**Clostridium difficile** Toxins Facilitate Bacterial Colonization by Modulating the Fence and Gate Function of Colonic Epithelium

Magdalena Kasendra, Riccardo Barrile, Rosanna Leuzzi, and Marco Soriani

Novartis Vaccines and Diagnostics, Via Fiorentina 1, Siena, Italy

The contribution of *Clostridium difficile* toxin A and B (TcdA and TcdB) to cellular intoxication has been studied extensively, but their impact on bacterial colonization remains unclear. By setting up 2- and 3-dimensional in vitro models of polarized gut epithelium, we investigated how *C. difficile* infection is affected by host cell polarity and whether TcdA and TcdB contribute to such events. Indeed, we observed that *C. difficile* adhesion and penetration of the mucosal barrier are substantially enhanced in poorly polarized or ethylene glycol tetraacetic acid–treated cells, indicating that bacteria bind preferentially to the basolateral (BL) cell surface. In this context, we demonstrated that sub-lethal concentrations of *C. difficile* TcdA are able to alter cell polarity by causing redistribution of plasma membrane components between distinct surface domains. Taken together, the data suggest that toxin-mediated modulation of host cell organization may account for the capacity of this opportunistic pathogen to gain access to BL receptors, leading to a successful colonization of the colonic mucosa.

**Keywords.** *C. difficile*; toxins; cell polarity; adhesion.

The mucosal surface of the human body represents a first line of defense from the environment and external pathogens. This barrier is mainly composed by polarized epithelial cells with distinct apical (AP) and basolateral (BL) membrane domains separated by tight junctions (TJs). TJs play pivotal roles in tissue integrity and maintenance of cell polarity by acting like a gate and a fence. In particular, they aim to regulate the paracellular passage of molecules, including pathogens (gate function), and restrict the movement of plasma membrane components between AP and BL regions (fence function) [1, 2]. The distinct composition of the cell surface, combined with the physical barrier that is formed by intercellular junctions, can deter bacterial colonization of the AP membrane of epithelial cells, translocation to underlying tissues, and further dissemination [3, 4]. Human mucosal pathogens have evolved different strategies for manipulating the epithelial gate/fence function and subverting host cell polarity in order to successfully colonize humans and cause disease [5–8].

*Clostridium difficile* is broadly recognized as the leading cause of nosocomial diarrhea. The pathogenesis initiates with destruction of intestinal microflora by antibiotic treatment, which enables *C. difficile* to colonize the gut, adhere to epithelium, and become pathogenic through the production of 2 potent toxins, TcdA and TcdB, which are essential for its virulence [9–11]. Both toxins belong to the family of large clostridial glucosylating toxins. These toxins inactivate host GTPases (including Rho, Rac, and Cdc42), leading to alteration of the epithelial barrier, damage to human intestinal mucosa, and inflammation of the colon [12–15]. The high rate of recurrent disease, even after repeated antimicrobial treatments [16], suggests that *C. difficile* has evolved mechanisms to persist in the intestinal tract. This long-term establishment is likely mediated by multiple colonization strategies. While the effects of toxins on epithelial cells have been largely described in vitro, little is known about how they might benefit bacteria during the host colonization process. The

Received 8 July 2013; accepted 3 October 2013; electronically published 22 November 2013.

Correspondence: Marco Soriani, PhD, Novartis Vaccines and Diagnostics Srl, Via Fiorentina 1, 53100, Siena, Italy (marco.soriani@novartis.com).

The Journal of Infectious Diseases 2014;209:1095–104

© The Author 2013. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com.

DOI: 10.1093/infdis/jit617
recognition of Rho family GTPases as master regulators of cell polarity in eukaryotes [17] led us to hypothesize that a toxin-mediated subversion of the epithelial polarity might serve as an important strategy for bacterial settlement in the gut.

In the present study, we postulate that C. difficile TcdA is able to perturb epithelial polarity by causing redistribution of plasma membrane components between distinct surface domains. As a consequence, subverted cell polarity enables bacteria to gain access to the BL surface where they display a preferential association. Therefore, TcdA might play an important role in colonization of colonic epithelium not only by disrupting its barrier function but also by perturbing epithelial polarity and promoting mucosal association.

MATERIALS AND METHODS

C. difficile Strains and Culture Conditions

C. difficile strains 630 and B1/NAP1/027 R20291 were kindly provided by Nigel Minton (University of Nottingham, Nottingham, UK). For infection studies, C. difficile strains were grown in brain heart infusion broth (Bacto) at 37°C in anaerobic conditions until an optical density measured at a wavelength of 600 nm (OD$_{600}$) reached 0.5 (early exponential phase).

2-Dimensional and 3-Dimensional Cell Cultures

The colon adenocarcinoma cell line Caco-2 (obtained from the American Type Culture Collection) was cultured in Dulbecco’s modified Eagle’s medium/F12 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), HEPES, nonessential amino acids, and penicillin/streptomycin. Cells were grown as 2-dimensional (2D) monolayers on collagen-coated Transwell inserts (3-μm pore size; BD Biosciences) and, unless stated otherwise, allowed to differentiate for 21 days. To disrupt calcium-dependent intercellular junctions, monolayers were transiently exposed to 4 mM ethylene glycol tetraacetic acid (EGTA) [18]. Cell polarity and TJ barrier function were determined in the initial experiments (data not shown). To test the effect of toxins on the capacity of C. difficile to infect epithelial cells, Caco-2 monolayers were preincubated with TcdA or TcdB for 16 hours.

Dextran Permeability and Membrane Lipid Diffusion Assays

Dextran permeability and membrane lipid diffusion assays were performed as described elsewhere [21]. Briefly, 2D Caco-2 monolayers were loaded with a 5-μM solution of BODIPY-FL-C5-sphingomyelin/bovine serum albumin (BSA) complex (Molecular Probes) for 10 minutes on ice, after which inserts were rinsed with ice-cold Hanks’ balanced salt solution/HEPES buffer, incubated for an additional hour on ice, and observed by confocal microscopy. Paracelluar permeability was quantified by measuring the transepithelial flux of a 4-kDa fluorescein isothiocyanate (FITC)–labeled dextran (Sigma-Aldrich) [19] and expressed as apparent permeability coefficient (Papp): Papp (cm/s) = $dQ/dt$ (1/AC0), where $dQ/dt$ is the permeability rate (μg/s), C0 is the initial concentration in the upper chamber (μg/mL), and A is the surface area of the membrane (cm$^2$) [22].

Immunofluorescence Microscopy

The 2D Caco-2 cultures pretreated with TcdA, TcdB, EGTA, or medium alone were rinsed with Dulbecco’s phosphate-buffered saline (DPBS), fixed with 4% paraformaldehyde, permeabilized, and blocked with 3% BSA in DPBS. Incubation with primary antibodies, including rabbit anti–zonula occludens-1 (ZO-1) antibody (61–7300; Invitrogen), mouse anti–E-cadherin (33–4000; Invitrogen), and goat anti-PKCζ antibody (sc-216-G; Santa Cruz Biotechnology), was performed either overnight at 4°C or for 2 hours at room temperature and followed by treatment with the appropriate Alexa fluor-conjugated secondary antibody. Samples were mounted using ProLong Gold Antifade Reagent with diamidino-2-phenylindole (Invitrogen) and analyzed by confocal microscopy using Zeiss LSM 710. Z-stacks 3D reconstructions were performed using Imaris software (BitPlane Inc.).

C. difficile Adhesion and Translocation Assay on 2D Caco-2 Monolayers

The 2D Caco-2 cultures were transferred into an anaerobic cabinet and infected for 1 hour with C. difficile from the exponential phase. Dextran solution was applied simultaneously to monitor monolayer integrity during infection. Bacterial translocation and adhesion were determined by cfu counting. Optimal multiplicity of infection (1:20) and maximum incubation time of Caco-2 monolayers in anaerobic conditions were determined in the initial experiments (data not shown). To test the effect of toxins on the capacity of C. difficile to infect epithelial cells, Caco-2 monolayers were preincubated with TcdA or TcdB for 16 hours.

C. difficile Infection of 3D Caco-2 Cysts

After treatment with 100 U/mL Collagenase VII (Sigma) for 15 minutes at 37°C to allow bacterial access to the cell surface, cysts were infected with 10^7 bacteria from exponential-phase cultures and incubated for 2 hours under anaerobic conditions. Cysts were then washed to remove unbound bacteria and subjected to immunofluorescence staining, as described above, with an anti-whole bacteria serum used to visualize C. difficile. Bacterial binding to 3D cysts was quantified using Imaris, as further described in the Supplementary data, on 3D reconstructions of images acquired with Zen2009 software.

Statistical Analyses

All experiments were performed at least 3 times in triplicate. Statistical analyses were performed using the nonparametric Mann-Whitney U test.
RESULTS

Cell Polarity Significantly Affects C. difficile Ability to Infect Caco-2 Cells

To dissect the impact of the host cell polarity on C. difficile infection, we used a Caco-2 cell line that closely resembles the natural physiology and architecture of human colonic tissue in vitro [23, 24]. Caco-2 cells were grown on a porous filter support and subsequently infected with C. difficile 630 strain either at its AP or BL side at increasing levels of cellular differentiation. The degree of epithelial polarity was monitored by TEER and dextran flux measurements (Figure 1A). After 1 hour of incubation in anaerobic conditions, the level of bacterial adhesion was determined by cfu counting. As shown in Figure 1B, bacteria adhere almost equally to nonpolarized Caco-2 cells (TEER 100 Ω·cm²) after infection from the AP or BL side. On the contrary, we observed a 20-times lower bacterial binding (P < .0001) to the AP surface of fully polarized Caco-2 cells (TEER 600 Ω·cm²) compared with nonpolarized cells. Overall, the increasing level of polarization paralleled a rise in bacterial adhesion to the BL side of the cells and a significant drop in their ability to infect the AP membrane of Caco-2 cells. Moreover, C. difficile displayed a preferential binding to the BL membrane, which was maximal when the monolayer was infected after 21 days of growth (Figure 1B). Together these data suggest that the efficiency of C. difficile infection of epithelial cells is dependent on the state of cellular differentiation. To further prove this relationship, a fully polarized Caco-2 monolayer was exposed to EGTA, a well-known calcium chelator that is able to disrupt E-cadherin–mediated cell–cell contact, followed by C. difficile infection from the AP side. As expected, EGTA treatment caused a gradual and significant increase (a fold increase from 2.6 ± 0.6 to 14.8 ± 3.7 compared with untreated cells) in bacterial association with Caco-2 cell surfaces (Figure 2B), which was consistent with the phenotype observed by confocal imaging (Figure 2C). This result further confirms that host cell susceptibility to C. difficile infection correlates with its polarity status. Moreover, the EGTA-mediated alteration of epithelial barrier integrity was found to promote an enhanced bacterial translocation through Caco-2 monolayers, resulting in a 3–10 times larger number of bacteria found to penetrate epithelium compared with those that remained in close contact with Caco-2 cells (Figure 2B).

3D Cyst Model Confirms Propensity of C. difficile to Target BL Membrane

To further demonstrate the preferential targeting of C. difficile to the BL surface of the mucosal barrier, a 3D intestinal epithelial cell culture system was used [20]. Caco-2 cells were grown in Matrigel to form hollow spheres consisting of a fully polarized monolayer surrounding a central lumen, with the AP side facing the lumen and the BL side facing the surrounding Matrigel (BL-side-out cysts). As illustrated in Figure 3A (lower panel), proteins that are typically expressed at the intestinal brush border (such as atypical protein kinase C [aPKC]) were observed to localize to the cell surface lining, that is, the lumen of the sphere. Staining of ZO-1 was observed at the level of TJs in the sub-AP region of cells. This indicates that the Caco-2 cyst model recapitulates the organization of human gut with structural cell polarity, as displayed by enterocytes in the intestine. Importantly, when an antibody against the extracellular domain of integrin β1 was added during growth, cysts with opposite polarity were formed (AP-side-out cysts; Figure 3A, upper panel). These spheres, while less well organized, were fully polarized as BL-side-out cysts. The use of Caco-2 cysts, which selectively expose outward their AP or BL membranes, allowed us to compare bacterial interactions with distinct cellular domains. C. difficile infection of AP- and BL-side-out cysts was carried out for 2 hours under anaerobic conditions and was followed by qualitative and quantitative examination of bacterial

Figure 1. Cell polarization state–dependent Clostridium difficile infection of Caco-2 cells. A, Kinetics of transepithelial electrical resistance and dextran permeability during 21 days of Caco-2 monolayer growth. B, Caco-2 cells at different stages of differentiation were infected either at apical (white bars) or basolateral (black bar) side with C. difficile 630 strain. After 1 hour of incubation, the number of bacteria adhering to epithelium was quantified by cfu counting and expressed as cfu/100 cells. Nonspecific background binding of C. difficile to collagen-coated empty insert was subtracted from experimental sample counts before analysis. Results are the mean ± standard deviation from 3 independent experiments performed in triplicate.
Figure 2. Increase in *Clostridium difficile* capacity to colonize and penetrate epithelium induced by ethylene glycol tetraacetic acid (EGTA) treatment. 

A, Effect of EGTA on transepithelial electrical resistance (TEER) and permeability to fluorescein isothiocyanate-dextran (FD4) in Caco-2 monolayer. TEER is expressed as the percentage of the baseline value (before EGTA treatment). Each value represents the mean ± standard deviation (SD; n = 3). 

B, Fully polarized Caco-2 monolayers were preincubated with 4 mM EGTA for the indicated times followed by apical infection with *C. difficile* 630. After 1 hour of incubation, the number of bacteria that adhered (white bar) or translocated across (black bar) the epithelium was quantified by cfu counting and expressed as cfu/100 cells. Values are the mean ± SD from 3 independent experiments performed in triplicate. *P < .05; **P < .01; ***P < .001. 

C, Maximum projection (extended focus) images of adherent *C. difficile* 630 bacteria on control and EGTA-treated Caco-2 cells. Bacteria were labeled with an anti-whole bacteria serum and a secondary fluorescent antibody (green), cellular actin was stained with phalloidin-Alexa Fluor 568. Scale bar = 10 µm.
binding to the AP side vs the BL side (Figure 3B and 3C). As shown in Figure 3B, a significantly larger number of bacteria was found to adhere to BL-side-out compared with AP-side-out cysts \((P < .0001)\), suggesting that receptors localized basolaterally are a preferential target of \(C.\) difficile during colonization of the human gut.

**TcdA and TcdB Modulate the Gate Function of Colonic Epithelium**

Having established that host cell polarity might influence the capacity of \(C.\) difficile to colonize and penetrate colonic epithelium, we determined whether its toxins, which are known to disrupt epithelial barrier integrity [25–28], are also able to modulate the epithelial fence function. Alteration of the TJ gate function in Caco-2 monolayers preexposed to subtoxic concentrations of TcdA and TcdB, ranging between 100 and 1000 ng/mL (as determined by measurement of ATP in metabolically active cells), was confirmed by detecting the paracellular passage of fluorescently labeled 4 kDa dextran (FD4). In accordance with previously published observations [25–28], a dose–time-dependent increase in paracellular permeability for nonionic molecules was observed in toxin-treated cells (Figure 4A and 4B). Intriguingly, the extent of this enhancement differed significantly between TcdA and TcdB, amounting to an increase of 8–25-fold vs 3–12-fold, respectively, after 24 hours of exposure.

**TcdA, But Not TcdB, Is Able to Modify the Fence Function of Colonic Epithelium**

Next, we determined whether \(C.\) difficile toxins influence the polarity of colonic mucosa by perturbing the distribution of plasma membrane components between distinct surface domains. Initially, diffusion of fluorescently labeled sphingomyelin, a lipid component of plasma membrane, was visualized by confocal imaging in control and toxin-treated monolayers. As expected, in control cells the fluorescent lipid complex added apically was found to easily intercalate and diffuse within the outer leaflet of the bilayer, giving an evident staining of the AP membrane. In contrast, upon EGTA (positive control) or TcdA treatment, diffusion of the lipid was no longer limited, leading to lateral and BL membrane labeling, as shown in confocal z sections (Figure 5A). Slight staining of lateral membranes was
also detected in cells treated with TcdB (500 and 1000 ng/mL), presumably as a result of lipid diffusion in solution across disrupted TJs. These data suggest that although both toxins are able to affect epithelial permeability, alteration in TJ fence function is predominantly induced by TcdA. To further examine this specific modulation of cellular organization, changes in the polarized distribution of plasma membrane proteins in monolayers exposed to C. difficile toxins were assessed by immunofluorescence staining of AP and BL proteins (aPKC and E-cadherin, respectively). Additionally ZO-1 labeling was simultaneously performed to reveal the precise localization of TJs. As shown in Figure 5B, Caco-2 monolayers exposed to EGTA or TcdA displayed a significant displacement of cell membrane constituents, including expansion of aPKC to lateral cell membranes and E-cadherin staining that was localized around the cells. Also, the distribution of ZO-1 was evidently altered. No changes in the distinct composition of plasma membrane proteins were observed in response to TcdB. Together these observations demonstrate that subtoxic concentrations of TcdA, but not TcdB, are able to affect barrier function (gate function) of the epithelium and to subvert cell polarity (fence function). Of importance, by checking for caspase-3 activation and DNA fragmentation, we demonstrated that TcdA-mediated subversion of host cell polarity is not due to apoptosis (Supplementary data and Supplementary Figure 1).

**TcdA-Mediated Subversion of Cell Polarity Facilitates C. difficile Adhesion and Translocation of Caco-2 Monolayer**

Since our results suggested that cell polarity was a deterrent to C. difficile colonization of the host cell surface and that TcdA was able to alter this epithelial function, we set out to determine whether this activity could play a beneficial role in establishment of C. difficile infection. Because we were unable to compare C. difficile wild-type and toxin mutant strains in our infection assay due to the drastically limited time of Caco-2 survival in anaerobic condition (<2 hours), we tested the effect of purified toxins on C. difficile capacity to infect human cells. Briefly, fully polarized Caco-2 monolayers were preexposed to different concentrations of TcdA or TcdB for 16 hours followed by incubation with C. difficile 630 at the AP side. In order to monitor toxin action in our in vitro assay, FITC-dextran was added simultaneously to the inner chamber of the Transwell system (Figure 6A). As a result, striking differences were observed in the ability of the C. difficile 630 strain to colonize cells in vitro in the presence or absence of TcdA (Figure 6B and 6C). A statistically significant increase in bacterial adhesion rates was noticed in Caco-2 monolayers preexposed to 500 and 1000 ng/mL of TcdA. The influence of the highest concentration tested on the number of adherent bacteria was comparable to the effect promoted by 30 minutes of EGTA treatment (44.4 ± 5 bacteria/100 cells in toxin-treated monolayers vs 40.7 ± 7 bacteria/100 cells in EGTA-exposed monolayers). However, as shown in Figure 6B, bacterial translocation was only slightly enhanced by subtoxic doses of TcdA without reaching the levels observed upon EGTA treatment (Figure 2B). Similar results were obtained by incubating cells with C. difficile R20291 (Stoke Mandeville), which is characterized as an epidemic and hypervirulent strain (Supplementary Figure 2), indicating that this phenotype is not strain specific. Strikingly, no differences in bacterial adhesion between control and TcdA-treated cells were observed when only partially polarized (4-day-old) monolayers were infected, confirming again that epithelial polarity is an important target of C. difficile pathogenesis (Supplementary Figure 3). Moreover, polarized monolayer exposure to TcdB was not able to facilitate colonization of Caco-2 cells (Supplementary Figure 4); this is in line with evidence that this toxin is not able to interfere with polarization status.

**DISCUSSION**

An important aspect in the understanding of microbial pathogenesis is recognition of the different strategies evolved by
bacteria and viruses to circumvent or disrupt the mucosal barrier in order to facilitate colonization and/or dissemination to distal tissues. Some of these strategies involve complicated and ingenious pathogen-mediated subversion of the host cell organization and functions [29]. In particular, a number of mucosal pathogens, including *Pseudomonas aeruginosa*, *Helicobacter pylori*, and *enteropathogenic Escherichia coli*, have been shown to directly target various components of the polarity regulation network [6–8]. In our study, by using colonic Caco-2 cells grown on Transwell inserts to mimic natural architecture and tissue polarity in vitro, we found that the establishment of *C. difficile* infection strongly depends on the state of cellular differentiation, further reinforcing studies previously undertaken by Cerquetti and colleagues [30]. The use of the Transwell system allowed us to precisely distinguish between bacterial adhesion and translocation rates and to demonstrate that both processes are significantly enhanced in monolayers whose fence and gate functions are not fully established or have been altered by calcium chelation. This is consistent with the preferential tropism of *C. difficile* to the basal cell surface observed in 2D and 3D Caco-2 cell models. Taken together, our findings suggest that *C. difficile* might exploit a nutrient-rich niche located directly beneath the epithelium to persist in the human colon.

Although the effect of TcdA and TcdB, recognized as the primary virulence factors of *C. difficile*, on the structure of cytoskeleton and the function of TJs has been a topic of intense study, the fundamental question of how they might benefit bacteria during host colonization remains unanswered [9, 10]. Moreover, our current understanding of the molecular mechanism of *C. difficile* toxin action is mainly based on in vitro studies that use highly toxic concentrations, thus leading to increased paracellular permeability and apoptotic or necrotic cell death [26–28, 31–33]. Since toxin production in vitro usually ranges from 50 to 2000 ng/mL within 24 hours of *C. difficile* growth [34], it is likely that the local concentration of TcdA and TcdB in the colon of *C. difficile*-infected patients may reach similar levels. Based on the evidence reported in this study, we hypothesize that *C. difficile* may initiate its association with the colonic mucosa by producing low amounts of toxin A, which could facilitate the first steps of colonization. Indeed, the alteration of plasma membrane component distribution between distinct surface domains of TcdA-treated Caco-2 cells, together with the previously mentioned *C. difficile* preference for the BL membrane, suggests that subversion of host cell polarity might act as a key mechanism and lead to increased bacterial binding. This notion might also explain why in vivo adhesion of an

![Figure 5](image-url)
Figure 6. Effect of TcdA on 630 *Clostridium difficile* colonization of colonic mucosa. 

A. Effect of TcdA on epithelial polarity determined by transepithelial electrical resistance (TEER) and dextran flux measurements. TEER is expressed as the percentage of the baseline value (before TcdA treatment). Each value represents the mean ± standard deviation (SD; n = 3). 

B. Fully polarized Caco-2 monolayers were preincubated with indicated concentrations of TcdA for 16 hours followed by apical infection with *C. difficile* 630 strain. After 1 hour of incubation, the number of bacteria that adhered (white bar) or translocated across (black bar) the epithelium was quantified by cfu counting and expressed as cfu/100 cells. Values are the mean ± SD from 3 independent experiments performed in triplicate. **P < .01; ***P < .001. 

C. Maximum projection (extended focus) images of adherent *C. difficile* bacteria on control and TcdA-treated Caco-2 cells. Bacteria were labeled with an anti-whole bacteria serum, and a secondary fluorescent antibody (green) and cellular actin was stained with phalloidin-Alexa Fluor 568 (red). Scale bars = 10 µm.
avirulent nontoxinogenic strain is facilitated by coadministration of *C. difficile* toxins in a hamster model [35]. In this context, the elevated rate of intestinal colonization in healthy adults infected by toxigenic *C. difficile* suggests that a minimal level of toxin production could facilitate the colonization state without the occurrence of clinical symptoms [36, 37]. On the contrary, toxin B, in addition to its expected influence on bacterial translocation rates, does not play a role in the establishment of the AP surface association, which is consistent with its inability to modulate epithelial fence function.

Segregation of AP and BL receptors in the polarized epithelium and compartmentalization of different cytoplasmic and membrane-associated signaling molecules are crucial for regulation of an innate immune response against pathogens and for prevention of unrestrained or prolonged inflammation. Under normal healthy circumstances, several Toll-like receptors (TLRs), such as TLR5, which recognize pathogen-associated molecular patterns (PAMPs) and activate innate immune pathways, are physically separated from the luminal content. This allows epithelial cells to mount a rapid proinflammatory response only if surface or secreted components from commensal or pathogenic bacteria have breached the epithelial barrier [38]. Therefore, alterations in cell polarity induced by *C. difficile* toxins may allow PAMPs to access the BL surface and trigger the production of inflammatory cytokines and chemokines. Activation of different TLRs, including TLR4 and TLR5, by several *C. difficile* components (ie, surface layer proteins and flagellin) is known to play a pivotal role in determining the final outcome of infection [39, 40]. Notably, Yoshino and colleagues [40] recently demonstrated the contribution of clostridial toxins in this process, showing that breaching of epithelial barrier function by *C. difficile* toxin might lead to more severe inflammation present at the site of infection. Therefore, as postulated in this study, modulation of cell architecture by TcdA may not only facilitate colonization of the gut mucosa but also play a role in landscaping the immune response.

Taken together, our concept of *C. difficile* pathogenesis, which combines current knowledge with the results obtained in this study and depicted in Figure 7, proposes that different local levels of *C. difficile* toxins, as well as the time of exposure, might serve to fine-tune the cell epithelial barrier, ranging from mild alterations of its polarity to induction of acute inflammation, apoptotic signaling, and cell death. This specific modulation of mucosal organization and function might facilitate *C. difficile* settlement in the human gut and development of chronic and persistent infection. In conclusion, we suggest TcdA-mediated subversion of epithelial polarity as a novel strategy used by *C. difficile* to enhance its ability to reside in a very competitive environment of the human gut.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

*Acknowledgments.* We thank Caroline H. Damsky (University of California, San Francisco, College of Dentistry, Department of Cell and Tissue Biology) for providing us with A2I8 monoclonal antibody, which was obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biology. We are grateful to Matthew Bottomley for critical reading of the manuscript.

*Financial support.* This work was supported by internal funding from Novartis Vaccines and by the European Community’s Seventh Framework.
10. Lyras D, O
11. Kuehne SA, Cartman ST, Minton NP. Both, toxin A and toxin B, are
14. Popoff MR, Geny B. Rho/Ras-GTPase-dependent and -independent ac-
1104
References
Programme “CLOSTNET” [PEOPLE - Initial Training Network, PEOPLE-ITN-2008-237942]. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Potential conflicts of interest. The authors do not have a commercial or other association that might pose a conflict of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

14. Vllasaliu D, Fowler R, Garnett M, Eaton M, Stolnik S. Barrier character-
19. Villassaldu D, Fowler R, Garnett M, Eaton M, Stolnik S. Barrier character-
32. Chen ML, Pothoulakis C, LaMont JT. Protein kinase C signaling regulates ZO-1 translocation and increased paracellular flux of T84 colonocytes exposed to Clostridium difficile toxin A. J Biol Chem 2002; 277: 4247–54.