimurium (strain SL1344), the *S*. Typhimurium SL1344 effector mutants Δ*sipA*, and Δ*sopB*/*sopE*/*sopE2* (Boyle et al., 2006), wild-type EPEC (strain E2348/69), wild-type EPEC (strain JPN15), and the JPN15 Tir mutants (including complement rescues) Δ*tir*, Δ*tir* + *tir*, and Δ*tir* + *tir*Y474F. All bacterial strains were grown at 37°C using LB agar and LB broth (BD Biosciences).

Bacterial Infections

For EPEC infections, 1 μL of 12–16 hr overnight broth culture was used to infect individual wells of cultured cells contained within six well format plates for 6–8 hr at an MOI of 10:1. For *S*. Typhimurium infections, 12–16 hr shaking cultures of the bacteria were subcultured at 37°C (shaking)

in LB broth for 3–4 hr to activate the bacteria. Cultured cells (in well plates) were infected with a 1:100 dilution (MOI of 50:1) the bacteria (diluted in DMEM +10% FBS) for 15 min to observe membrane ruffle formation. Cells were washed 5× with Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS [–/–]) (Gibco, Thermo Fisher Scientific) 3 hr post-infection and 2 mL of prewarmed DMEM +10% FBS was added back to the wells (EPEC only).

Antibodies and Reagents

Antibodies and reagents used in this study included: Alexa Fluor 594- and 488-conjugated phalloidin (Invitrogen); Alexa Fluor 594- and 488-conjugated goat anti-rabbit antibodies (2 μg/mL, Invitrogen); mouse anti-CD147 (10 μg/mL for immunofluorescence and 2 μg/mL for Western blotting, Abcam, ab666); mouse anti-α tubulin (1:1,000 for Western blot, Developmental Studies Hybridoma Bank, 12G10); normal mouse IgG (10 μg/mL for immunofluorescence, R&D Systems) and HRP-conjugated goat anti-mouse antibodies (1 μg/mL, Invitrogen).

Immunolocalization

Cells (on coverslips) were fixed at room temperature for 15 min using 37°C 3% paraformaldehyde (made in 150 mM NaCl, 4 mM Na/K PO4, 5.0 mM KCl, pH 7.3) and then rinsed 3× with PBS [–/–]. Cells were permeabilized by submerging coverslips in −20°C acetone for 10 min. Following this, the coverslips were air-dried at room temperature for 30 min. Samples were blocked using 5% normal goat serum (prepared in PBS [–/–]) for 20 min after which samples were incubated overnight at 4°C with primary antibodies prepared in TPBS/BSA (PBS [–/–], 0.5% Tween-20, 0.1% bovine serum albumin [BSA]). The next day, coverslips were rinsed 3× for 10 min with TPBS/BSA, then treated with secondary antibodies (Alexa Fluor 594- or 488-conjugated goat anti-rabbit antibodies) at room temperature in the dark for 2 hr. To visualize F-actin, samples were incubated using Alexa Fluor 594- or 488-conjugated phalloidin (prepared in PBS [–/–]) for 15–20 min. Samples were finally rinsed 3× with PBS [–/–] and then mounted onto glass microscope slides using Prolong Diamond antifade mounting medium containing DAPI (Invitrogen).

Lysate Preparation and Western Blotting

Lysates were prepared and proteins were resolved via gel electrophoresis as described previously (Dhanda et al., 2018b).

CD147 RNAi

A premixed smart pool (ON-Targetplus siRNA) of 4 CD147-targeting and four nontargeting control siRNA were purchased from Dharmacon (Horizon Discovery). SiRNA transfections were carried out using the siRNA transfection reagent INTERFERin (Polyplus transfection). Briefly, a ~0.1 mL master mix of serum-free DMEM containing 50 nM siRNA duplexes and 3 μL INTERFERin was added (dropwise) over cells contained within clear polystyrene 24-well culture plates. Transfected cells were incubated for 72–96 hr (posttransfection) in order to obtain cells with undetectable levels of CD147 protein.

Microscopy

Images were acquired on a Leica DMI4000B (Leica Microsystems) inverted fluorescent microscope fitted with a Hamamatsu Orca R2 CCD camera (Hamamatsu Photonics). All microscopy components were controlled by MetaMorph Imaging System software (Universal Imaging). Images were evaluated using MetaMorph Imaging System software or ImageJ.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.01. All results involving immunofluorescence microscopy were obtained from experiments performed at least three times (n = 3) and all presented images are representative of the experiments performed. For data in Figure 1E, an unpaired parametric two-tailed *t* test (with Welch's correction) was performed to determine if means were significantly different. In Figure 1E, 230 and 212 total bacteria were enumerated from 17 to 19 infected HeLa cells treated with nontargeting [control] or CD147-targeting siRNA sequences respectively, from two independent experiments. Results were normalized to controls and plotted as a bar graph of the percent efficiency of pedestal formation (± standard deviation [SD]).

RESULTS

CD147 is Recruited to the Basolateral Surface of EPEC Pedestals but is Dispensable for Their Formation

When EPEC interact with their host cells, they cause the distention of the host plasma membrane by recruiting copious actin filaments beneath the regions of bacterial attachment, leaving the microbes atop the cells on "pedestals" (Moon et al., 1983). To investigate the potential involvement of CD147 at pedestals, we immunolocalized CD147 in HeLa cells infected with wild-type EPEC (strain E2348/69). Immunostaining revealed an enrichment of CD147 (compared to regions of the host cell without attached bacteria) at sites of EPEC bacterial attachment (Fig. 1A). Upon closer examination, CD147 did not localize to the actin-rich cores of the pedestals but rather surrounded them in an irregular puncta-like pattern (Fig. 1A, inset). To examine CD147 in more precise detail at pedestals, we utilized a strain of EPEC (JPN15) that generates larger and easily resolvable pedestals as it lacks the bundle-forming pili genetic information in EPEC. Using this strain of EPEC, we again saw that CD147 clearly localized at the periphery of the pedestals (Fig. 1A), extending along the lateral surfaces of the structures as well as the basal surface beneath the actin core forming a cup-like shape (Fig. 1B). Interestingly, we did not observe an enrichment of CD147 at the apical surface of pedestals (Fig. 1B).

EPEC utilizes the T3SS effector Tir, in conjunction with the bacterial surface ligand intimin to firmly attach to the surface of host epithelial cells (Kenny et al., 1997). EPEC strains that do not express Tir, can attach to the surface of host cells and efface microvilli, but, do not generate pedestals and are associated with diminished infectivity of infected animals (Kenny et al., 1997; Deng et al., 2003). Because CD147 localizes around pedestals, we thought that CD147 recruitment at bacterial attachment sites might potentially represent an early signaling

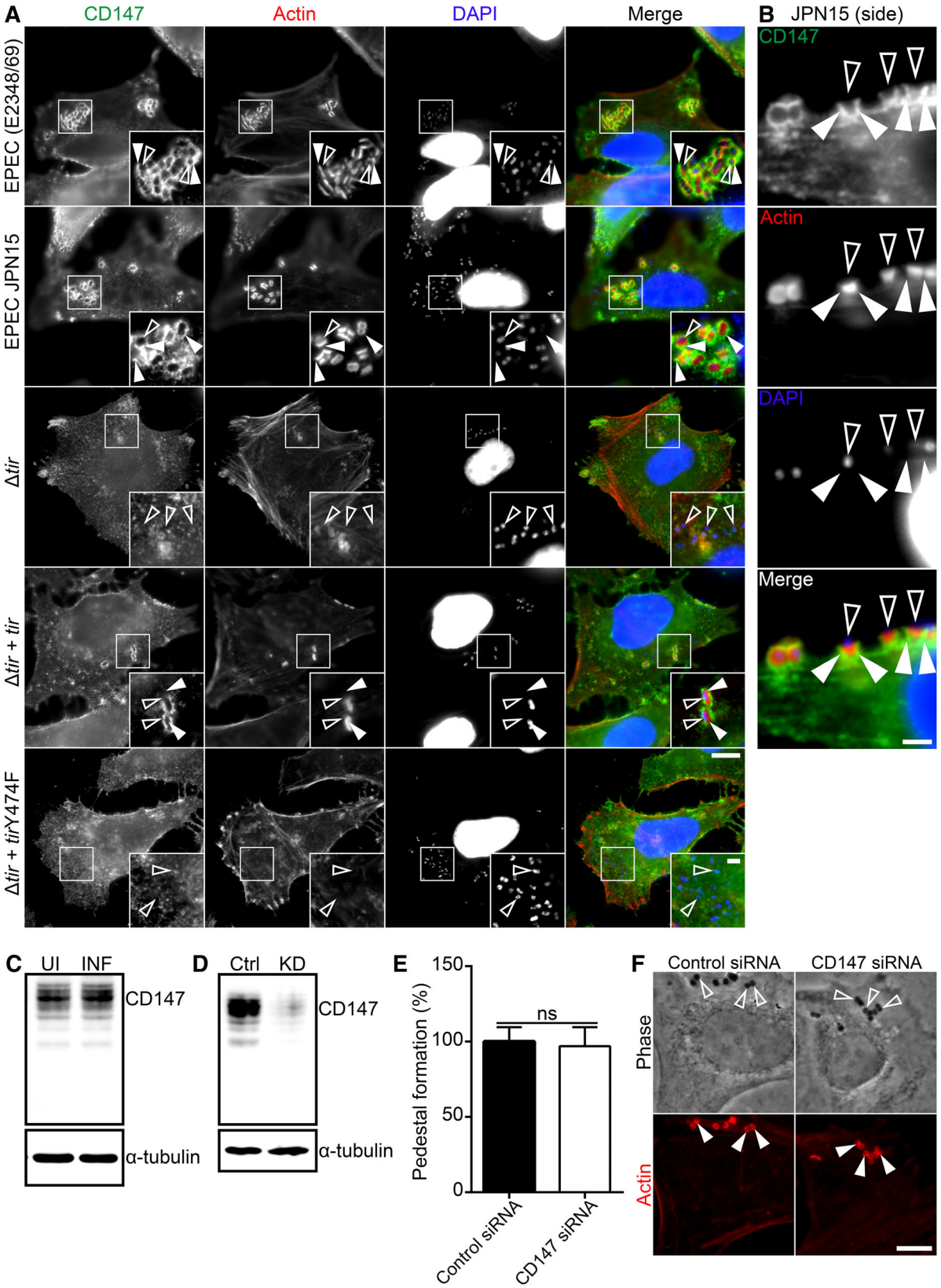

Fig. 1. Legend on next page.

event that precedes the actin polymerization step of pedestal generation. However, we saw no evidence of CD147 enrichment at bacterial attachments sites when we infected HeLa cells with EPEC JPN15 Δ*tir* (Fig. 1A). Using an complemented EPEC JPN15 strain (Δ*tir* + *tir*), CD147 localization was restored and surrounded pedestals in an indistinguishable manner as when we infected with the wild-type EPEC JPN15 (Fig. 1A). Mechanistically, the Arp2/3-mediated generation of pedestals primarily hinges on the phosphorylation of tyrosine 474 of Tir by host tyrosine kinases (Rosenshine et al., 1992; Kalman et al., 1999; Kenny, 1999; Gruenheid et al., 2001). To test if the phosphorylation of this residue was essential for CD147 recruitment to pedestals, we infected HeLa cells with an EPEC JPN15 Δ*tir* mutant complemented with a variant of Tir that is non-phosphorylatable at residue 474 (Δ*tir* + *tir*Y474F). We saw that CD147 was absent at those bacterial attachment sites (Fig. 1A). Despite CD147 localization to the perimeter of pedestals, our findings suggest that its recruitment to these regions is still dependent upon actin polymerization beneath the bacteria and not their firm attachment to the host cell surface. CD147 is a well-known asparagine-linked glycosylated protein (Bai et al., 2014). Gao et al. (2013) recently found that the EPEC effector NleB functions as a glycosyltransferase. This prompted us to examine whether EPEC infection of HeLa cells would alter the levels of any of the various glycosylated forms of CD147 found in those cells. When compared to uninfected samples, we observed no obvious alteration to the protein levels of any of the various glycosylated forms of CD147 during the wild-type EPEC JPN15 infections (Fig. 1C).

To examine the functional role of CD147 at EPEC pedestals, we depleted CD147 in HeLa cells using short interfering RNA (siRNA; Fig. 1D) and utilized immunofluorescence microscopy counts to quantify the pedestal formation efficiency (percentage of surface-associated bacteria with pedestals) of JPN15 infected HeLa cells treated with nontargeting (control) or CD147-targeting siRNA sequences. We found that EPEC pedestal formation was unperturbed in cells with depleted of CD147 when compared to control cells treated with nontargeting (control) siRNA (Fig. 1E,F).

CD147 is Recruited to *S.* Typhimurium Membrane Ruffles

Salmonella serovar Typhimurium, like EPEC, co-opt the host actin cytoskeleton as a crucial stage of their infectious process. Although EPEC generally remains extracellular atop actin-rich pedestals, *S.* Typhimurium utilize T3SS effectors to rapidly enter the host epithelia. Effectors delivered through this T3SS cause membrane ruffling of the host cell surface. As these membrane ruffles are also actin-rich membrane associated structures, we hypothesized that they may also be enriched with CD147. When we examined HeLa cells infected with wild type *S.* Typhimurium (strain SL1344), endogenous CD147 was visibly enriched at the ruffles when compared to its normal cellular distribution (Fig. 2A). Primary antibody controls lacked any specific localization to the structures (Fig. 2B). When we examined the co-distribution of CD147 and F-actin at the structures, we noticed intense puncta of CD147 at the F-actin rich regions of the ruffles; despite this, the two proteins did not colocalize completely.

A number of injected T3SS effectors, that include SipA, SopB, SopE, and SopE2, control the host cytoskeletal remodeling, that is, required for *S.* Typhimurium epithelial invasion (Galyov et al., 1997; Hardt et al., 1998; Zhou et al., 1999a; Bakshi et al., 2000; Stender et al., 2000). Because these effectors directly regulate host cytoskeletal proteins and enzymes such as actin, Cdc42, Rac1, MYO6, and T-plastin (Fu and Galán, 1999; Zhou et al., 1999b; Brooks et al., 2017), we examined whether CD147 localization was also under a similar mode of control. To do this, we infected cultured cells with various *S.* Typhimurium effector mutants (Δ*sipA* and Δ*sopB*/*E*/*E2*) and assessed endogenous CD147 at the bacterial attachment sites or membrane ruffles.

Salmonella serovar Typhimurium SipA augments bacterial invasion efficiency through its ability to enhance F-actin bundling in one of two ways: (1) by directly binding actin or (2) by modulating the activity of host F-actin bundling proteins such as T-plastin (Zhou et al., 1999a, 1999b; Lilic et al., 2003). Despite this, SipA expression is dispensable for membrane ruffle formation and bacterial invasion (Zhou et al., 1999a). Immunostaining of HeLa cells infected with the *S.* Typhimurium Δ*sipA* mutant revealed there was no obvious alteration in the localization of CD147 at membrane ruffles as compared to the wild-type infections (Fig. 2A).

The *S.* Typhimurium effectors SopB, SopE, and SopE2 usurp host Rho GTPases in order to activate the Arp2/3-mediated actin polymerization machinery needed for membrane ruffle generation (Galyov et al., 1997; Hardt et al., 1998; Bakshi et al., 2000; Stender et al., 2000). *Salmonella* serovar Typhimurium mutants that are deficient in all three effectors fail to generate membrane ruffles and thus do not invade the host epithelia (Jolly et al., 2014). To assess whether CD147 recruitment was dependent on membrane ruffle formation, we infected cultured

Fig. 1. CD147 localizes to the basolateral edges of EPEC pedestals but its expression is not essential for their formation. (**A**) Immunolocalization of CD147 at bacterial attachment sites during 7 hr infections of HeLa cells with wild-type EPEC (strains E2348/59 and JPN15) and JPN15 effector mutants (including complement rescues): Δ*tir*, Δ*tir* + *tir* and Δ*tir* + *tir*Y474F. Samples were stained using a mouse monoclonal CD147 targeting antibody (green), Alexa594-phalloidin (red) to visualize F-actin and DAPI (blue) to visualize DNA. Insets depict pedestals or bacteria-host adhesion sites (boxes). Solid arrowheads indicate CD147 surrounding pedestals while open arrowheads point to adherent bacteria. Scale bars = 10 and 2 μm (insets). (**B**) CD147 localizes to the basolateral edges of pedestals. Hela cells were infected with EPEC JPN15 and pedestals extending laterally from the cell periphery were observed. Solid arrowheads indicate CD147 surrounding pedestals while open arrowheads point to adherent bacteria. Scale bars is 2 μm. (**C**) CD147 protein levels are unaltered during EPEC JPN15 infections. Whole HeLa cell lysate from uninfected (UI) and 8 hr EPEC infections (INF) were probed for endogenous CD147 using a mouse monoclonal anti-CD147 antibody. α-tubulin was used as the loading control. (**D**) CD147 siRNA-mediated knockdown in HeLa cells. HeLa cells were treated with nontargeting [control] (Ctrl) or CD147-targeting (KD) siRNA sequences and whole-cell lysates were probed for endogenous CD147 using a mouse monoclonal CD147-tageting antibody. α-tubulin was used as the loading control. (**E** and **F**) EPEC pedestal formation does not require CD147. (E) Bar graph of the percent efficiency of pedestal formation of infected HeLa cells treated with nontargeting [control] (Control siRNA) or CD147-targeting (CD147 siRNA) siRNA sequences. There was a ~3% decrease (not significantly different) in pedestal formation in the absence of CD147. (F) Immunofluorescent micrograph representative of the data depicted in (E). Solid arrowheads indicate pedestals while open arrowheads point to adherent bacteria. Scale bar is 10 μm.

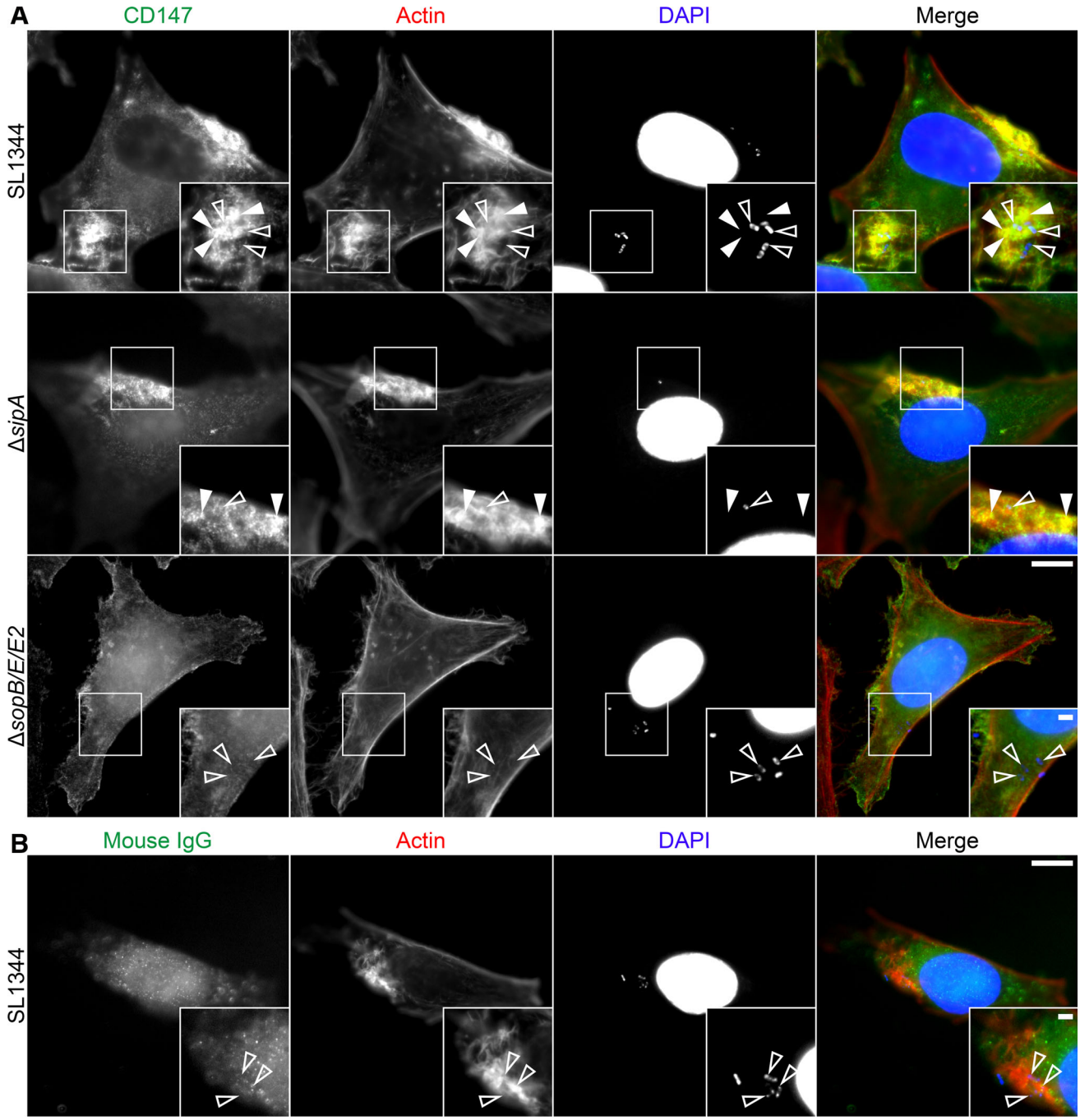

Fig. 2. Redistribution of CD147 during *S*. Typhimurium infections depends on membrane ruffle formation. (**A**) CD147 at membrane ruffles does not completely colocalize with F-actin. HeLa cells infected with wild type *S*. Typhimurium or *S*. Typhimurium effector mutants (Δ*sipA* and Δ*sopB/sopE/sopE2*) were fixed then stained with a mouse monoclonal CD147-targeting antibody (green), Alexa594-phalloidin (red) to visualize F-actin and DAPI (blue) to visualize DNA. Solid arrowheads within insets (enlargement of boxed regions) indicate CD147 at some membrane ruffles (top and middle). *S*. Typhimurium bacteria are indicated by open arrowheads. CD147 recruitment also depends on membrane ruffle formation (bottom). Scale bars, 10 and 2 μm (insets). (**B**) Primary antibody controls do not show enrichment at *S*. Typhimurium membrane ruffles. HeLa cells infected with wild-type *S*. Typhimurium were fixed then stained with normal mouse IgG antibody (green), Alexa594-phalloidin (red) to visualize F-actin and DAPI (blue) to visualize DNA. *S*. Typhimurium bacteria are indicated by open arrowheads. Scale bars = 10 and 2 μm (insets).

HeLa cells with the *S*. Typhimurium Δ*sopB/E/E2* mutant. We found no obvious CD147 enrichment at sites where the bacteria had maintained their adherence to the host cell surface, suggesting that membrane ruffle generation is a key signal for the recruitment of host CD147 to the structures (Fig. 1A).

DISCUSSION

The current repertoire of host proteins that are recruited to EPEC pedestals exceeds 50 and is continually growing (Law and Guttman, 2014). These proteins and their localizations are generally described as occupying one of four major regions of the pedestal: (1) the apical region directly beneath the structures (clathrin, Veiga et al., 2007; N-WASP, Kalman et al., 1999; Hsc70, Walker et al., 2018), (2) the stalk-like actin-rich region (transgelin, Chua et al., 2018a), (3) the basal region which extends beyond the actin-rich parts of the stalk (spectrin, Ruetz et al., 2011; Ruetz et al., 2012), and (4) the edges of the pedestal that surround the actin core (adducin and protein 4.1; Ruetz et al., 2011, 2012).

In this manuscript, our most significant finding was our identification of CD147 at the basolateral edges of pedestals. Although we showed that CD147 expression is unchanged during the infections and that CD147 is not required for pedestal formation, its localization at the periphery of the structures might lead to clues to its associations with other proteins within the structures. The cup-like localization pattern of CD147 at pedestals resembles very closely that of the spectrin-associated protein adducin (Ruetz et al., 2011, 2012). Thus, could CD147 and the spectrin cytoskeleton represent a new functional system at pedestals? Despite limited literature on the subject, there is evidence that CD147 could in fact control spectrin cytoskeletal organization as Besse et al. (2007) reveal a role for *Drosophila melanogaster* CD147 in organizing spectrin and actin at neuromuscular junctions.

Our RNAi studies demonstrate that CD147 is not required for pedestal generation and suggest that an unknown functional redundancy may be at play during these infections. If CD147 is not crucial for the formation of pedestals, what else could it be doing at these structures? Hints to its role at pedestals may lie in its normal cellular role during signaling events. CD147 can interact with CD147 molecules expressed on neighboring cells (trans interactions) as well as those on the same cell surface (cis interactions; Yurchenko et al., 2010). One potential explanation for CD147 clustering at the edges and base of pedestals may be to "communicate" or signal to nearby bacteria at their CD147-rich pedestal edges. As multiple EPEC bacteria generally collect into clusters (microcolonies) on the surface of the host cell, the lateral edges of pedestals could represent an easily accessible region for neighboring bacteria to signal to one another. Recent findings by Velle and Campellone (2017) revealed the presence of EPEC "macrocolonies" or large foci of bacteria, which span several host epithelial cells. Could CD147 signaling promote this expansion of EPEC bacteria across multiple host cells? One potential mechanism, with its roots derived from the normal cellular role of CD147 in promoting the proper formation and activity of actin-rich lamellipodia, focal adhesion, and invadopodia is that CD147 signaling promotes the actin-based motility/formation of EPEC macrocolonies. Whether CD147 does in fact possess functional activity at pedestals will require further investigation.

We were unable to functionally assess the role of CD147 during *S*. Typhimurium infections as a CD147 null cell line was not available. Consequently, further studies into whether CD147 influences bacterial infection dynamics and the organization of actin or spectrin-based components such as adducin requires further scrutiny.

In conclusion, we have shown that CD147 is a new component of the actin-rich structures generated by EPEC and *S*. Typhimurium during their infectious processes. Although not essential for EPEC pedestal formation, our morphological findings present the possibility of CD147 interacting with spectrin-associated components. The investigation into the role of CD147 at these structures and those generated by other actin-hijacking microbes (Till et al., 2008; Bernard et al., 2014) is ongoing as clues to its precise function at these sites may reveal themselves through comparative work.

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AUTHOR CONTRIBUTIONS

A.S.D. and J.A.G. conceived the study. A.S.D. and C.Y. performed the experiments. All authors analyzed the data and wrote the manuscript.

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