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The Secreted Effector Protein EspZ Is Essential for Virulence of Rabbit Enteropathogenic *Escherichia coli*

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Attaching and effacing (A/E) pathogens adhere intimately to intestinal enterocytes and efface brush border microvilli. A key virulence strategy of A/E pathogens is the type III secretion system (T3SS)-mediated delivery of effector proteins into host cells. The secreted protein EspZ is postulated to promote enterocyte survival by regulating the T3SS and/or by modulating epithelial signaling pathways. To explore the role of EspZ in A/E pathogen virulence, we generated an isogenic *espZ* deletion strain (Δ*espZ*) and corresponding *cis*-complemented derivatives of rabbit enteropathogenic *Escherichia coli* and compared their abilities to regulate the T3SS and influence host cell survival *in vitro*. For virulence studies, rabbits infected with these strains were monitored for bacterial colonization, clinical signs, and intestinal tissue alterations. Consistent with data from previous reports, *espZ*-transfected epithelial cells were refractory to infection-dependent effector translocation. Also, the Δ*espZ* strain induced greater host cell death than did the parent and complemented strains. In rabbit infections, fecal Δ*espZ* strain levels were 10-fold lower than those of the parent strain at 1 day postinfection, while the complemented strain was recovered at intermediate levels. In contrast to the parent and complemented mutants, Δ*espZ* mutant fecal carriage progressively decreased on subsequent days. Δ*espZ* mutant-infected animals gained weight steadily over the infection period, failed to show characteristic disease symptoms, and displayed minimal infection-induced histological alterations. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining of intestinal sections revealed increased epithelial cell apoptosis on day 1 after infection with the Δ*espZ* strain compared to animals infected with the parent or complemented strains. Thus, EspZ-dependent host cell cytoprotection likely prevents epithelial cell death and sloughing and thereby promotes bacterial colonization.

The attaching and effacing (A/E) group of bacteria includes Gram-negative enteric pathogens that colonize the intestine and cause disease in diverse animals (1, 2). A/E pathogens adhere intimately to intestinal epithelial cells and efface the brush border microvilli (3). Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are human-specific A/E pathogens that cause diarrhea and contribute to significant morbidity. EPEC is an important agent of diarrheal disease in children, particularly in developing countries (4). EHEC, a foodborne pathogen that causes watery or bloody diarrhea, is often associated with outbreaks (5). The mouse pathogen *Citrobacter rodentium* and rabbit EPEC (REPEC) have been used to model EPEC- and EHEC-induced human disease (6–8). *C. rodentium* is a nonmotile enteric pathogen that causes colonic hyperplasia, but typically not diarrhea, in mice. On the other hand, REPEC causes diarrhea in rabbits and elicits disease that more closely resembles human A/E infections.

All A/E pathogens harbor a type III secretion system (T3SS) that is essential for inducing effacing lesions and causing disease. The T3SS conveys a diverse array of bacterial effector molecules directly into intestinal epithelial cells, where these proteins engage and subvert host cell structural proteins and signaling pathways (9). Studies from several laboratories have uncovered specific host cell-interacting partners of various secreted proteins, and the consequent intestinal cell signaling and structural alterations have been characterized in some detail. *In vivo* studies have shown the essentiality of the T3SS for virulence, although the requirement for specific secreted effectors is variable (10). While some effector molecules, such as Tir, are critical for virulence (11, 12), deletion mutants lacking other effectors exhibit a colonization defect only in competition studies with the isogenic parent wild-type (WT) strain (13). Colonization by strains mutated for several other effectors, however, is not significantly different from that by the parent strain either alone or in competition studies (14, 15).

EspZ is a 99- to 100-amino-acid secreted effector molecule that was first implicated in virulence in the *C. rodentium*-mouse model of infection (16, 17). *C. rodentium* Δ*espZ* strains colonized the intestines of NIH Swiss mice but failed to cause the characteristic colonic hyperplasia (17). Furthermore, while WT *C. rodentium* strains caused 100% mortality in the more susceptible C3H/HeJ mice, all animals infected with isogenic Δ*espZ* strains survived infection (17). Since those observations constituted only a minor

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aspect of a broader study of *C. rodentium* virulence factors, complementation data were not provided, and the mechanisms of EspZ action were not explored in that study.

The precise contribution of EspZ to A/E virulence is currently unknown. We and others have demonstrated that EspZ is cytoprotective and that infection with an EPEC Δ*espZ* mutant, relative to WT EPEC, results in increased epithelial cell death *in vitro* (18–21). Two mechanisms, perhaps not mutually exclusive, have been proposed to account for the cytoprotective roles of EspZ. The first proposed mechanism is that EspZ, possibly via interactions with host proteins, engages epithelial cell cytoprotective signaling pathways or inhibits processes that trigger the death of infected cells (18–20, 22). The alternate mechanism, proposed by Berger et al., suggests that EspZ may directly modulate the translocation of effector proteins into host cells and that the increased death of Δ*espZ* mutant-infected cells results from the unregulated delivery of secreted proteins (including cytotoxic effectors) into host cells (21). Furthermore, strain specificity was noted for the EspZ-dependent regulation of type III secretion: EHEC EspZ was shown to block both EPEC- and EHEC-mediated translocation, while EPEC EspZ was able to inhibit translocation from EPEC but not EHEC.

Since A/E pathogen infection in rabbits most closely approximates human disease, the goal of this study was to use this model to determine the role of EspZ in virulence. First, we verified that the *in vitro* phenotypes attributed to EPEC EspZ held true for the REPEC strain (serotype O103:H2; also known as E22) and that REPEC *espZ* could complement the EPEC Δ*espZ* mutant and vice versa. To examine the role of EspZ in virulence, rabbits were mock infected or were infected with the following REPEC strains: the WT, the Δ*espZ* strain, and single-copy *cis*-complemented Δ*espZ* strains (complemented with EPEC or REPEC *espZ*). Bacterial burden, disease parameters, tissue histological alterations, apoptotic cell death, and host responses of intestinal tissues were monitored for the duration of the study. Our observations reveal that REPEC *espZ* is essential for virulence in the rabbit model and that the EPEC and REPEC *espZ* genes exhibit functional similarity under both *in vitro* and *in vivo* conditions. We propose that EspZ limits host intestinal epithelial cell death, thereby preserving the substratum and promoting bacterial colonization.

(Portions of this work were presented at the 2012 Digestive Diseases Week in San Diego, CA, and at the 2013 FASEB Summer Conference on Gastrointestinal Mucosa in Steamboat Springs, CO.)

MATERIALS AND METHODS

Bacterial strains and plasmids. The strain designated WT REPEC in this study refers to a Nal^r derivative of the sequenced O103:H2 REPEC strain, also known as strain E22 (11). The isogenic nonpolar REPEC Δ*espZ* strain was generated by using a SacB-based counterselection strategy (23). Fragments flanking the upstream and downstream regions of REPEC *espZ* were amplified by using primer pairs NoEspZ2/NiEspZR and CoEspZR2/CiEspZ (Table 1), respectively, fused via crossover PCR, and cloned into the SalI site of pTOF24 (23). The resulting plasmid was digested with SmaI and ligated with a similarly digested nonpolar kanamycin resistance cassette amplified from MK41 (16), using primer pair KanF/KanR (Table 1). The final construct was transformed into REPEC E22, and the disruption and loss of *espZ* in kanamycin-resistant, sucrose-resistant, chloramphenicol-sensitive colonies were verified by PCR.

For *cis*-complementation of the REPEC Δ*espZ* strain, REPEC *espZ*, along with its native promoter, was amplified by using primer pair RespZF/RespZR and cloned into pGEM-T Easy (Promega, Madison, WI). The *espZ*-containing fragment was excised by using NotI and cloned into similarly digested pGRG36 (24) to generate pJSW201. A similar plasmid harboring EPEC *espZ*, pJSW101, was generated previously (18). To generate REPEC and EPEC *espZ* complements (VK581 and VK582, respectively), the REPEC Δ*espZ* strain was transformed with pJSW201 or pJSW101 and screened for the Tn*7*-mediated transposition of *espZ* into the neutral site downstream of *glmS* (24).

To generate a TEM-1 β-lactamase reporter plasmid for the translocation assays, the region encoding the type III secretion signal (first 20 amino acids) of EspZ, along with the native locus of enterocyte effacement 2 (LEE2) promoter, was amplified from EPEC (strain E2348/69) by using primer pair SR15/SR16, digested with KpnI and EcoRI, and cloned into similarly digested pMB196, in frame with β-lactamase (25), to generate plasmid pSR1. Plasmid pMB201 was used as the vector control (25).

For transient expression of *espZ* in eukaryotic cells, the corresponding REPEC and EPEC isoforms were amplified by using primer pairs SR3/SR4 and SR1/SR2, respectively, and cloned into pGEM-T Easy (Promega, Madison, WI). The *espZ* fragments were then excised with XbaI and BspEI and cloned into the corresponding sites in pCIP, in frame with the hemagglutinin (HA) tag and upstream of the internal ribosome entry site (IRES) sequence, to generate plasmids pSR2 and pSR3, respectively. pCIP, derived from the lentiviral expression plasmid pCIG (26) by the insertion of a puromycin resistance cassette downstream of the IRES sequence, was a kind gift from Felicia Goodrum, University of Arizona.

Cell lines and *in vitro* infection studies. Caco-2 BBE ($C2_{BBE}$) cells (as well as HeLa and T84 cells [not shown]) were used to monitor infection-induced host cell death, while the translocation assays were performed with HeLa cells. Death of infected host cells was assessed via propidium iodide (PI) uptake assays as described previously (18).

Translocation assays. Translocation assays were performed as previously described (21, 27), with some modifications. For the experiments described in the legend of Fig. 2A, HeLa cells were first transiently transfected with pSR2 (harboring REPEC *espZ*), pSR3 (harboring EPEC *espZ*), or a lentiviral expression plasmid backbone (pSR4) (control), and translocation of TEM-1 β-lactamase from a subsequent infection with the REPEC strain harboring pSR1 (REPEC/pSR1) was monitored by using a fluorescence-based assay. For the experiments described in the legend of Fig. 2B, HeLa cells were infected with various REPEC strains ("first-wave infection"), and translocation of TEM-1 β-lactamase from a subsequent infection with REPEC/pSR1 was monitored by using the same fluorescence-based assay as the one described above. For transfection studies, HeLa cells seeded into 6-well plates were transfected with pSR2 or pSR3 (REPEC or EPEC *espZ*) or with pSR4 (vector control) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 h, the transfected cells were seeded into 96-well, black-walled, clear-bottom microplates (PerkinElmer, Waltham, MA) and grown to confluence at 37°C in the presence of 5% CO_2. The cells were treated overnight with serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with mannose and HEPES and then infected with REPEC/pSR1 to monitor translocation. Bacterial cells grown overnight in LB broth were diluted 1:100 in Casamino Acids-DMEM (cDMEM) supplemented with 2.5 mM probenecid until they reached an optical density at 600 nm (OD_{600}) of 0.2 to 0.35. cDMEM is a tissue culture medium with low autofluorescence (28), and probenecid, a nonspecific anion inhibitor, facilitates CCF2-AM [coumarin-cephalosporin-fluorescein (2)-acetoxymethyl] loading by blocking its active efflux from cells (29). HeLa cells preloaded with CCF2-AM (GeneBLAzer In Vivo detection kit; Life Technologies, Carlsbad, CA) were infected with ~10^6 bacteria (multiplicity of infection [MOI] of 500 to 700). The cultures were diluted as needed to normalize OD levels. Infection was monitored with a plate reader (Synergy HT; Biotek) set at 37°C for 120 min. Cells were excited at 400/10 nm, and emission was recorded at 460/40 nm and 520/10 nm, respectively, for 2 h at 5-min intervals.

For the two-wave infections, HeLa cells were seeded into 96-well,

TABLE 1 Bacterial strains, plasmids, and primers

Strain, plasmid, or primer	Genotype and/or description or sequence (5′–3′)	Reference
Strains		
ECB480	E22 (WT REPEC; serotype O103:H2) (Nal^r)	11
VK550	E22 Δ*espZ* (Nal^r Kan^r)	This work
VK581	E22 Δ*espZ*Ω*espZ*^{REPEC} (Nal^r Kan^r); REPEC Δ*espZ cis*-complemented with REPEC *espZ*	This work
VK582	E22Δ*espZ*Ω*espZ*^{EPEC} (Nal^r Kan^r); REPEC Δ*espZ cis*-complemented with EPEC *espZ*	This work
Plasmids		
pMB196	Vector backbone for cloning TEM-1 fusions	25
pMB201	Vector control for translocation assays	25
pSR1	Expresses the first 20 amino acids of EspZ fused to TEM-1 β-lactamase; used for translocation assays	This work
pSR2	Plasmid for transient expression of REPEC *espZ* in eukaryotic cells; REPEC *espZ* allele cloned into pSR4	This work
pSR3	Plasmid for transient expression of EPEC *espZ* in eukaryotic cells; EPEC *espZ* allele cloned into pSR4	This work
pSR4	Lentiviral expression plasmid based on the pCIP-TTL-HA backbone[a]	This work
pGRG36	Plasmid used to generate pJSW201 and pJSW101	24
pJSW101	Harbors EPEC *espZ*; used to generate VK582	18
pJSW201	Harbors REPEC *espZ*; used to generate VK581	This work
Primers		
Ni-EspZR	AGCGCAGTAACCCGGGGCTGCATCCATTAACTTTTCTCCA	
No-EspZ2	TTTTGTCGACAGCGGGTCAATAACGCCTGC	
Ci-EspZ	TGGATGCAGCCCCGGGTTGGCGGCGGATTAGCGATGAAATATGC	
Co-EspZR2	GTTCGTCGACGCCGTTAGCCAGTCACTATC	
KanF	CCCGGCATGCTAACTAGGAGGAATAAATG	
KanR	ATTACCCGTCCTGTCCTGAG	
RespZF	TGCAGGCTCTGAAGTAAGTAT	
RespZR	TTAGGCATATTTCATCGCTA	
SR1	TCTAGAATGGAAGCAGCAAATTTAAGCCCTT	
SR2	TCCGGAGGCATATTTCATCGCTAATCCGC	
SR3	TCTAGAATGGATGCAGCAAATTTAAGCCCTT	
SR4	TCCGGAGGCATATTTCATCGCTAATCCGC	
SR15	TCATGAATTCCTGCAGGCTCTGAAGTAAG	
SR16	TTCCGGTACCATTGATTGTGGCTGCCAGTG	

[a] Kind gift from Felicia Goodrum, University of Arizona.

black-walled, clear-bottom microplates (PerkinElmer) and allowed to grow to confluence. The cells were treated overnight with serum-free DMEM supplemented with mannose and HEPES. Bacterial cells grown overnight in LB broth were diluted 1:100 in cDMEM supplemented with probenecid until they reached an OD_{600} of 0.2 to 0.35. HeLa cells preloaded with CCF2-AM were infected with ~10^6 first-wave bacteria and incubated at 37°C for 30 min. The cells were then washed with cDMEM to remove unattached bacteria and infected with ~10^6 bacteria (MOI of between 500 and 700) of the second-wave REPEC/pSR1 infection. Translocation of TEM-1 β-lactamase was monitored as described above.

Rabbit infection studies. All animal experiments and procedures were conducted with IACUC approval from the New Mexico VA Healthcare System (NMVAHCS) IACUC Committee. Groups of 6- to 8-week-old, specific-pathogen-free, New Zealand White (NZW) male rabbits (Charles River, Wilmington, MA), weighing 1.4 to 1.8 kg, were infected with WT REPEC or the Δ*espZ* or *cis*-complemented Δ*espZ* strains (with either REPEC or EPEC *espZ*) and monitored for a total period of up to 7 days. Briefly, the animals were administered 2 ml 10% sterile sodium bicarbonate via an orogastric tube, followed by 2 ml of a bacterial solution (bacteria suspended in phosphate-buffered saline [PBS]). Bacteria remaining in the orogastric tube were flushed with an additional 1 ml of sterile PBS. Animal weight, clinical signs, and mortality/morbidity were monitored on a daily basis, and rabbits were scored for disease as described in Table S1 in the supplemental material; fecal swabs and/or pellets were monitored for bacterial loads on a daily basis and are reported as CFU/g feces. At the end of the study, infected animals were euthanized by the intramuscular administration of ketamine (35 mg/kg of body weight) and xylazine (7 mg/kg of body weight), followed by an overdose of intracardiac pentobarbital-sodium and phenytoin sodium (Euthasol) (1 ml per rabbit). Animals were necropsied on days 1, 3, and 7, and several samples were collected for subsequent analysis. Intestinal contents and tissues (ileum, cecum, and colon) were homogenized and plated for an estimation of the bacterial burden (intralumenal and attached). For hematoxylin-and-eosin (H&E) staining, intestinal tissues (ileum, cecum, and colon) were fixed in 10% buffered formalin (4% formaldehyde). The sections for histopathology were obtained from identically sampled sites within ceca of all rabbits.

Cytokine measurements. Cytokine expression in intestinal mucosal scrapings was quantified by quantitative reverse transcription-PCR (qRT-PCR) using rabbit-specific primers for proinflammatory cytokines, as described previously (30). Briefly, intestinal mucosal scrapings were collected on days 3 and 7 postinfection and frozen in RNAlater solution (Life Technologies, Grand Island, NY) at −80°C. Total RNA was extracted by using an RNeasy minikit (Qiagen, Valencia, CA) and reverse transcribed with an RT kit (Qiagen, Valencia, CA), and mRNA was quantitated by using qRT-PCR for interleukin-1β (IL-1β), IL-6, and gamma interferon (IFN-γ). Normalization to the hypoxanthine phosphoribosyltransferase (HPRT) gene was performed.

TUNEL staining of rabbit tissues for evaluation of epithelial apoptosis. Rabbit ileal tissues were collected and stored in 3% formalin during necropsy. Five-micrometer-thick sections of paraffin-embedded tissues were attached to glass slides. The DeadEnd fluorometric terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) system (Promega), which labels DNA with fluorescein-12-dUTP at the 3′ end, was used to stain apoptotic cells, according to the manufacturer's protocol. Cells were also stained with 4′,6-diamidino-2-

phenylindole (DAPI) to visualize DNA and localize both healthy and apoptotic nuclei. Images were collected with a Zeiss 510 confocal microscope and processed by using ImageJ64. The only manipulation of the raw data was changing color of the DAPI pixels to red and the color of fluorescein to green for better image contrast.

Statistical analyses. A Microsoft-based software package was used for statistical analyses. Significance was determined by using two-tailed Student's *t* tests (*P* values of ≤0.05 were considered significant) as well as analysis of variance (ANOVA), where goodness of fit and standardized coefficients of variance were derived in order to facilitate comparisons between multiple groups of independently collected and continuous numerical data. For the translocation studies, and since the number of observations over the various time points was very large (660 total), goodness-of-fit statistical analyses were performed by using ANOVA followed by a stringent Tukey test. *P* values of <0.05 were considered significant.

RESULTS

REPEC and EPEC EspZ proteins delay death of infected host intestinal epithelial cells. To begin exploring the role of EspZ in the rabbit model of infection, we generated a nonpolar *espZ* deletion mutant (Δ*espZ*) of REPEC; this mutant was *cis*-complemented with REPEC or EPEC *espZ* by the insertion of the respective genes, along with their native promoter sequences, into the site adjacent to *glmS* via Tn*7* transposition (to generate the Δ*espZ*/*espZ*$_R$ and Δ*espZ*/*espZ*$_E$ strains, respectively). RT-PCR analyses confirmed near-WT levels of *espZ* expression in the complemented Δ*espZ*/*espZ*$_R$ strain (Fig. 1A). Similarly, *espZ* expression in the complemented Δ*espZ*/*espZ*$_E$ strain was also verified (not shown). Furthermore, comparable expression levels were observed for the gene downstream of *espZ* (*escI*) in the WT, Δ*espZ*, and complemented strains, confirming that the *espZ* deletion/disruption did not result in transcriptional polarity (data not shown).

We previously demonstrated that EPEC *espZ* delays the death of infected human intestinal epithelial cells (18). To determine if EspZ played a similar role in the REPEC strain background, C2$_{BBE}$ intestinal epithelial cells were infected with the REPEC WT, Δ*espZ*, Δ*espZ*/*espZ*$_R$, or Δ*espZ*/*espZ*$_E$ strain, and host cell survival was quantitated by monitoring propidium iodide uptake. As described above for the corresponding EPEC strains, WT REPEC induced marked death of infected host epithelial cells compared to mock-infected cells. Δ*espZ* strains induced a greater degree of host cell death than did the isogenic parent strain, and this was reversed by *cis*-complementation with either REPEC or EPEC *espZ* (Fig. 1B). Furthermore, both EPEC and REPEC *espZ*-transfected intestinal epithelial cells were protected from staurosporine-induced apoptosis, suggesting a direct engagement of cytoprotective signaling pathways by these proteins (18) (data not shown).

REPEC EspZ inhibits type III secretion system-mediated effector translocation. Using a complex experimental setup, Berger et al. observed that the EPEC and EHEC EspZ proteins could interfere with the bacterial T3SS-mediated delivery of effector molecules into host cells (21). Those investigators demonstrated that large amounts of EPEC EspZ in host cells, either expressed from a transfected construct or delivered via a first-wave infection (at a high multiplicity of infection [MOI] of >500), could inhibit T3SS-dependent translocation from a subsequent second wave of EPEC infection. To determine if this function was conserved in REPEC EspZ and if EPEC and REPEC *espZ* molecules could cross-complement for this phenotype, we performed similar translocation assays. Consistent with the observations for EPEC, REPEC strains were impaired for the translocation of a TEM-1 β-lactamase reporter protein into host cells transfected with REPEC or EPEC *espZ* compared to vector-transfected controls (Fig. 2A).

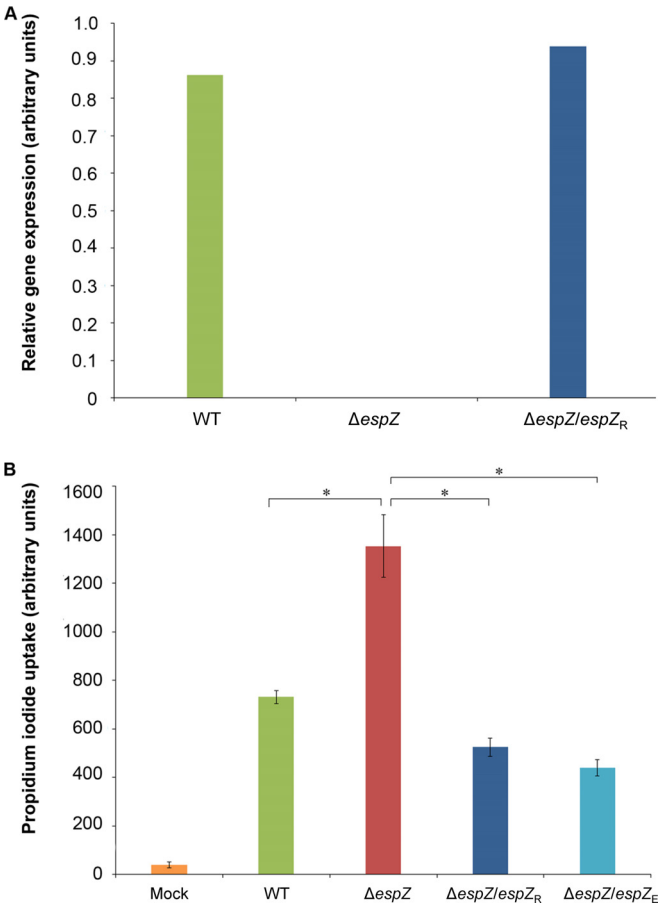

FIG 1 Both the REPEC and EPEC EspZ proteins protect epithelial cells from REPEC-induced death. (A) Reverse transcription analyses demonstrating comparable *espZ* expression levels in the REPEC parental (WT) strain and the *cis*-complemented REPEC Δ*espZ* strains. Relative gene expression levels are shown and were derived via densitometric analyses of the linear phase of *espZ* amplicon accumulation. Data are normalized to the expression levels of the *E. coli* genes *escI* and *groE*. (B) PI uptake cell death assay using C2$_{BBE}$ host cells infected with the parent REPEC strain (green bar), the Δ*espZ* mutant (red bar), and the Δ*espZ* mutant *cis*-complemented with *espZ* from REPEC (Δ*espZ*/*espZ*$_R$) (dark blue bar) or from EPEC (*espZ*/*espZ*$_E$) (light blue bar) for 4.5 h. Data are presented as average PI uptake rates ± standard errors of the means for eight technical replicates. Asterisks indicate significant differences ($P \leq 0.05$).

A similar conservation of function between the EPEC and REPEC EspZ proteins was observed in the two-wave infection system, which employed a net MOI (number of bacteria/host cell) of >500. The rate of REPEC T3SS-dependent translocation of the reporter protein into epithelial cells (second wave) was significantly lower when host cells were infected previously (first wave) with WT REPEC than when host cells were treated with a first-wave Δ*espZ* strain infection (Fig. 2B). Surprisingly, however, *cis*-complementation with either REPEC or EPEC *espZ* failed to restore the translocation phenotype of the Δ*espZ* strain (Fig. 2B), even though *espZ* expression in the Δ*espZ*/*espZ*$_R$ strain was verified to be comparable to that of the parent WT strain (Fig. 1A). The lack of complementation was also specific for the transloca-

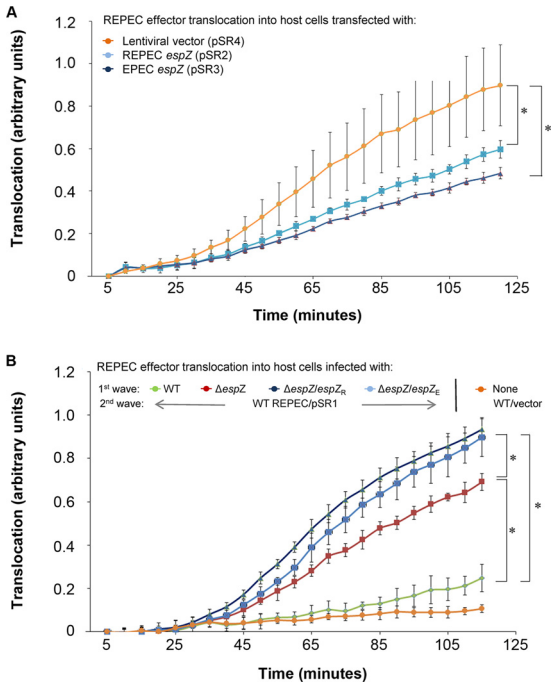

FIG 2 The presence of EspZ in host cells can limit T3SS-dependent effector translocation. A TEM-1 β-lactamase (encoded on pSR1) reporter system was used to monitor effector translocation from WT REPEC into host cells transiently transfected with an REPEC *espZ*-containing (pSR2), EPEC *espZ*-containing (pSR3), or control (pSR4) plasmid (A) or previously infected (first wave) with WT REPEC, the REPEC Δ*espZ* mutant, or the REPEC Δ*espZ* mutant complemented with REPEC EspZ (*espZ*/*espZ*$_R$) or EPEC EspZ (*espZ*/*espZ*$_E$) (B). TEM-1 β-lactamase translocation-dependent cleavage of the β-lactam-containing compound CCF2 in host cells was monitored via fluorescence measurements over a 120-min time period. Asterisks indicate significant differences ($P \leq 0.05$).

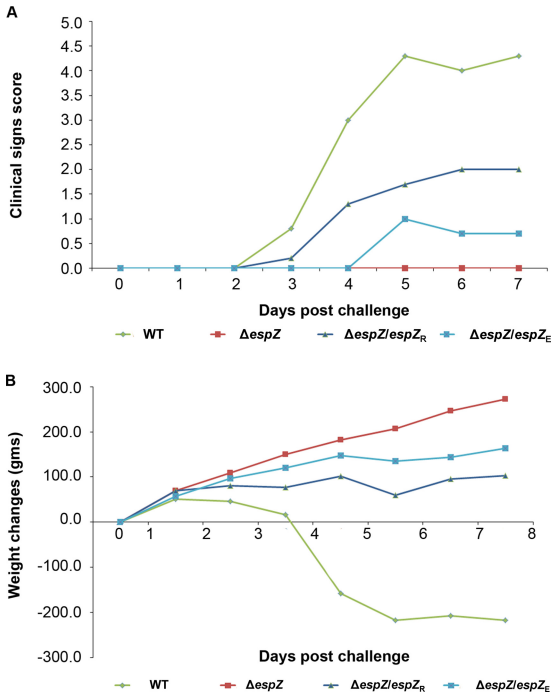

FIG 3 Rabbits infected with the REPEC Δ*espZ* strain are clinically indistinguishable from mock-infected animals. Data shown are representative of data from a total of three independent animal studies, each performed with a minimum of eight animals per group on day 1. (A) Clinical sign scores of the animals over 7 days of infection with the parent WT strain, the Δ*espZ* mutant, and the Δ*espZ* mutant *cis*-complemented with REPEC *espZ* (*espZ*/*espZ*$_R$) or EPEC *espZ* (*espZ*/*espZ*$_E$). Scores are based on four parameters, as detailed in Table S1 in the supplemental material (with "0" referring to no clinical signs), and are for an average of at least three animals per group per time point. The number of animals decreased during the course of the study due to death/euthanasia; therefore, standard statistical analyses were not performed. (B) Weight change(s) of animals over the course of 7 days of infection with the parent WT strain, the Δ*espZ* mutant, and the Δ*espZ* mutant *cis*-complemented with REPEC *espZ* (*espZ*/*espZ*$_R$) or EPEC *espZ* (*espZ*/*espZ*$_E$).

tion phenotype, since complementation was successful with the same strains in the cell death assays (MOI = 100) (Fig. 1B). Notably, the original report by Berger et al. did not provide complementation data in connection with the EPEC Δ*espZ* strain's influence on effector translocation (21). The use of extremely high MOIs and the lack of complementation highlight the need for caution in interpreting the results of two-wave infections. Overall, the results suggest that REPEC *espZ*, like EPEC *espZ*, may, under certain circumstances (such as transfection-mediated high-level expression), influence the translocation of effector proteins.

EspZ is essential for causing disease in a rabbit model of infection. To assess the role of EspZ in A/E pathogen virulence, New Zealand White rabbits were infected with WT REPEC or the Δ*espZ*, Δ*espZ*/*espZ*$_R$, or Δ*espZ*/*espZ*$_E$ isogenic derivative and monitored for various parameters over 7 days. Clinical signs of disease were pronounced, and the scores progressively increased in WT REPEC-infected animals (Fig. 3A; see also Table S1 in the supplemental material); these signs included moderate-to-severe diarrhea and weight loss (Fig. 3B) over the 7-day period. In contrast, Δ*espZ* strain-infected animals were clinically indistinguishable from control, uninfected animals and gained weight normally for the duration of the study (Fig. 3). *cis*-complementation with either REPEC or EPEC *espZ* partially restored the virulence of the Δ*espZ* strains, with the animals displaying moderate clinical signs (Fig. 3A) but not the dramatic weight loss noted for WT-infected animals (Fig. 3B). This finding suggests that EspZ is an essential determinant of A/E pathogen virulence and that the REPEC and EPEC EspZ proteins display considerable functional congruence in the rabbit model of infection.

EspZ is essential for intestinal REPEC colonization. To determine if disease progression correlated with intestinal colonization of the different strains, stool and cecal tissue samples from infected animals were processed and plated onto selective media. We focused on cecal colonization since a pilot experiment revealed maximal bacterial burden at this site compared to the ileum and the rectum (not shown). WT colonization and expansion were evident in ceca of infected animals (Fig. 4C), and bacteria were shed at high levels in the stool throughout the duration of the study (Fig. 4A and B). For the Δ*espZ* strain-colonized animals, stool Δ*espZ* cell counts were >10-fold lower than those of the WT on day 1 postinfection and steadily decreased to a level ~100-fold lower than those of the WT by day 7 postinfection. Cecal Δ*espZ* cell counts were comparable to those of the parent WT strain only on day 1 postinfection and decreased to levels ~15-fold lower than those of WT REPEC on day 7 postinfection (Fig. 4C). *cis*-complementation of the mutant with REPEC or EPEC *espZ* increased bacterial loads in both the cecum and the stool relative to

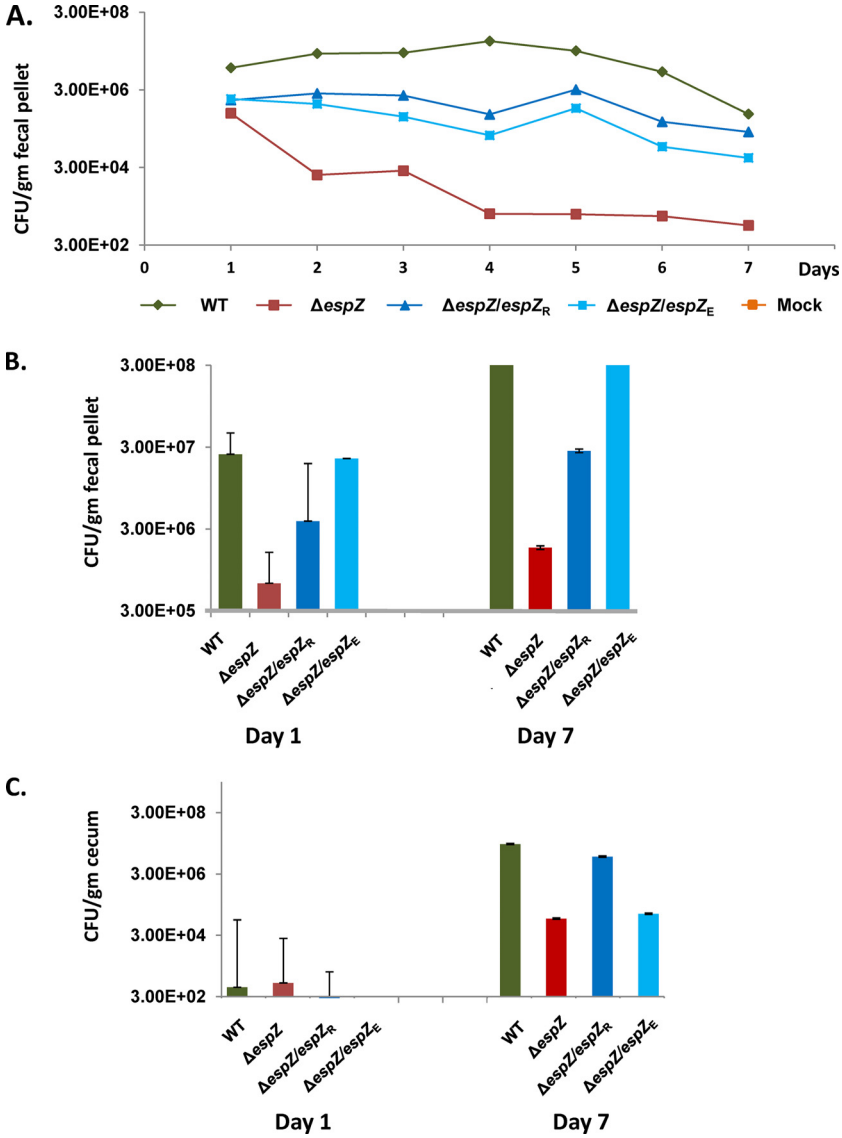

FIG 4 The REPEC Δ*espZ* strain does not expand in, and is rapidly cleared from, infected rabbits. The data shown are representative of a total of three independent animal studies, each performed with a minimum of eight animals per group on day 1. (A) The bacterial burden throughout rabbit infection with the parent WT strain, the Δ*espZ* mutant, or the Δ*espZ* mutant *cis*-complemented with REPEC *espZ* (*espZ*/*espZ*$_R$) or EPEC *espZ* (*espZ*/*espZ*$_E$) or in mock-infected animals was enumerated. Fecal pellets were collected from a minimum of three animals per group per day and plated onto medium selective for the infecting REPEC strain, as described in Materials and Methods. Data shown are average CFU per gram of fecal matter for each group of animals over time. (B) Bacterial burden data from panel A highlighting pathogen accumulation at the first (day 1) and last (day 7) time points following infection of rabbits with the parent WT strain, the Δ*espZ* mutant, or the Δ*espZ* mutant *cis*-complemented with *espZ* from REPEC (*espZ*/*espZ*$_R$) or EPEC (*espZ*/*espZ*$_E$). Data shown are average CFU per gram of fecal matter for each group of animals. (C) Enumeration of bacteria adherent to the cecum over time for rabbits infected with the parent WT strain, the Δ*espZ* mutant, or the Δ*espZ* mutant *cis*-complemented with REPEC *espZ* (*espZ*/*espZ*$_R$) or EPEC *espZ* (*espZ*/*espZ*$_E$). Ceca from euthanized animals were dissected, and REPEC bacteria were recovered as described in Materials and Methods. Data shown are average CFU per gram of cecal tissue for each group of animals. The limit of detection was 100 CFU/mg tissue. Since the number of animals in each group decreased variably over time due to the need to euthanize ill or moribund animals, statistical analyses were not performed on these data.

the Δ*espZ* strain, although colonization was less robust than that by the parent WT strain. Taken together, these data suggest that EspZ is essential for REPEC intestinal colonization and expansion and that both the REPEC and EPEC *espZ* genes can partially restore function to a REPEC Δ*espZ* strain *in vivo*.

EspZ is essential for REPEC to cause heightened and sustained tissue injury. Histopathological examination of hematoxylin-and-eosin (H&E)-stained sections of the ceca of rabbits infected with REPEC, the complemented mutants, or the Δ*espZ* strain revealed mild hyperemia and a mild heterophilic infiltrate in the lamina propria at day 1 postinfection (Fig. 5). Slight to moderate multifocal enterocyte death with mild mucosal attenuation was also observed for the Δ*espZ* strain-infected rabbits. At day 3 postinfection, the severity of inflammation had progressed in the ceca of rabbits infected with REPEC or the complemented mutants but not in the Δ*espZ* strain-infected rabbits. By day 7 postinfection, there was a moderate to multifocally severe heterophilic and lymphoplasmacytic infiltrate in the lamina propria and

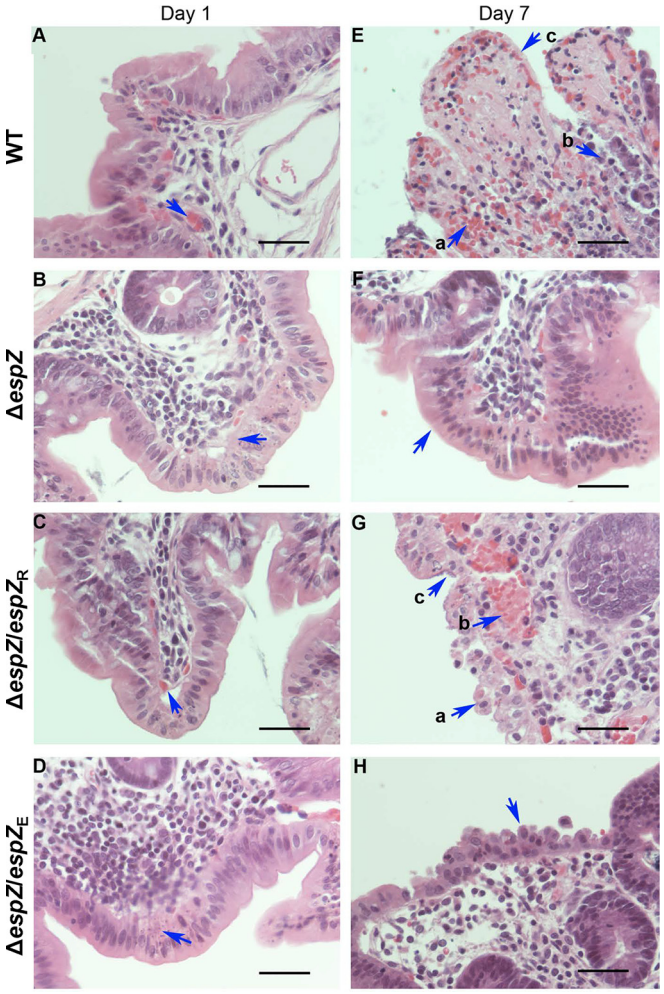

FIG 5 Infection with the Δ*espZ* REPEC strain causes minimal and unsustained gut tissue damage. (A to D) H&E-stained sections of rabbit cecum on day 1 postinfection. Shown are representative images from a minimum of three animals per group infected with WT REPEC (A), the Δ*espZ* mutant (B), or the Δ*espZ* mutant *cis*-complemented with REPEC *espZ* (*espZ*/*espZ*$_R$) (C) or EPEC *espZ* (*espZ*/*espZ*$_E$) (D). Arrows are positioned over sites of proprial or submucosal hemorrhage (a to c) or over apoptosing enterocytes (d). Bar, 50 μm. (E to H) H&E-stained sections of rabbit cecum on day 7 postinfection. Shown are representative images from a minimum of three animals per group infected with either WT REPEC (E), the Δ*espZ* mutant (F), or the Δ*espZ* mutant *cis*-complemented with REPEC *espZ* (*espZ*/*espZ*$_R$) (G) or EPEC *espZ* (*espZ*/*espZ*$_E$) (H). Arrows are positioned over sites of hemorrhage (Ea, Ec, and Gb), breached or damaged epithelial cells (Eb, Ga, and H), epithelial cells coated with bacteria (Gc), or epithelial cells with normal morphology (F). Bar, 50 μm. Tissue alterations were scored by an American College of Veterinary Pathologists board-certified pathologist (M. W. Riggs) blind to treatment conditions.

submucosa, accompanied by multifocal proprial and submucosal microhemorrhage in the ceca of rabbits infected with REPEC or the complemented mutants. Multifocal mucosal attenuation progressing to ulceration with fibrinous exudation and accumulation of cellular debris in cecal crypts were also evident in these animals (Fig. 5). In contrast, by day 7 postinfection, cecal damage was considerably mitigated in the Δ*espZ* strain-infected animals, with only mild hyperemia, scattered proprial microhemorrhage, and a mild heterophilic and lymphoplasmacytic infiltrate limited to the lamina propria being observed. Mild multifocal enterocyte death was also observed for the Δ*espZ* strain-infected animals, but unlike for the WT- and complemented mutant-infected animals, no ulceration was identified.

EspZ is essential for inducing proinflammatory cytokine production from infected tissues *in vivo*. We next sought to determine if the impaired colonization and decreased tissue injury observed for Δ*espZ* strain-infected animals correlated with altered proinflammatory cytokine production. Infection with the WT, but not the Δ*espZ* strain, resulted in significantly elevated levels of IL-6, but not IL-1β or IFN-γ, as early as day 1 postinfection (Fig. 6). By day 7 postinfection, however, WT REPEC induced significant increases in the levels of all three cytokines. In contrast, the production of each of these cytokines by Δ*espZ* strain-infected animals was indistinguishable from that in the PBS-treated control animals. Taken together, these data suggest that EspZ promotes intestinal bacterial colonization, which in turn results in increased epithelial tissue injury and increased cytokine production.

EspZ limits intestinal epithelial cell apoptosis during the early stages of infection. Based on the observed *in vitro* cytoprotective phenotype for EspZ, we hypothesized that the Δ*espZ* strain induced heightened enterocyte death in the rabbit intestine early (days 1 to 2) in infection. We further hypothesized that the sloughing of apoptotic epithelial cells, along with the attached bacteria, would limit colonization. To test this hypothesis, cecal tissues from WT-infected rabbits at day 1 of infection, as well as from PBS-treated control animals, were subjected to semiquantitative terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. Compared to PBS-treated animals, intestinal tissue from WT-infected animals displayed a modest increase in the number of TUNEL-positive cells (Fig. 7). In Δ*espZ* mutant-infected animals, despite a bacterial burden comparable to (or lower than) that in the WT-infected animals (Fig. 4), there was a marked increase in the number of apoptotic epithelial cells; consistent with the disease phenotype, epithelial cells from animals infected with the complemented strains displayed an intermediate level of TUNEL-positive cells. Thus, consistent with the *in vitro* observations, EspZ has a cytoprotective role in infected intestinal epithelial tissues of rabbits.

DISCUSSION

The primary findings of this study are that EspZ promotes host epithelial cell survival *in vitro* and *in vivo* and that it is essential for virulence in a rabbit model of infection. REPEC EspZ recapitulates the *in vitro* functional attributes of EPEC EspZ, and both isoforms partially complement the *in vivo* virulence defects of a REPEC Δ*espZ* strain. The differential abilities of the two isoforms to complement the REPEC Δ*espZ* strain, particularly *in vivo*, could be due to differences in the primary sequence, although a potential contribution of expression variability cannot be ruled out. The two isoforms display 82% identity and 92% similarity at the amino acid level.

EspZ is unique to A/E pathogens and does not display similarity to proteins outside this group. The EPEC and REPEC EspZ proteins promote the survival of infected intestinal epithelial cells *in vitro* and *in vivo* (in rabbit intestines). Accordingly, Heczko et al. reported decreased apoptosis in the ileum and ileal Peyer's patches of rabbits with severe REPEC infection relative to uninfected control animals (31).

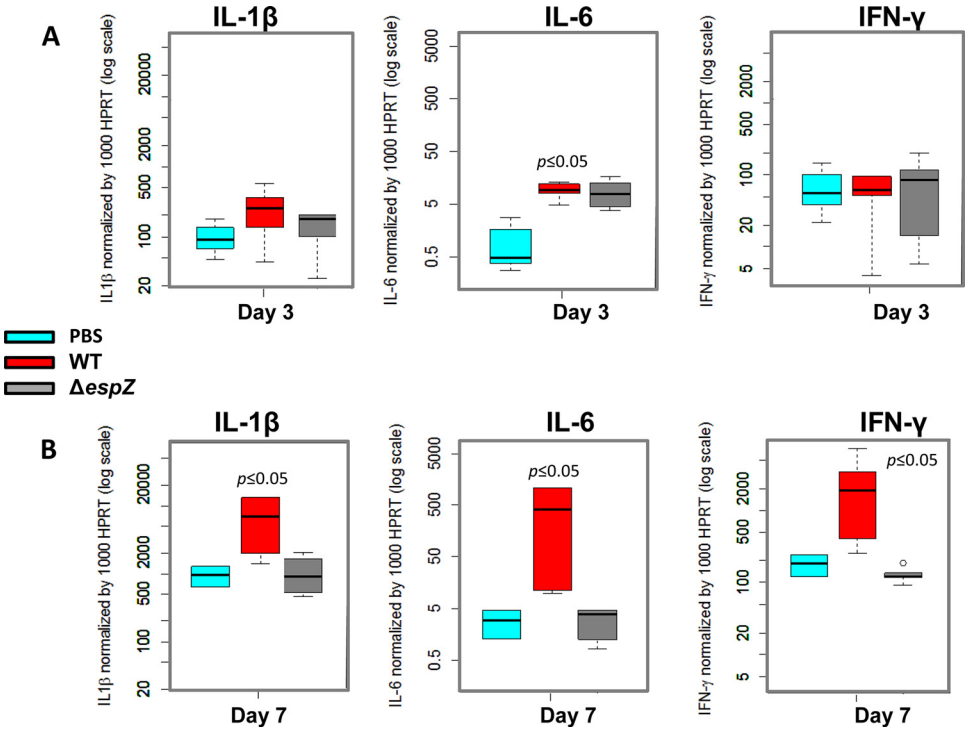

FIG 6 Proinflammatory cytokine levels remain unaltered over time in rabbits infected with the Δ*espZ* REPEC strain. Intestinal mucosal scrapings were used for quantitation of multiple cytokines on day 3 (A) or day 7 (B) postinfection in rabbits gavaged with PBS or the WT or isogenic Δ*espZ* strain. RNA was isolated from the samples as described in Materials and Methods and subjected to reverse transcription and quantitative PCR to determine IL-1β, IL-6, and IFN-γ levels. The relative gene expression level was obtained by normalizing the specific cytokine gene expression level to that of the control hypoxanthine-guanine phosphoribosyltransferase gene.

Two nonexclusive mechanisms have been proposed to explain the cytoprotective effect of EspZ. Roxas et al. and Shames et al. presented data to suggest that EspZ directly engages epithelial cell proteins and influences host signaling pathways to prevent the premature death of infected cells (18–20). Importantly, we demonstrated that transfected REPEC *espZ* protected both $C2_{BBE}$ and HeLa cells from staurosporine-induced intrinsic apoptosis (18). Shames et al. identified two EspZ-interacting epithelial cell proteins, the transmembrane glycoprotein CD98 and the inner mitochondrial protein TIM17b, and implied a role for these proteins in the survival of infected host cells. The EspZ interaction with CD98 was hypothesized to induce focal adhesion kinase (FAK)- and phosphatidylinositol 3-kinase (PI3K)/Akt-dependent prosurvival signaling (19); however, we reported that the inhibition of FAK phosphorylation decreased the survival of EPEC-infected HeLa cells but not that of intestinal epithelial cell lines (18). For TIM17b, consistent with a role in EspZ-dependent cytoprotection, small interfering RNA (siRNA)-mediated depletion of this protein resulted in an increased death of WT EPEC-infected, but not Δ*espZ* strain-infected, HeLa cells (20). A role for TIM17b in infected intestinal epithelial cells remains to be established.

On the other hand, Berger et al. demonstrated that EspZ limits the translocation of bacterial effector proteins into host epithelial cells, possibly via interactions with components of the T3SS (21). According to this model, the protective effects of EspZ can be explained by its ability to limit the translocation of cytotoxic effector proteins during an infection. Our studies similarly confirmed that REPEC and EPEC *espZ*-transfected host cells were refractory to translocation from infecting REPEC strains. While we were also able to confirm that a first wave of WT infection was more effective (than the isogenic Δ*espZ* strain) in inhibiting translocation from a subsequent second wave of REPEC infection (net MOI of >500), the failure of complementation of the Δ*espZ* strain infection suggests caution in interpreting this phenotype.

Notably, since complementation was demonstrated for the cell death phenotype with the same strains (even at a "low" MOI of ~100), the increased cytotoxicity of the Δ*espZ* strain is likely not due to an enhanced translocation of other effectors. Additionally, our studies call into question the implicit assumption that the first-wave infection with the WT and Δ*espZ* strains differed in only one aspect: the presence or absence of EspZ. We noted clear differences in cell death between the two conditions, consistent with the cytoprotective role of EspZ (data not shown). The fluorescence readout for the translocation reporter does not factor into such alterations.

Irrespective of the underlying mechanisms, the cytoprotective effects of EspZ likely contribute to enhanced colonization *in vivo*. Thus, while the REPEC Δ*espZ* strain induces heightened enterocyte death in rabbits on day 1, bacterial colonization is inefficient relative to that of the WT strain, and the animals do not display the characteristic pathologies associated with infection. Previous studies by Mills et al. suggest that EspZ is secreted into host cells early in infection (27). Protection of infected epithelial cells during the initial stages of infection could therefore facilitate bacterial colonization by stabilizing the underlying epithelial substratum. Indeed, such protection may be achieved via cooperative, or ad-

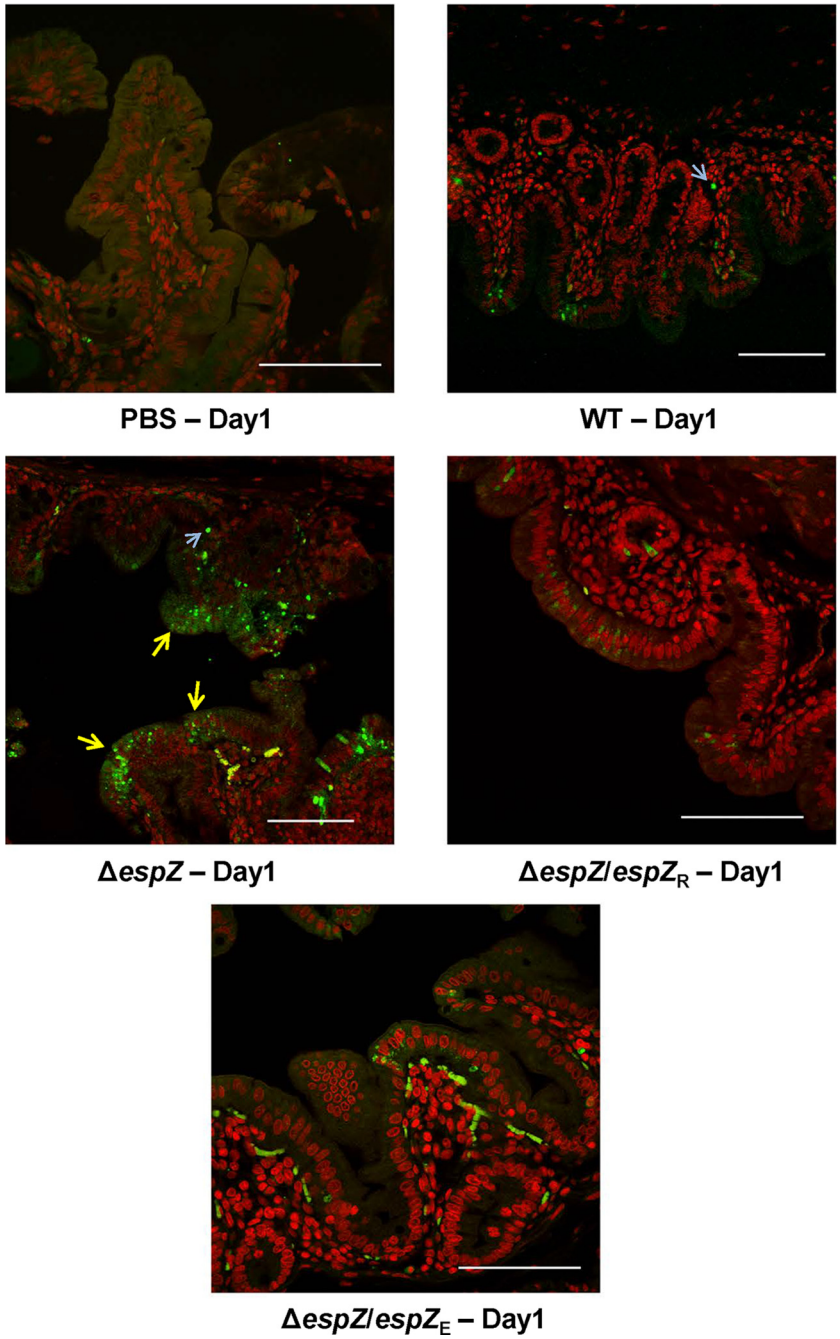

FIG 7 Infection with the REPEC Δ*espZ* strain results in significant intestinal cell apoptosis *in vivo*. Shown are images from TUNEL staining of cecal tissues from rabbits mock gavaged with PBS or infected with the parent WT strain, the Δ*espZ* mutant, or the Δ*espZ* mutant *cis*-complemented with REPEC *espZ* (*espZ*/*espZ*$_R$) or EPEC *espZ* (*espZ*/*espZ*$_E$). Images are representative of those from multiple fields and multiple animals and highlight the intestinal cell apoptotic status on day 1 postinfection (yellow arrows), when histopathological observations revealed alterations for all groups (Fig. 5). Host cell nuclei were stained red by using the fluorescent marker DAPI, and TUNEL-positive apoptotic cells were stained green by using resorcinol phthalein (fluorescein). Light blue arrows pointing to diffuse staining are subepithelial autofluorescing erythrocytes. Bar, 100 μm.

ditive, actions of many effectors. NleB1, another effector protein common to A/E pathogens, inhibits death receptor signaling by engaging and modifying host cell death domain-containing proteins (32, 33). *C. rodentium* mutants lacking NleB1 were impaired for colonization of mouse intestines.

More broadly, other pathogens also appear to employ the strategy of host epithelial cell preservation as a means to increase the efficiency of colonization. The *Shigella flexneri* effector protein OspE binds to host cell integrin-linked kinase (ILK), promotes FAK phosphorylation, and inhibits the detachment of infected cells from the basement membrane (34). OspE-deficient bacteria fail to colonize or cause disease in a guinea pig model of infection.

The significantly diminished proinflammatory cytokine responses in Δ*espZ* strain-infected animals throughout the course of

infection suggest that the decreased virulence of this strain results from impaired colonization, which is supported by the decreased bacterial burdens observed, rather than heighted bacterial clearance via host immune responses. We cannot, however, rule out a broader role for EspZ in limiting host immune responses, particularly during a prolonged infection. Interleukin-17-producing T helper 17 (Th17) cells play an essential role in the clearance of the A/E pathogen *C. rodentium* (35). Phagocytosis of infected apoptotic cells by dendritic cells uniquely triggers the release of both interleukin-6 and transforming growth factor β, which is essential for Th17 development. Blocking apoptosis during *C. rodentium* infection impairs Th17 development. It is possible, therefore, that the EspZ-dependent inhibition of apoptosis delays Th17 development and the consequent clearance of the bacteria.

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We confirm that we do not have any conflict of interest related or relevant to the manuscript.

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