IAI Journals.ASM.org

Host Immunity to *Clostridium difficile* PCR Ribotype 017 Strains

Nazila V. Jafari,[a] Mario Songane,[a] Richard A. Stabler,[b] Mamoun Elawad,[c] Brendan W. Wren,[b] Elaine Allan,[d] Mona Bajaj-Elliott[a]

Infection, Immunity, Inflammation and Physiological Medicine Programme, Institute of Child Health, University College London, London, United Kingdom[a]; Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, London, United Kingdom[b]; Gastroenterology Department, Great Ormond Street Hospital, London, United Kingdom[c]; Research Department of Microbial Diseases, Eastman Dental Institute, University College London, London, United Kingdom[d]

Clostridium difficile **is an important nosocomial pathogen and the leading cause of antibiotic-associated diarrhea. Multilocus sequence typing indicates that *C. difficile* strains belong to five distinct genetic clades encompassing several PCR ribotypes (RT). Since their emergence in 2003, hypervirulent RT027 strains have been a major focus of research; in contrast, our current understanding of RT017-mediated disease pathogenesis lags far behind. In this study, we aimed to characterize host immunity to CF5 and M68, two genetically well-defined RT017 strains. Both strains engaged with host Toll-like receptor 2/6 (TLR2/6), TLR2-CD14, and TLR5 to similar extents in a model cell line. Despite this, CF5 mediated significantly greater dendritic cell (DC) interleukin-12 (IL-12), IL-27, and IL-10 immunity than M68. Both strains elicited similar IL-1β mRNA levels, and yet only M68 caused a marked increase in secretory IL-1β. A CF5 cocultured-DC cytokine milieu drove an equipotent Th1 and Th17 response, while M68 promoted greater Th17 immunity. Human gastrointestinal *ex vivo* cytokine responses to both strains were characterized. Taken together, our data suggest that *C. difficile* strains mediate overlapping and yet distinct mucosal and DC/T cell immunity. Finally, toxin-driven IL-1β release supports the hypothesis that this cytokine axis is a likely target for therapeutic intervention for *C. difficile* infection.**

Clostridium difficile, a Gram-positive, spore-forming anaerobe, is the leading cause of hospital and community-acquired diarrhea in the elderly (1–3). *C. difficile* infection (CDI) mediates a spectrum of clinical symptoms ranging from mild diarrhea to fatal pseudomembranous colitis (4). CDI often occurs following broad-spectrum antibiotic treatment, an intervention that causes dysbiosis of the intestinal microbiota allowing *C. difficile* endospores to germinate and grow (5). Restoration of the biodiversity of the bacterial microbiota is one promising therapeutic avenue currently being explored for CDI (6, 7).

In the last decade, the global incidence of CDI has increased dramatically due to the emergence and spread of a number of PCR ribotypes (RT) (8). Although the increased rates of CDI have been primarily attributed to RT027, other emerging PCR ribotypes, such as RT001, RT017, and RT078, have been implicated in recent outbreaks which, in common with CDI due to RT027 strains, also show an increase in disease severity (1, 9, 10). RT027 strains produce three toxins, including two monoglucosylating exotoxins—toxin A (TcdA) and toxin B (TcdB)—and a binary toxin (CDT) with ADP-ribosylating activity (11, 12). Most pathogenic strains produce TcdA and TcdB; however, due to deletions and/or insertions in the *tcdA* gene, some strains release only a functional TcdB (13). The first A^- B^+ strain reported was 8864 (toxinotype X) that contains a 5.9-kb deletion plus a 1.1-kb insertion that disrupts TcdA production (14). Serogroup F strains (toxinotype VIII; RT017) were the second A^- B^+ group to be identified (15, 16). RT017 strains represent a lineage of clinical significance (17, 18) since they have been responsible for CDI outbreaks in many countries, including the United States (19), Ireland (20), Netherlands (21), Germany (22), and China (23, 24). Murine studies by Lawley et al. highlight how antibiotic treatment inadvertently promotes RT017 M68 carriers to become super spore shredders enhancing host-to-host transmission (5). Despite increasing appreciation of the disease-causing potential of RT017 toxin A-negative lineage, our understanding of the interaction of this lineage with the host lags behind the well-studied RT027 strains.

In the present study, we characterized host immunity to strain CF5, which was isolated in Belgium in 1995 from an asymptomatic patient (19), and strain M68, which was isolated in 2003 during a large CDI outbreak in Ireland (20). Interestingly, whole-genome sequencing indicates that *C. difficile* CF5 and M68 (RT017 strains that emerged 8 years apart) occupy a distinct phylogenetic lineage (25). The availability of genetic information made CF5 and M68 the strains of choice for further investigation in this study. Both strains elicited similar cytokine responses in HEK293 cells, a model cell line stably transfected with either one or two Toll-like receptor (TLR) genes, suggesting similar engagement with this family of pattern recognition receptors (PRRs). Despite this similarity, the two strains mediated an overlapping and yet distinct cytokine milieu in murine bone marrow-derived dendritic cells (BMDCs); this was particularly evident for bacterium-driven interleukin-1β (IL-1β) immunity. The infected BMDC cytokine milieu yielded an equipotent Th1 but significantly divergent Th17 axis in response to the two infectious agents. The two strains showed marked variation in their toxin-secreting capacities despite 100% sequence identity in the toxin gene locus (25). Overall, our study raises the hypothesis that *C. difficile* RT017 strains exert virulence by targeting the host IL-1β axis, leading to greater cytotoxicity, cytokine release, and potent Th17 immunity—cellular events that may contribute to immunopathology seen in *C. difficile*-mediated diseases.

Received 9 September 2014 **Accepted** 10 September 2014
Published ahead of print 15 September 2014
Editor: S. R. Blanke
Address correspondence to Mona Bajaj-Elliott, m.bajaj-elliott@ucl.ac.uk.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.02605-14.

doi:10.1128/IAI.02605-14

MATERIALS AND METHODS

Ethics statement. Ethical approval for obtaining mucosal biopsy specimens from patients undergoing routine endoscopic procedure was granted by the Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee (06/Q0508/26). Written informed consent was provided by the legal guardians of the study participants.

C57BL/6 wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the Institute of Child Health animal facilities. Approval for animal studies was obtained from University College London Ethics Committee (70/7326). All experiments were performed according to the United Kingdom Home Office guidelines.

Reagents. Brain heart infusion (BHI) agar and broth, *C. difficile* selective supplement, and defibrinated horse blood were purchased from Oxoid, Basingstoke, United Kingdom. Cysteine, NCTC-135 medium, gentamicin, red blood cell lysing buffer, and lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 were obtained from Sigma-Aldrich, Poole, United Kingdom. Dulbecco modified Eagle medium (DMEM) with GlutaMAX-I, Iscove modified Dulbecco medium (IMDM), Roswell Park Memorial Institute (RPMI) 1640, phosphate-buffered saline (PBS), trypsin-EDTA, 2-mercaptoethanol, and recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) were obtained from Invitrogen/Gibco, Paisley, United Kingdom. All cell culture media were supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin solution, and 1% nonessential amino acids (also obtained from Invitrogen/Gibco).

Cells transfected with TLR2/1, TLR2/6, TLR5, and TLR9 were grown in complete DMEM supplemented with 100 μg of normocin/ml and 10 μg of blasticidin S/ml (eBioscience, Hatfield, United Kingdom). Blasticidin S was replaced with puromycin (510 μg/ml) for the TLR2-CD14 and TLR4-CD14 cells.

Primers for cytokine mRNA detection were from Eurofins MEG Operon, Ebersberg, Germany (26), and SYBR green JumpStart *Taq* ReadyMix was from Sigma-Aldrich. Gene expression of IL-23 p19 subunit was analyzed by using *Taq*Man probe-based PCR from Applied Biosystems.

Bacterial strains and growth conditions. *C. difficile* strains CF5 and M68 ($TcdA^-$ $TcdB^+$ CDT^-, RT017) (19, 20) were cultured on BHI agar supplemented with 5% defibrinated horse blood or preequilibrated BHI broth containing *C. difficile* selective supplement and 0.05% cysteine. All bacterial cultures were grown in an anaerobic chamber (Don Whitley Scientific, Shipley, United Kingdom) in an atmosphere of 10% CO_2, 10% H_2, and 80% N_2 at 37°C. In coculture studies, bacterial cultures were grown by inoculating preequilibrated BHI broth with a single colony grown on a BHI agar plate. A stationary-phase bacterial culture was used to infect a particular cell line at a predetermined multiplicity of infection (MOI). To prepare the supernatants used in the cytotoxicity assay, a bacterial culture was pelleted by centrifugation (10,000 × *g*, 15 min) and then filter sterilized using 0.22-μm-pore-size filter.

Cell cytotoxicity assay. African green monkey kidney cells (Vero cells, ATCC CCL-81) were seeded at a concentration of 0.5×10^6/ml. Confluent cells were cocultured with 2-fold serially diluted filter-sterilized bacterial supernatants. The cytopathic effect (CPE) was determined by comparing infected cells to uninfected control cells and was scored on a scale of 0 to 4. The endpoint was determined as the last dilution that caused 100% or scale 4 CPE.

***C. difficile*-TLR engagement.** Human TLR-transfected-HEK293 cells were kindly provided by David Guiliano (University College London, United Kingdom). Cells were seeded in duplicate at a density of 3.5×10^4/ml in 96-well plates and cocultured with bacteria at an MOI of 10. Specific ligands for each TLR-transfected cell were used as a positive control (see Fig. S1 in the supplemental material). At 8 h postculture, secreted IL-8 (a marker for TLR-mediated NF-κB activation) was quantified by using an enzyme-linked immunosorbent assay (ELISA).

Generation and coculture of murine BMDCs. Bone marrow from the femurs and tibias of C57BL/6 WT mice was flushed with PBS–2% fetal calf serum containing 10 μg of gentamicin/ml. The cell population was depleted of red blood cells by using 1 ml of red blood cell lysing buffer/pair of legs. Cells were resuspended in complete IMDM containing 50 μM 2-mercaptoethanol, gentamicin at 10 μg/ml, and GM-CSF at 20 ng/ml. Cells were washed and resuspended in complete RPMI 1640 without antibiotics and seeded at a density of 10^6/ml prior to coculture with *C. difficile* strains prepared as described above (MOI of 10).

ELISA. Tissue and cell culture supernatant cytokine protein secretion was measured using ELISA kits (eBioscience) according to the manufacturer's instructions. Pro-IL-1β release in cell lysates (treated with NP-40 lysis buffer [Abcam, Cambridge, United Kingdom]) and supernatants was assessed by ELISA (eBioscience). CF5 and M68 bacterial supernatants were obtained by filter sterilization as described above, and secreted TcdB levels were quantified by ELISA (TechLab, Orlando, FL) according to the manufacturer's instructions.

T cell proliferation assay. Splenocytes were harvested from WT mice by passing the splenic contents through a cell strainer (70 μm; BD Biosciences, Hatfield, United Kingdom). Cells were washed with PBS, and red blood cells were depleted by using red blood cell lysing buffer. Next, splenocytes at 0.5×10^6/ml (in PBS) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience) to a final concentration of 10 μM. Dynabeads mouse T-activator CD3/CD28 were added to CFSE-labeled splenocytes at a bead/cell ratio of 1:5 and then cocultured with *C. difficile*-stimulated BMDCs in a 96-well plate at a DC/splenocyte ratio of 1:10 at 37°C for 72 to 96 h. Cells were costained with anti-mouse CD4–PE-Cy5 (eBioscience) and analyzed by flow cytometry gated on $CD4^+$ cells.

IVOC. Colonic pinch biopsy specimens from individuals ($n = 30$; mean age, 10.4 ± 4.7 [the standard deviation]) undergoing routine endoscopy for gastrointestinal (GI) symptoms (e.g., constipation and allergy) were obtained. Macroscopically uninflamed tissue was oriented with the mucosal surface upward and mounted on sterile foam supports in 12-well plates. The foams were saturated with *in vitro* organ culture (IVOC) media consisting of complete DMEM supplemented at a ratio of 1:1 with NCTC-135 medium (27). The explants were inoculated with 5×10^8 *C. difficile* cells at 37°C in 5% CO_2 humidified incubator for 3 to 6 h.

Statistical analysis. Statistical analysis was performed by using a two-way analysis of variance (ANOVA), followed by a Bonferroni posttest. A nonparametric *t* test (Mann-Whitney U test) was performed on data from *ex vivo* cocultures. The data were considered significant if P was <0.05 as determined using Prism version 5.00 (GraphPad, San Diego, CA).

RESULTS

CF5 and M68 RT017 cross talk with human TLRs. Prior to the commencement of coculture studies, bacterial growth kinetics and survival in aerobic conditions were investigated. No significant difference between CF5 and M68 strains was observed in these assays, indicating that a comparison of host interactions elicited by these two infectious agents was a viable option (see Fig. S2 and S3 in the supplemental material). In addition, the effects on host cell cytotoxicity were similar in response to both CF5 and M68 up to 8 h postexposure (see Fig. S4 in the supplemental material). Finally, since bacterial cocultures induced significantly more IL-8 than filter-sterilized supernatants (see Fig. S5 in the supplemental material), all experiments were performed with bacterial cocultures at a defined MOI.

The potential engagement of strains CF5 and M68 with human TLRs was determined by coculturing stably transfected HEK293 cells expressing individual TLRs and CD14 (a coreceptor) or a combination of TLRs, involved in bacterial recognition (Fig. 1). Although the TLR4-CD14 complex is the receptor for bacterial LPS, *C. difficile* surface layer proteins have been implicated as potential ligands for the TLR4 receptor (28). Both strains mediated statistically significant IL-8 secretion in TLR2/6- and TLR2-

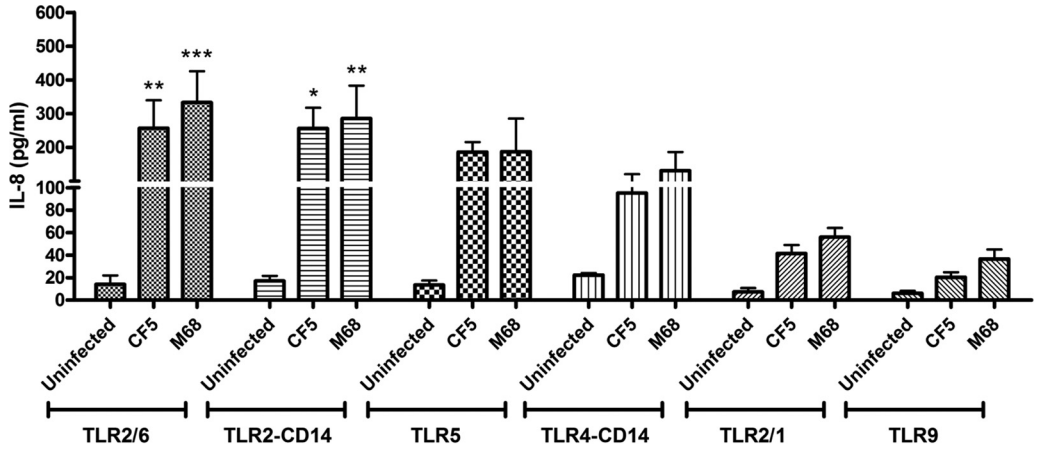

FIG 1 *C. difficile* RT017 CF5 and M68 mediate TLR activation and IL-8 production. HEK293 cells expressing homo- or heterologous TLRs were cocultured with RT017 CF5 and M68 (MOI = 10) for 8 h. Postinfection, IL-8 was quantified by ELISA. The data represent the means ± the standard errors of the means (SEM) from three independent experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (significant differences from uninfected cells). *P* values were obtained by using ANOVA, followed by a Bonferroni posttest analysis.

CD14-transfected cells, and no significant difference between the two was observed. TLR5 engagement was similar to that of TLR4. Interaction with the TLR2/1 heterodimer and the TLR9 receptor was the least potent. Overall, no significant difference in IL-8 release was noted between CF5- and M68-mediated TLR activation, suggesting that they engage with this family of PRRs to similar extents.

Defining CF5- and M68-mediated effects on murine BMDC activation. CF5- and M68-mediated effects on DC/T cell outcome were investigated in the murine system since it provides a genetically homogenous model. Modulation of BMDC cell surface markers in response to CF5 and M68 was examined. Both strains induced similar increases in HLA-DR, although strain-specific effects on CD80, CD86, and CD40 were recorded (see Fig. S6 in the supplemental material).

The time-dependent effect of infection on BMDC cytokine gene and protein expression was investigated. IL-12 family members, including IL-12, IL-23, and IL-27, are critical determinants of downstream T cell response(s). Heterodimeric IL-12 comprises subunits p35/p40, IL-23 p19/p40, and IL-27 p28/EBI3. Both strains induced p35, p40, p19, and p28 mRNA expression in a time-dependent manner (Fig. 2A to D). CF5 mediated a significantly greater p35 and p28 response than M68 at 6 h postinfection (Fig. 2A and D). Both strains expressed comparable levels of EBI3 mRNA (Fig. 2E), and the strains were similar in elicitation of IL-10 mRNA for the first 6 h postinfection with divergence noted at 8 h (Fig. 2F). Similar profiles for pro-IL-1β gene expression were observed in response to both strains (Fig. 2G).

Strain CF5 caused a significant increase in IL-12 and IL-27 mRNA (Fig. 2A and D) and protein levels (Fig. 3A and B). CF5 also mediated a potent IL-10 response compared to the M68 strain (Fig. 3C). Interestingly, both strains induced similar amounts of IL-23 and tumor necrosis factor alpha (TNF-α) (Fig. 3D and E).

Although no difference in pro-IL-1β gene expression was observed (Fig. 2G), M68 mediated a marked release of IL-1β compared to the CF5 strain (Fig. 3F). Since the ELISA utilized was unable to distinguish between pro-IL-1β and cleaved IL-1β, an ELISA specific for pro-IL-1β was performed (Fig. 3G). Significantly greater pro-IL-1β in CF5-infected versus M68-infected cell lysates was measured, with no detectable presence of the precursor in the supernatants (data not shown). Taken together, these data suggested that CF5 and M68 mediated similar levels of pro-IL-1β gene and protein expression and that the loss of cellular pro-IL-1β in response to M68 paralleled an increase in secreted cleaved cytokine (Fig. 3F).

RT017 CF5 and M68 show significant variation in TcdB secretion. *C. difficile* TcdA and TcdB are known inflammasome activators, a cellular pathway that culminates in IL-1β secretion and pyroptosis (26, 29). Since CF5 and M68 TcdBs share 100% predicted sequence identity (25), a difference in toxin secretion offered an alternative explanation for the observed differential effect on BMDC IL-1β release. We confirmed the lack of TcdA protein expression by the two strains (data not shown). The presence of secretory TcdB in CF5 and M68 bacterial supernatants was investigated by using a TOX A/B test (Fig. 4A). The M68 strain secreted significant amounts of toxin; in contrast, CF5 TcdB was not detectable by this assay. Next, the functional properties of TcdB were examined by measuring its cytotoxicity potential on Vero cells, which are known to be TcdB sensitive (30). Although undetectable by ELISA (Fig. 4A), CF5 TcdB cytotoxicity was recorded, suggesting the presence of low levels of active toxin (Fig. 4B). Collectively, these experiments indicated that in addition to TcdB tertiary structure, a strain's toxin secretory capacity also has an impact on its intoxication potential.

RT017 CF5 and M68 generate differential T cell immunity. The T cell proliferative capacity and effector function in response to paraformaldehyde (PFA)-fixed CF5- and M68-stimulated BMDCs were studied. Infected BMDCs were cocultured with CFSE-labeled splenocytes in the presence of anti-CD3/CD28 (31). At 96 h after coculture, CF5- and M68-mediated T cell proliferation was quantified, and similar increases between the two were recorded (Fig. 5A and B). Both infectious agents also caused an increase in IFN-γ-expressing T cells, with CF5 showing a trend of greater increase. In contrast, strain M68 caused a significant increase in IL-17A-expressing $CD4^+$ T cells (see Fig. S7B in the supplemental material) and IL-17A induction compared to CF5 (Fig. 5D). Levels of IL-10 production

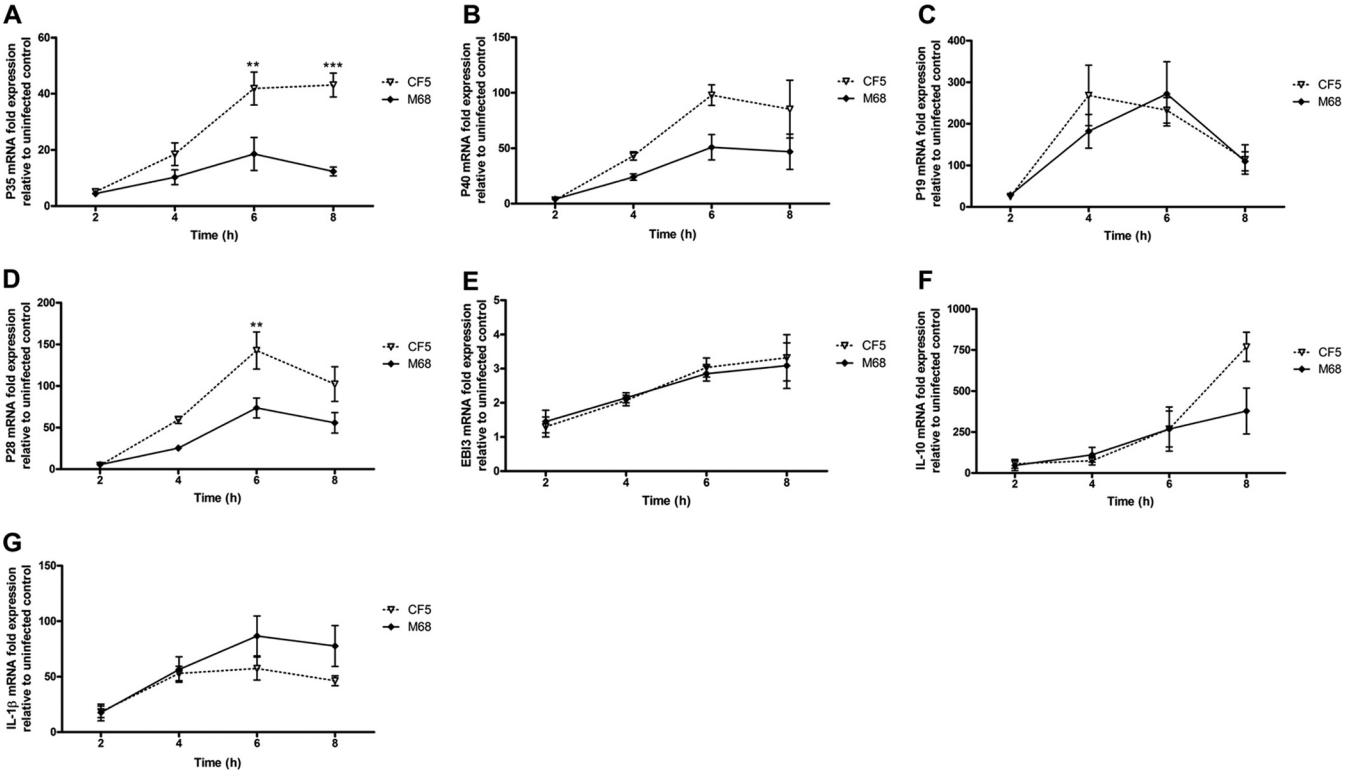

FIG 2 Time-dependent effect of *C. difficile* RT017 CF5 and M68 on BMDC cytokine mRNA expression. BMDCs were stimulated with CF5 and M68 at an MOI of 10, and the mRNA expression of IL-12 family members—p35 (A), p40 (B), p19 (C), p28 (D), EBI3 (E) IL-10 (F), and IL-1β (G)—was quantified by real-time PCR. The data are presented as the fold increase compared to expression in uninfected control cells. The data represent the means ± the SEM from three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (significant interstrain differences). *P* values were obtained by using ANOVA with a Bonferroni posttest analysis.

were similar in response to both infectious agents (Fig. 5E), whereas strain CF5 showed a greater propensity for IL-4 (Fig. 5F) and IL-2 (Fig. 5G). Significant differences in T cell IL-17A, IL-4, and IL-2 levels emphasized the capacity of *C. difficile* to fine-tune host immunity in a strain-specific manner.

***Ex vivo* mucosal cytokine responses to *C. difficile* strains CF5 and M68.** To improve our understanding of the human mucosal cytokine milieu generated in response to strains of RT017, colonic tissue biopsy specimens were cocultured with strains CF5 and M68 for 6 h. In control uninfected tissue, IL-6 was detectable (median, ~80 pg/ml), and coculture caused an ~2-fold increase (Fig. 6A). Both infections elicited similar significant increases in IL-8 (Fig. 6B). Interestingly, as noted in stimulated BMDCs (Fig. 3F), M68 elicited significant IL-1β secretion compared to strain CF5 (Fig. 6C). It is also worth noting that M68 showed a trend toward greater IFN-γ levels, whereas IL-17A release reached statistical significance (Fig. 6D and E). Both strains mediated similar IL-22 responses (Fig. 6F). Collectively, both strains caused significant increase in mucosal cytokine production within the first few hours of coculture. Elicitation of similar IL-6, IL-8, IFN-γ, and IL-22 levels but varied IL-1β and IL-17A levels suggests that the GI mucosal immune system can sense and respond to *C. difficile* infection in a strain-specific manner.

DISCUSSION

CDI is a significant nosocomial pathogen that in recent years has also been increasingly implicated in community-acquired infection (3, 32, 33). CDI constitutes a global health burden, exacerbated by the recent emergence of more-virulent strains that exhibit increased resistance to antibiotics and promote greater disease severity (34–36).

In the present study, we focused on *C. difficile* RT017 and chose strains CF5 and M68 as two representatives primarily because of the availability of their genome sequences. In addition, the two strains offered an opportunity to investigate the role of a single toxin (TcdB) in the context of a coculture, and it is important to note that most studies to date have investigated the effects of purified or recombinant TcdB on the host. TLRs are major innate PRRs (37); the data implicate TLR4 and TLR5 in *C. difficile* recognition and host defense (28, 38). At present, the potential engagement of other TLR members with *C. difficile* remains ill defined. We observed significant bacterial engagement with TLR2/6, TLR2-CD14, and TLR5, whereas engagement with TLR4-CD14, TLR2/1, and TLR9 was less potent (Fig. 1). This series of experiments suggested that CF5 and M68 strains interact with human TLRs to similar extents.

The role of *C. difficile* in mediating DC activation and maturation and subsequent T cell immunity was investigated. Cocultures mediated similar increases in major histocompatibility complex class II expression; interestingly, the effect on CD80, CD86, and CD40 expression varied among the two agents. Overall, infections led to overlapping and yet distinct BMDC cytokine profiles. Strain CF5 mediated significantly greater IL-12, IL-27, and IL-10

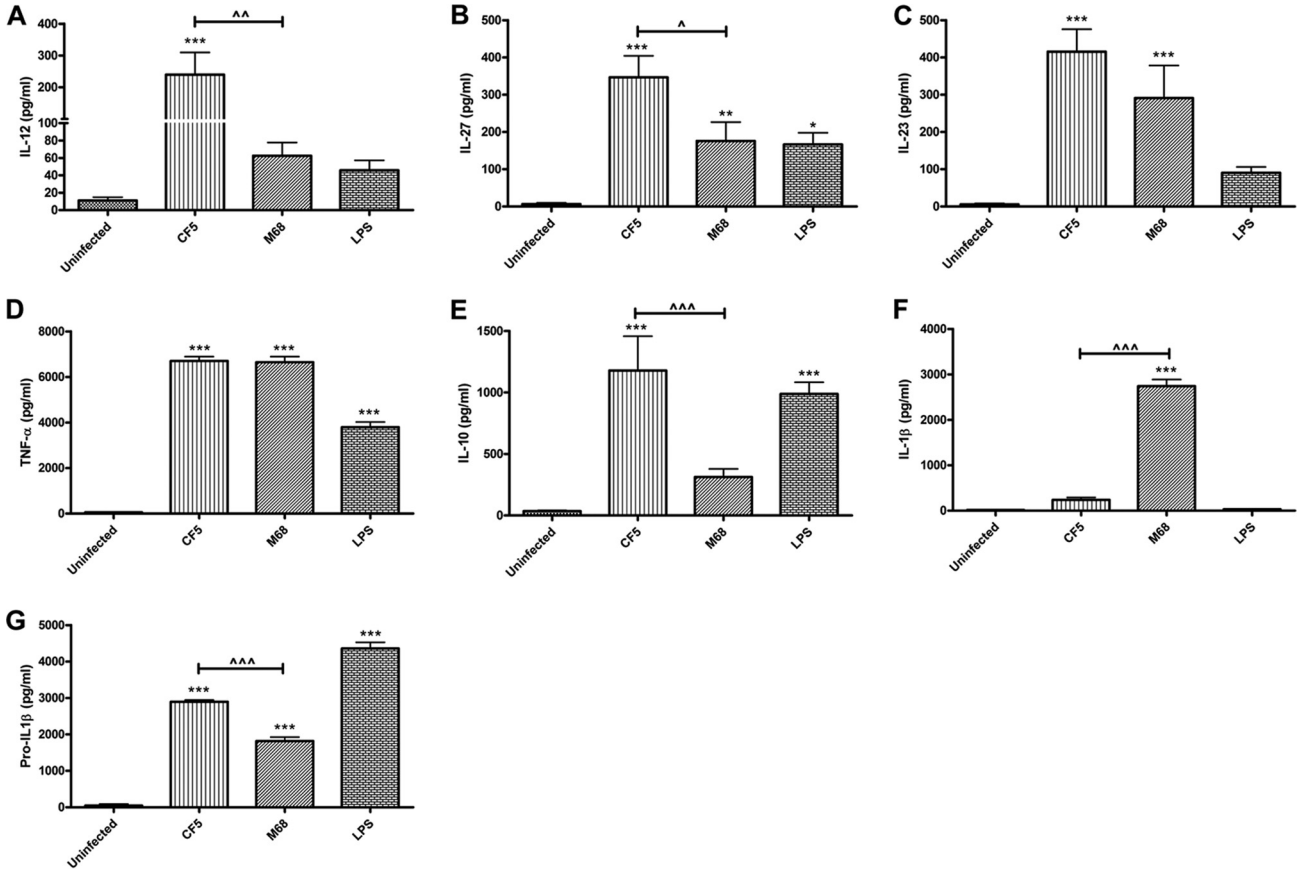

FIG 3 *C. difficile* RT017 CF5- and M68-mediated effects on BMDC cytokine production. BMDCs were stimulated with CF5 and M68 (MOI = 10) for 8 h. IL-12 (A), IL-27 (B), IL-23 (C), TNF-α (D), IL-10 (E), IL-1β (F), and cellular pro-IL-1β (G) were measured by ELISA. Pro-ILβ was undetectable in the cell culture supernatants. LPS at 1 μg/ml served as a positive control. The data represent the means ± the SEM of three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (significant differences from uninfected cells). ^, $P < 0.05$; ^^, $P < 0.01$; ^^^, $P < 0.001$ (significant interstrain differences). *P* values were obtained by using ANOVA, followed by a Bonferroni posttest analysis.

(Fig. 3A to C), whereas both CF5 and M68 drove an equipotent IL-23 and TNF-α response (Fig. 3D and E).

Among the cytokines tested, M68-mediated effects on IL-1β release were the most significant. CF5 caused minimal IL-1β secretion, whereas M68 was a very potent inducer (Fig. 3F). CF5 and M68 TcdB share complete sequence identity; in light of this knowledge, the differential IL-1β response was intriguing and led us to hypothesize that the amount of toxin secreted may be a key

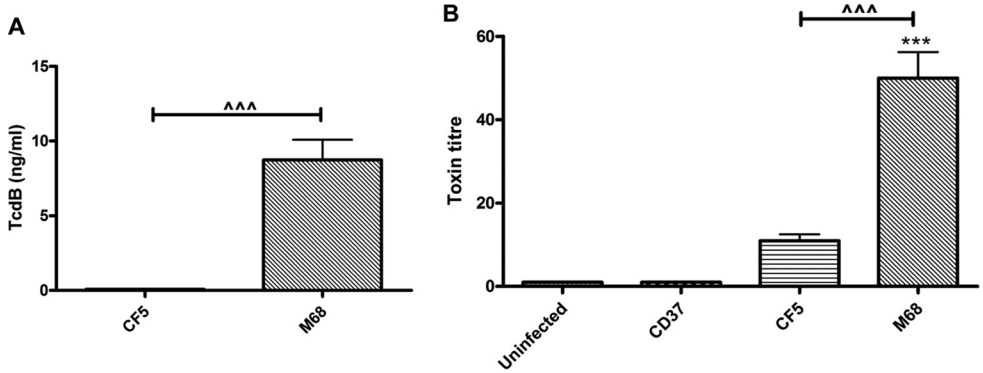

FIG 4 Secretion and cytotoxic potential of *C. difficile* RT017 CF5 and M68 TcdB. (A) Stationary-phase CF5 and M68 culture supernatants were filtered, and TcdB protein was measured by using the TOX A/B test. The data represent means ± the SEM from four independent experiments. (B) Confluent Vero cells were cocultured with serial dilutions of bacterial supernatants, and cytotoxicity was scored at 8 h postinfection. CD37, a nontoxigenic strain, served as a control. The data are presented as the means ± the SEM of three independent experiments performed in duplicate. ***, $P < 0.001$ (significant difference compared to uninfected controls); ^^^, $P < 0.001$ (a significant interstrain difference). *P* values were obtained by using ANOVA with a Bonferroni posttest analysis.

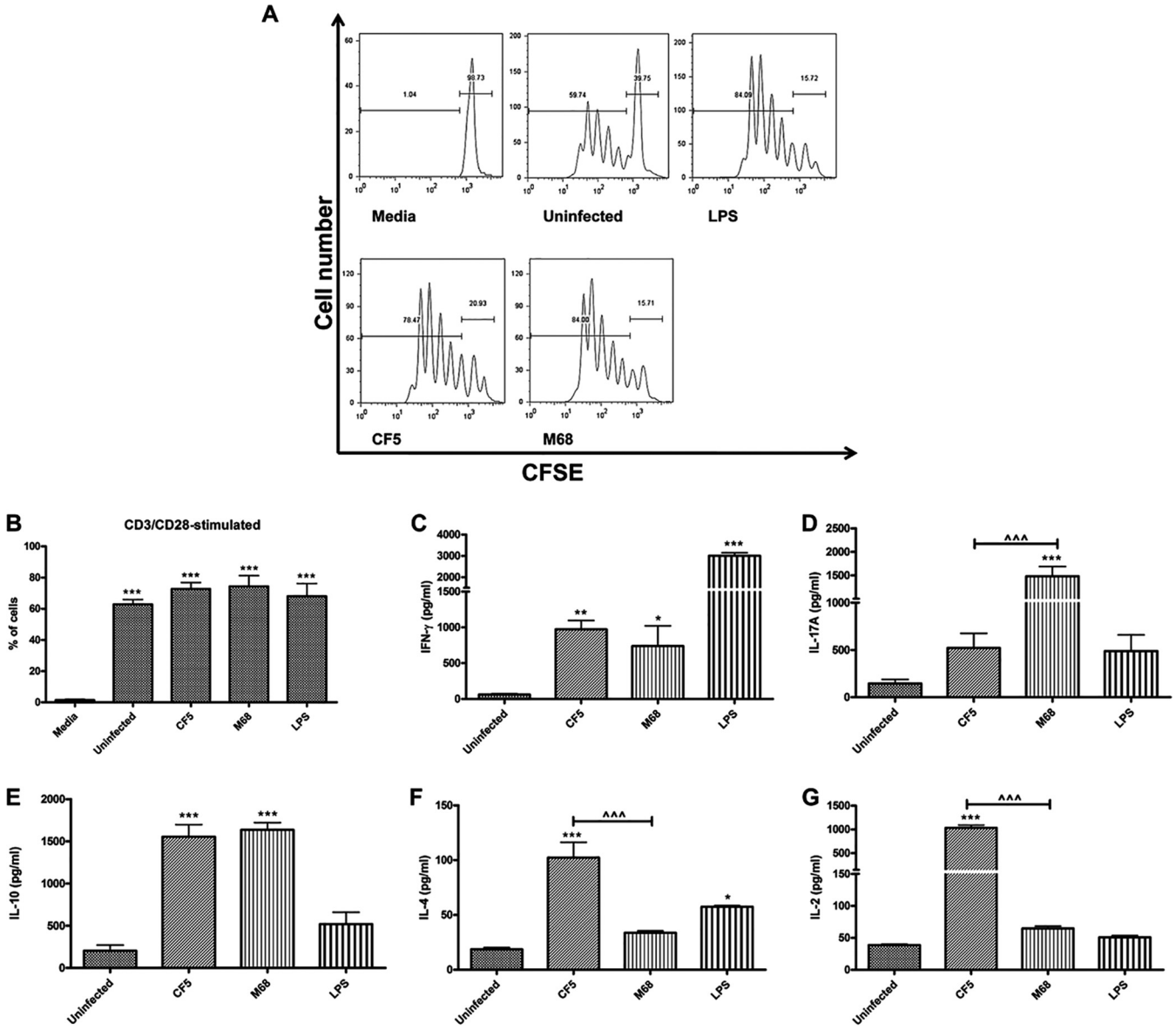

FIG 5 *C. difficile* RT017 CF5- and M68-mediated T cell proliferation and cytokine immunity. (A and B) WT BMDCs were stimulated with PFA-fixed *C. difficile* at an MOI of 50. At 24 h postinfection, stimulated BMDCs were cocultured with CFSE-labeled splenocytes in the presence of anti-CD3/CD28 and medium. At 96 h poststimulation, T cell proliferation was quantified by gating on $CD4^+$ T cells. (A) A representative flow cytometric plot is shown. (B) The data are representative of three independent experiments. Quantification is presented as the percentages of proliferating cells. The data represent the means ± the SEM of three independent experiments. ***, $P < 0.001$ (a significant difference compared to control media). (C to G) The secretion of cytokines IFN-γ (C), IL-17A (D), IL-10 (E), IL-4 (F), and IL-2 (G) was measured by ELISA. LPS at 1 μg/ml was used as a reference stimulus. The data represent the means ± the SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.0$; ***, $P < 0.001$ (significant differences compared to uninfected control cells). ^, $P < 0.05$; ^^, $P < 0.01$ (significant interstrain differences). *P* values were obtained by using ANOVA with a Bonferroni posttest analysis.

determinant impacting on the inflammasome axis and IL-1β levels. This was indeed the case since M68 produced significant amounts of TcdB, whereas CF5 TcdB was undetectable by ELISA. Interestingly, CF5 TcdB intoxication was detectable in a Vero cell cytotoxicity assay, indicating that the cytotoxicity assay is more sensitive than the currently commercially available ELISA kit for toxin quantification. Our observations indicated that CF5 is markedly impaired in its ability to secrete TcdB compared to the M68 strain, suggesting that strains with identical TcdB protein sequences may not necessarily exhibit equivalent intoxication potentials.

Understanding the bacterium-driven host IL-1β axis is crucial because this cytokine is a pleiotropic immune mediator that contributes to neutrophil recruitment during CDI (39). One may propose a role for this axis in the pathophysiology seen in M68 murine models of infection (40). Research on how CF5 TcdB secretion is impaired may offer insights into novel therapeutics that may target and block toxin secretion.

The potential of *C. difficile*-stimulated DCs to prime and influence T cell immunity was examined. Both strains induced strong IFN-γ immunity (Fig. 5C and F). In addition, a robust IL-17A response to M68 indicated that this strain promoted a skewed

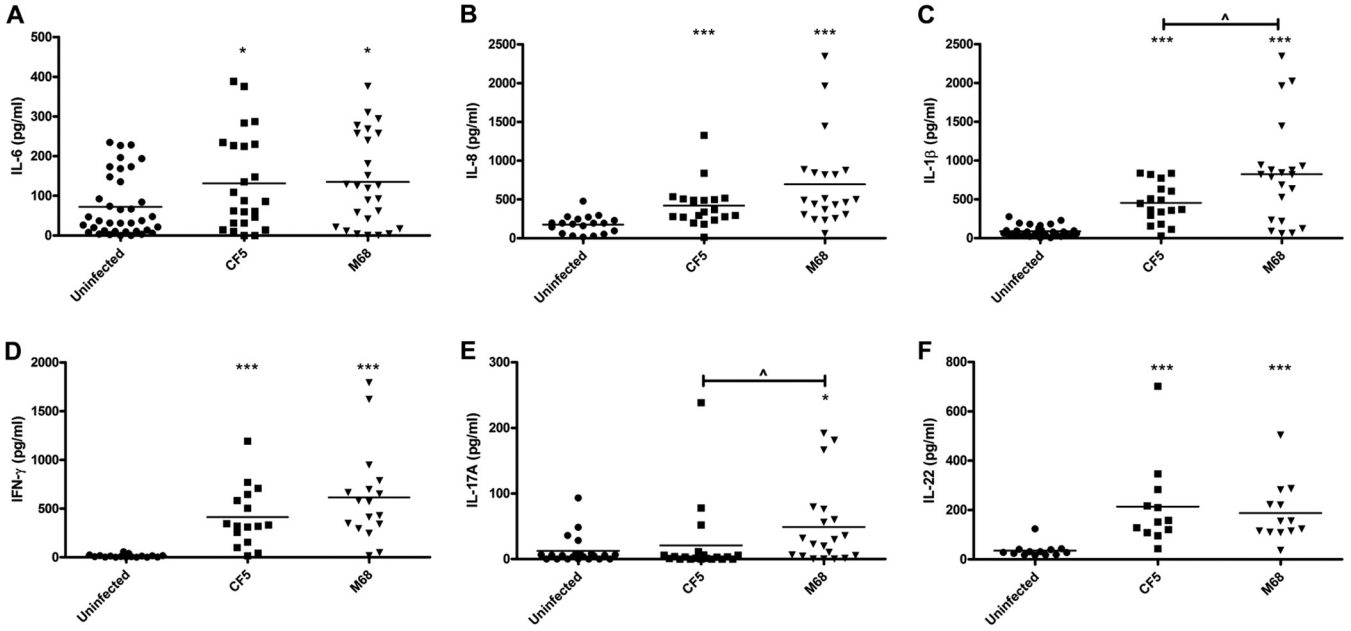

FIG 6 *Ex vivo* mucosal cytokine response(s) to *C. difficile* RT017 CF5 and M68. Macroscopically normal and matched multiple colonic biopsy specimens (30 individuals with mean age of 10.4 ± 4.7 [the standard deviation]) were cocultured with *C. difficile* CF5 and M68 (5×10^8) for 6 h. Postinfection, IL-6 (A), IL-8 (B), IL-1β (C), IFN-γ (D), IL-17A (E), and IL-22 (F) were quantified by ELISA in duplicate. The bars represent the median levels. *, $P < 0.05$; ***, $P < 0.001$ (significant differences from uninfected controls). ^, $P < 0.05$ (a significant interstrain difference). The data were analyzed by using a Mann-Whitney U test.

Th17 axis (Fig. 5D). The role of IL-1β as a Th17 differentiation factor is well established; one may therefore hypothesize that the M68-mediated BMDC IL-1β release contributes to the observed Th17 response. Interestingly, both strains mediated similar IL-23 (a Th17 effector cytokine) levels (41), adding credence to the notion that IL-1β is a likely key determinant of Th17 immunity. Future studies utilizing neutralizing antibodies should define the cytokines responsible for the observed T cell responses. Th2 immunity, as seen with an increase in IL-4 during CF5 infection (Fig. 5F), may be crucial in CDI, since IgG antibodies assist in toxin neutralization. Combinatorial T cell immunity, with specific targeting of IL-1β, is likely to be pivotal in defining immune protection versus immunopathology during CDI.

Analysis of GI mucosal immunity revealed that *C. difficile* RT017 mediated an inflammatory mucosal cytokine milieu (Fig. 6). Both strains caused significant IL-8 induction accompanied by a comparatively weak IL-17A response, suggesting that IL-8 may play a crucial role in mediating the early neutrophil recruitment necessary for containment of the infection. Interestingly, both strains caused robust induction of IL-22 (Fig. 6F). IL-22 exerts antimicrobial and regenerative effects in the GI epithelia. As seen previously for innate DC activation (Fig. 3F), M68 mediated a significant increase in mucosal IL-1β, further supporting the notion that this pathway may be an attractive target for therapeutic intervention in CDI (Fig. 6C and D).

Comparative analysis of host immunity to two RT017 strains has highlighted how this pathogen has targeted innate IL-1β, a cytokine that promotes neutrophil recruitment and Th17 immunity and pathology. Improved understanding of how phylogenetically distinct lineages interact with the host may offer potential insight(s) that may not only aid in the design of better future therapeutics but also help to reduce the emergence of highly virulent strains.

REFERENCES

1. **Rupnik M, Wilcox MH, Gerding DN.** 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat. Rev. Microbiol. **7:**526–536. http://dx.doi.org/10.1038/nrmicro2164.
2. **Deshpande A, Pasupuleti V, Thota P, Pant C, Rolston DD, Sferra TJ, Hernandez AV, Donskey CJ.** 2013. Community-associated *Clostridium difficile* infection and antibiotics: a meta-analysis. J. Antimicrob. Chemother. **68:**1951–1961. http://dx.doi.org/10.1093/jac/dkt129.
3. **Burke KE, LaMont JT.** 2014. *Clostridium difficile* infection: a worldwide disease. Gut Liver **8:**1–6. http://dx.doi.org/10.5009/gnl.2014.8.1.1.
4. **Johal SS, Hammond J, Solomon K, James PD, Mahida YR.** 2004. *Clostridium difficile* associated diarrhoea in hospitalized patients: onset in the community and hospital and role of flexible sigmoidoscopy. Gut **53:**673–677. http://dx.doi.org/10.1136/gut.2003.028803.
5. **Lawley TD, Clare S, Walker AW, Goulding D, Stabler RA, Croucher N, Mastroeni P, Scott P, Raisen C, Mottram L, Fairweather NF, Wren BW, Parkhill J, Dougan G.** 2009. Antibiotic treatment of *clostridium difficile* carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. Infect. Immun. **77:**3661–3669. http://dx.doi.org/10.1128/IAI.00558-09.
6. **Lawley TD, Clare S, Walker AW, Stares MD, Connor TR, Raisen C, Goulding D, Rad R, Schreiber F, Brandt C, Deakin LJ, Pickard DJ, Duncan SH, Flint HJ, Clark TG, Parkhill J, Dougan G.** 2012. Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing *Clostridium difficile* disease in mice. PLoS Pathog. **8:**e1002995. http://dx.doi.org/10.1371/journal.ppat.1002995.
7. **Britton RA, Young VB.** 2014. Role of the intestinal microbiota in resistance to colonization by *Clostridium difficile*. Gastroenterology **146:**1547–1553. http://dx.doi.org/10.1053/j.gastro.2014.01.059.
8. **Bartlett JG.** 2006. Narrative review: the new epidemic of *Clostridium difficile*-associated enteric disease. Ann. Intern. Med. **145:**758–764. http://dx.doi.org/10.7326/0003-4819-145-10-200611210-00008.
9. **Cheknis AK, Sambol SP, Davidson DM, Nagaro KJ, Mancini MC, Hidalgo-Arroyo GA, Brazier JS, Johnson S, Gerding DN.** 2009. Distribution of *Clostridium difficile* strains from a North American, European and Australian trial of treatment for *C. difficile* infections: 2005-2007. Anaerobe **15:**230–233. http://dx.doi.org/10.1016/j.anaerobe.2009.09.001.
10. **Hunt JJ, Ballard JD.** 2013. Variations in virulence and molecular biology

among emerging strains of *Clostridium difficile*. Microbiol. Mol. Biol. Rev. **77:**567–581. http://dx.doi.org/10.1128/MMBR.00017-13.
11. **Popoff MR, Rubin EJ, Gill DM, Boquet P.** 1988. Actin-specific ADP-ribosyltransferase produced by a *Clostridium difficile* strain. Infect. Immun. **56:**2299–2306.
12. **Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC.** 2005. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet **366:**1079–1084. http://dx.doi.org/10.1016/S0140-6736(05)67420-X.
13. **Voth DE, Ballard JD.** 2005. *Clostridium difficile* toxins: mechanism of action and role in disease. Clin. Microbiol. Rev. **18:**247–263. http://dx.doi.org/10.1128/CMR.18.2.247-263.2005.
14. **Borriello SP, Wren BW, Hyde S, Seddon SV, Sibbons P, Krishna MM, Tabaqchali S, Manek S, Price AB.** 1992. Molecular, immunological, and biological characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile*. Infect. Immun. **60:**4192–4199.
15. **von Eichel-Streiber C, Zec-Pirnat I, Grabnar M, Rupnik M.** 1999. A nonsense mutation abrogates production of a functional enterotoxin A in *Clostridium difficile* toxinotype VIII strains of serogroups F and X. FEMS Microbiol. Lett. **178:**163–168. http://dx.doi.org/10.1016/S0378-1097(99)00327-4.
16. **Rupnik M, Kato N, Grabnar M, Kato H.** 2003. New types of toxin A-negative, toxin B-positive strains among *Clostridium difficile* isolates from Asia. J. Clin. Microbiol. **41:**1118–1125. http://dx.doi.org/10.1128/JCM.41.3.1118-1125.2003.
17. **Dingle KE, Griffiths D, Didelot X, Evans J, Vaughan A, Kachrimanidou M, Stoesser N, Jolley KA, Golubchik T, Harding RM, Peto TE, Fawley W, Walker AS, Wilcox M, Crook DW.** 2011. Clinical *Clostridium difficile*: clonality and pathogenicity locus diversity. PLoS One **6:**e19993. http://dx.doi.org/10.1371/journal.pone.0019993.
18. **Stabler RA, Dawson LF, Valiente E, Cairns MD, Martin MJ, Donahue EH, Riley TV, Songer JG, Kuijper EJ, Dingle KE, Wren BW.** 2012. Macro and micro diversity of *Clostridium difficile* isolates from diverse sources and geographical locations. PLoS One **7:**e31559. http://dx.doi.org/10.1371/journal.pone.0031559.
19. **Johnson S, Sambol SP, Brazier JS, Delmee M, Avesani V, Merrigan MM, Gerding DN.** 2003. International typing study of toxin A-negative, toxin B-positive *Clostridium difficile* variants. J. Clin. Microbiol. **41:**1543–1547. http://dx.doi.org/10.1128/JCM.41.4.1543-1547.2003.
20. **Drudy D, Harnedy N, Fanning S, O'Mahony R, Kyne L.** 2007. Isolation and characterisation of toxin A-negative, toxin B-positive *Clostridium difficile* in Dublin, Ireland. Clin. Microbiol. Infect. **13:**298–304. http://dx.doi.org/10.1111/j.1469-0691.2006.01634.x.
21. **Kuijper EJ, van den Berg RJ, Debast S, Visser CE, Veenendaal D, Troelstra A, van der Kooi T, van den Hof S, Notermans DW.** 2006. *Clostridium difficile* ribotype 027, toxinotype III, the Netherlands. Emerg. Infect. Dis. **12:**827–830. http://dx.doi.org/10.3201/eid1205.051350.
22. **Arvand M, Hauri AM, Zaiss NH, Witte W, Bettge-Weller G.** 2009. *Clostridium difficile* ribotypes 001, 017, and 027 are associated with lethal *C. difficile* infection in Hesse, Germany. Euro Surveill. **14:**19403.
23. **Collins DA, Hawkey PM, Riley TV.** 2013. Epidemiology of *Clostridium difficile* infection in Asia. Antimicrob. Resist. Infect. Control **2:**21. http://dx.doi.org/10.1186/2047-2994-2-21.
24. **Huang H, Fang H, Weintraub A, Nord CE.** 2009. Distinct ribotypes and rates of antimicrobial drug resistance in *Clostridium difficile* from Shanghai and Stockholm. Clin. Microbiol. Infect. **15:**1170–1173. http://dx.doi.org/10.1111/j.1469-0691.2009.02992.x.
25. **He M, Sebaihia M, Lawley TD, Stabler RA, Dawson LF, Martin MJ, Holt KE, Seth-Smith HM, Quail MA, Rance R, Brooks K, Churcher C, Harris D, Bentley SD, Burrows C, Clark L, Corton C, Murray V, Rose G, Thurston S, van Tonder A, Walker D, Wren BW, Dougan G, Parkhill J.** 2010. Evolutionary dynamics of *Clostridium difficile* over short and long time scales. Proc. Natl. Acad. Sci. U. S. A. **107:**7527–7532. http://dx.doi.org/10.1073/pnas.0914322107.
26. **Jafari NV, Kuehne SA, Bryant CE, Elawad M, Wren BW, Minton NP, Allan E, Bajaj-Elliott M.** 2013. *Clostridium difficile* modulates host innate immunity via toxin-independent and -dependent mechanism(s). PLoS One **8:**e69846. http://dx.doi.org/10.1371/journal.pone.0069846.
27. **Hicks S, Candy DC, Phillips AD.** 1996. Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro. Infect. Immun. **64:**4751–4760.
28. **Ryan A, Lynch M, Smith SM, Amu S, Nel HJ, McCoy CE, Dowling JK, Draper E, O'Reilly V, McCarthy C, O'Brien J, Ni ED, O'Connell MJ, Keogh B, Morton CO, Rogers TR, Fallon PG, O'Neill LA, Kelleher D, Loscher CE.** 2011. A role for TLR4 in *Clostridium difficile* infection and the recognition of surface layer proteins. PLoS Pathog. **7:**e1002076. http://dx.doi.org/10.1371/journal.ppat.1002076.
29. **Ng J, Hirota SA, Gross O, Li Y, Ulke-Lemee A, Potentier MS, Schenck LP, Vilaysane A, Seamone ME, Feng H, Armstrong GD, Tschopp J, Macdonald JA, Muruve DA, Beck PL.** 2010. *Clostridium difficile* toxin-induced inflammation and intestinal injury are mediated by the inflammasome. Gastroenterology **139:**542–552. http://dx.doi.org/10.1053/j.gastro.2010.04.005.
30. **Torres J, Camorlinga-Ponce M, Munoz O.** 1992. Sensitivity in culture of epithelial cells from rhesus monkey kidney and human colon carcinoma to toxins A and B from *Clostridium difficile*. Toxicon **30:**419–426. http://dx.doi.org/10.1016/0041-0101(92)90538-G.
31. **Shi M, Lin TH, Appell KC, Berg LJ.** 2009. Cell cycle progression following naive T cell activation is independent of Jak3/common gamma-chain cytokine signals. J. Immunol. **183:**4493–4501. http://dx.doi.org/10.4049/jimmunol.0804339.
32. **Cartman ST, Heap JT, Kuehne SA, Cockayne A, Minton NP.** 2010. The emergence of "hypervirulence" in *Clostridium difficile*. Int. J. Med. Microbiol. **300:**387–395. http://dx.doi.org/10.1016/j.ijmm.2010.04.008.
33. **Freeman J, Bauer MP, Baines SD, Corver J, Fawley WN, Goorhuis B, Kuijper EJ, Wilcox MH.** 2010. The changing epidemiology of *Clostridium difficile* infections. Clin. Microbiol. Rev. **23:**529–549. http://dx.doi.org/10.1128/CMR.00082-09.
34. **Goorhuis A, Bakker D, Corver J, Debast SB, Harmanus C, Notermans DW, Bergwerff AA, Dekker FW, Kuijper EJ.** 2008. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin. Infect. Dis. **47:**1162–1170. http://dx.doi.org/10.1086/592257.
35. **Kuehne SA, Cartman ST, Minton NP.** 2011. Both toxin A and toxin B are important in *Clostridium difficile* infection. Gut Microbes **2:**252–255. http://dx.doi.org/10.4161/gmic.2.4.16109.
36. **Khanna S, Pardi DS, Aronson SL, Kammer PP, Orenstein R, St Sauver JL, Harmsen WS, Zinsmeister AR.** 2012. The epidemiology of community-acquired *Clostridium difficile* infection: a population-based study. Am. J. Gastroenterol. **107:**89–95. http://dx.doi.org/10.1038/ajg.2011.398.
37. **Wells JM, Rossi O, Meijerink M, van BP.** 2011. Epithelial crosstalk at the microbiota-mucosal interface. Proc. Natl. Acad. Sci. U. S. A. **108:**4607–4614. http://dx.doi.org/10.1073/pnas.1000092107.
38. **Jarchum I, Liu M, Lipuma L, Pamer EG.** 2011. Toll-like receptor 5 stimulation protects mice from acute *Clostridium difficile* colitis. Infect. Immun. **79:**1498–1503. http://dx.doi.org/10.1128/IAI.01196-10.
39. **Hasegawa M, Yamazaki T, Kamada N, Tawaratsumida K, Kim YG, Nunez G, Inohara N.** 2011. Nucleotide-binding oligomerization domain 1 mediates recognition of *Clostridium difficile* and induces neutrophil recruitment and protection against the pathogen. J. Immunol. **186:**4872–4880. http://dx.doi.org/10.4049/jimmunol.1003761.
40. **Buckley AM, Spencer J, Maclellan LM, Candlish D, Irvine JJ, Douce GR.** 2013. Susceptibility of hamsters to *Clostridium difficile* isolates of differing toxinotype. PLoS One **8:**e64121. http://dx.doi.org/10.1371/journal.pone.0064121.
41. **Buonomo EL, Madan R, Pramoonjago P, Li L, Okusa MD, Petri WA, Jr.** 2013. Role of interleukin 23 signaling in *Clostridium difficile* colitis. J. Infect. Dis. **208:**917–920. http://dx.doi.org/10.1093/infdis/jit277.