Protection from *Clostridium difficile* Infection in CD4 T Cell- and Polymeric Immunoglobulin Receptor-Deficient Mice

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Clostridium difficile rivals methicillin-resistant *Staphylococcus aureus* as the primary hospital-acquired infection. *C. difficile* infection (CDI) caused by toxins A and/or B can manifest as mild diarrhea to life-threatening pseudomembranous colitis. Although most patients recover fully from CDI, ~20% undergo recurrent disease. Several studies have demonstrated a correlation between anti-toxin antibody (Ab) and decreased recurrence; however, the contributions of the systemic and mucosal Ab responses remain unclear. Our goal was to use the CDI mouse model to characterize the protective immune response to *C. difficile*. C57BL/6 mice infected with epidemic *C. difficile* strain BI17 developed protective immunity against CDI and did not develop CDI upon rechallenge; they generated systemic IgG and IgA as well as mucosal IgA Ab to toxin. To determine if protective immunity to *C. difficile* could be generated in immunodeficient individuals, we infected *CD4*^{-/-} mice and found that they generated both mucosal and serum IgA anti-toxin Abs and were protected from CDI upon rechallenge, with protection dependent on major histocompatibility complex class II (MHCII) expression; no IgG anti-toxin Ab was found. We found that protection was likely due to neutralizing mucosal IgA Ab. In contrast, *pIgR*^{-/-} mice, which lack the receptor to transcytose polymeric Ab across the epithelium, were also protected from CDI, suggesting that although mucosal anti-toxin Ab may contribute to protection, it is not required. We conclude that protection from CDI can occur by several mechanisms and that the mechanism of protection is determined by the state of immunocompetence of the host.

Over 500,000 cases of *Clostridium difficile* infection (CDI) occur annually in the United States, resulting in 15,000 to 20,000 deaths (1). CDI is becoming more prevalent, in part because the Gram-positive, anaerobic spore-forming bacterium is highly resistant to common disinfectants and an increased number of aged patients are receiving antibiotics in the health care setting (2). Colonization by *C. difficile* is dependent primarily on antibiotic disruption of the intestinal microbiota and can result in asymptomatic carriage or disease.

CDI ranges from mild diarrhea to life-threatening pseudomembranous colitis and results primarily from toxins A and/or B (1). Toxins A and B are encoded by *tcdA* and *tcdB*, respectively, carried on the pathogenicity locus in *C. difficile* (3). Toxins A and B enter the cell by receptor-mediated endocytosis, where acidification of the endosome results in exposure of hydrophobic residues that insert into the membrane. Proteolytic cleavage then results in release of the N-terminal domain from the endosome and glucosylation of rho-GTPases, resulting in disruption of the actin cytoskeleton (3). Disruption of the actin cytoskeleton on the epithelial barrier of the gastrointestinal tract leads to an increase in gut permeability, inflammation, and disease (4).

Standard treatment for CDI is antibiotic therapy, commonly vancomycin or metronidazole (2). For some patients, antibiotic treatment is effective in resolving diarrhea; however, 20% of individuals will develop recurrent episodes of CDI (5). A limited number of recurrent CDI patients have been treated successfully and cured with fecal transplants, presumably by restoring colonization resistance from the microbiota (6). Vaccines are under clinical development but are currently not available for CDI.

Several studies have demonstrated a connection between the immune response and protection from recurrent CDI. In active immunity, individuals who generated IgG anti-toxin A antibody (Ab) were found to asymptotically carry *C. difficile* and not

develop CDI (7). Consistent with this idea, patients infected with BI/NAP1/027 *C. difficile* with reduced levels of serum anti-toxin B Ab had more recurrent CDI than patients with high levels of this Ab (8). Passive immunity has also been shown to be effective in hamsters, as administration of anti-toxin A and B monoclonal Abs (MAbs) protected animals from CDI-associated mortality (9). Furthermore, in humans, CDI patients passively immunized with anti-toxin A and B MAbs had decreased recurrent CDI (10). These studies collectively suggest that an Ab response to *C. difficile* can be protective against CDI. In response to these studies, toxoid vaccination has been tested in hamsters and humans. Torres et al. found that a combination of parenteral and mucosal toxoid immunization protected hamsters from CDI (11), and in humans, toxoid immunization induced an anti-toxin Ab response that correlated with decreased recurrent CDI (12, 13). However, toxoid vaccination does not affect colonization (14). Because no vaccine for CDI is available, we think that an important step in developing a vaccine is to fully understand the nature of protective immune responses to *C. difficile*.

The standard CDI animal model for many years was the Syrian hamster; unfortunately, few immunologic reagents are available to study hamsters, and more importantly, they succumb to CDI-associated mortality upon infection, making it impossible to study

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FIG 1 Protection of WT C57BL/6 mice rechallenged with *C. difficile*. (A) Percentages of initial body weight 0 to 10 days after primary infection and rechallenge. (B) Numbers of *C. difficile* CFU/g of spores in feces at 10 days postinfection (d.p.i.) and 10 days postrechallenge (d.p.r.). (C) H&E-stained colon sections (7 μ m) at 3 d.p.i. and 3 d.p.r. Magnification, $\times 100$. Data are representative of 3 independent experiments with 4 to 8 mice per group. Student's *t* test and the Mann-Whitney test were used for analysis. *, $P < 0.05$; ns, no significance.

a protective immune response to infection. On the other hand, mice can survive CDI and are protected from reinfection (15), making the mouse model ideal for examining protective immune responses to *C. difficile*. In this study, we characterized the serum and mucosal immune responses to *C. difficile* in both immunocompetent and immunodeficient mice, and we show that protection is mediated by different immune responses dependent on the level of immunocompetence of the host.

MATERIALS AND METHODS

Mice. Mice were housed in the Comparative Medicine Facility at Loyola University Chicago and treated in accordance with the Institutional Animal Care and Use Committee. $CD4^{-/-}$ and major histocompatibility complex class II knockout ($MHCII^{-/-}$) mice from Jackson Laboratories (Bar Harbor, ME), polymeric immunoglobulin receptor knockout ($pIgR^{-/-}$) mice from the University of Missouri Mutant Mouse Regional Resource Center (Columbia, MO), and C57BL/6 wild-type (WT) mice from Charles River Laboratories (Wilmington, MA) were bred in-house.

Rechallenged CDI mouse model. Mice (8 to 12 weeks old) were treated with vancomycin (0.045 mg/ml), metronidazole (0.215 mg/ml), gentamicin (0.035 mg/ml), kanamycin (0.4 mg/ml), and colistin (850 U/ml) in sterile drinking water for 72 h and then with sterile drinking water for 48 h, followed by a single intraperitoneal injection of clindamycin (10 mg/kg of body weight). After 24 h, mice were challenged with 10^5 spores of epidemic strain BI17 *C. difficile* spores (also termed NAP1 by pulsed-field gel electrophoresis) by oral gavage. Mice were monitored for disease by the presence of diarrhea, weight loss, and fecal *C. difficile* CFU counts; colon histology was examined by using hematoxylin- and eosin (H&E)-stained formalin-fixed tissue sections (7 μ m). After recovery from primary infection, mice were given an antibiotic regimen identical to that for primary infection and rechallenged with 10^5 BI17 *C. difficile* spores at 5 weeks postinfection. For long-term immunity, mice were rechallenged at 63 or 135 days postinfection. Mice were monitored for disease as described above.

Spore preparation. BI17 *C. difficile* was cultured anaerobically overnight in reduced brain heart infusion (BHI) liquid medium supplemented with L-cysteine at 37°C. *C. difficile* was plated in a lawn on reduced blood agar plates and cultured anaerobically for 5 to 7 days at 37°C to induce sporulation. *C. difficile* was collected in sterile phosphate-buffered saline (PBS) and incubated at 68°C for 2 h to rid the culture of vegetative cells. Spores were washed 3 times with PBS, and numbers of CFU were determined by culturing serial dilutions on BHI agar containing taurocholate. Spore content was confirmed by phase-contrast microscopy.

CFU measurements. For enumeration of CFU, weighed fecal samples were homogenized in sterile PBS and heated at 68°C (2 h) to kill vegetative bacteria. Homogenates were centrifuged at $1,500 \times g$ for 30 s, and fecal supernatant was serially diluted and cultured anaerobically on reduced taurocholate-cefoxitin-D-cycloserine-fructose agar (TCCFA) (16) for 48 h at 37°C.

Purification of *C. difficile* toxin. Toxigenic *C. difficile* VPI 10463 was grown under anaerobic conditions as described above for 72 h, and toxin

FIG 2 Western blot analysis of serum and mucosal anti-toxin Ab in WT C57BL/6 mice. Western blots of DEAE-enriched toxin (toxin) and non-toxicogenic *C. difficile* supernatant control (NT) were probed with serum (10 d.p.r.) and colon homogenate (21 d.p.r.), followed by protein A/G to detect IgG Ab or anti-IgA Ab. Data are representative of 3 independent experiments with 4 to 8 mice per group.

FIG 3 Toxin neutralization titers of sera from rechallenged (10 d.p.r.) WT C57BL/6, $CD4^{-/-}$, and $pIgR^{-/-}$ mice. Bars represent means $\pm$ standard errors of the means (SEM). Data are representative of 3 independent experiments with 4 to 8 mice per group. *, $P < 0.05$; **, $P < 0.01$ (Student's t test). Prior to rechallenge, no neutralization activity was detected.

was precipitated from the supernatant with 55% saturated $(NH_4)_2SO_4$. The precipitate was dialyzed against 0.02 M phosphate buffer and enriched for toxin by using DEAE-cellulose in 0.02 M phosphate, pH 6.8; toxin was eluted with 0.2 M NaCl, as determined by Western blotting with mouse anti-toxin Ab followed by donkey anti-mouse IgG(H+L)-horse-radish peroxidase (HRP) (Jackson ImmunoResearch). Fractions were stored at $-20^\circ C$.

Analysis of anti-toxin Ab. For Western blots, DEAE-enriched toxin was electrophoresed in SDS-PAGE (4.5%) gels, transferred to nitrocellulose membranes (Bio-Rad), and probed with serum (1:250) or colon homogenate (1:10) followed by goat anti-mouse IgA-HRP (Southern Biotech), protein A/G-HRP (Thermo Scientific), or biotinylated anti-

mouse γ -heavy chain (Biolegend) and streptavidin-HRP (Vector Laboratories). For enzyme-linked immunosorbent assay (ELISA), microtiter plates were coated with DEAE-enriched toxin (5 $\mu g/ml$), and serum (1:100 to 1:204,800) or colon homogenate (undiluted to 1:2,048) was added. ELISA color was developed with goat anti-mouse IgA-HRP (Southern Biotech) or protein A/G-HRP (Thermo Scientific) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate, and the optical density was determined at 405 nm.

Fecal IgA Ab. Mucosal IgA levels were determined by an ELISA using rat anti-mouse IgA (BD Pharmingen)-coated microtiter plates. Fecal samples resuspended (0.1 g/ml) in PBS containing protease inhibitor cocktail (Sigma-Aldrich) were centrifuged at $18,000 \times g$ for 5 min and added (undiluted to 1:2,048) to wells, followed by goat anti-mouse IgA as described above.

Toxin neutralization assay. Caco-2 cells (5.5×10^4) were seeded in 96-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and incubated overnight in 5% CO_2 at $37^\circ C$. Toxin (twice the amount required to induce 100% cell rounding) was incubated with antiserum for 1 h at $37^\circ C$, and the mixture was added to Caco-2 cells for 48 h. Toxin-induced cell rounding was identified by microscopy, and the neutralization titer was determined as the reciprocal of the highest dilution of serum that inhibited $>50\%$ of cell rounding (17).

Immunoprecipitation. Rat anti-mouse IgA Ab (Biolegend) was bound to GammaBind G Sepharose (GE Healthcare) according to the manufacturer's instructions. After washing, IgA from colon homogenate or serum was bound to beads for 1 h at room temperature. Beads were then washed and resuspended in SDS sample buffer and loaded into a 4.5% SDS-PAGE gel. Western blot analysis was used to detect toxin as described above.

RESULTS

Protection of mice rechallenged with *C. difficile*. We utilized the CDI mouse model (15) to determine if mice develop a protective adaptive immune response to *C. difficile*. C57BL/6 mice were

FIG 4 Protection of $CD4^{-/-}$ mice rechallenged with *C. difficile*. (A) Percentages of initial body weight 0 to 10 days after primary infection and rechallenge. (B) H&E-stained colon sections (7 μm) at 3 d.p.i. and 3 d.p.r. Magnification, $\times 100$. (C) Numbers of *C. difficile* CFU/g of spores in feces at 10 d.p.i. and 10 d.p.r. Data are representative of 3 independent experiments with 4 to 8 mice per group. Student's t test and the Mann-Whitney test were used for analysis. *, $P < 0.05$; **, $P < 0.01$.

FIG 5 Western blot and ELISA of anti-toxin Ab in $CD4^{-/-}$ mice. (A) Western blot of DEAE-enriched toxin (toxin) and nontoxicogenic *C. difficile* supernatant control (NT) probed with colon homogenate (21 d.p.r.) and serum (10 d.p.r.), followed by anti-IgA or protein A/G. (B) ELISA of serially diluted colon homogenates (21 d.p.r.) from WT (○), $CD4^{-/-}$ (□), and preimmune (▲) mice, performed with toxin-coated microtiter plates. (C) ELISA of serially diluted sera (10 d.p.r.) from WT (○), $CD4^{-/-}$ (□), and preimmune (▲) mice, performed with toxin-coated microtiter plates. Data are representative of 3 independent experiments with 4 to 8 mice per group. O.D., optical density.

treated with antibiotics, infected with BI17 *C. difficile*, and monitored for disease. All mice survived but developed diarrhea and weight loss while actively colonized with *C. difficile* (Fig. 1A and B). Additionally, the colonic architecture from diseased mice was characteristic of CDI, including severe cellular infiltration, submucosal edema, and epithelial damage (Fig. 1C). By 7 to 10 days, mice were free from CDI symptoms, indicating that they had recovered from primary infection. To test for protective immunity, we rechallenged mice at 5 weeks postinfection after treating them with antibiotics to disrupt the intestinal microbiota. Rechallenged mice did not exhibit diarrhea or weight loss (Fig. 1A), despite colonization with *C. difficile* (Fig. 1B), and the colonic histologic architecture was normal (Fig. 1C), suggesting that rechallenged mice likely generated a protective adaptive immune response. Protection in rechallenged mice was not due to persistent *C. difficile* colonization following primary infection, as mice treated with vancomycin prior to rechallenge to clear *C. difficile* colonization were also protected from CDI (data not shown).

We tested sera from mice protected from CDI for binding to *C. difficile* cell surface antigens by immunofluorescence and to *C. difficile* lysate by Western blot analysis, and we found no detectable Ab. In contrast, these mice generated Ab against a protein corresponding to toxin A and/or toxin B (approximately 270 and 308 kDa, respectively); no reactivity was found with preimmune mouse serum. By using reagents specific for IgG and IgA, we found that the serum anti-toxin Ab was of both isotypes (Fig. 2). In contrast, we found that colon homogenates from each of 8 mice had IgA but not IgG anti-toxin Ab, as evidenced by the lack of binding to protein A/G (Fig. 2). We concluded that rechallenged mice generated systemic IgG and IgA and mucosal IgA anti-toxin Ab responses.

To determine if the anti-toxin Ab generated in the rechallenged mice is neutralizing, we incubated the antiserum with DEAE-enriched *C. difficile* toxin and subsequently added the mixture to Caco-2 cells. As determined by the lack of toxin-induced cell rounding, we found that the neutralization titer of WT C57BL/6 antiserum was $19.6 (\pm 5.7)$ (Fig. 3). Undiluted preimmune serum did not prevent toxin-induced cell rounding. We conclude that the rechallenged mice generated neutralizing serum anti-toxin Ab.

Protection from CDI in $CD4^{-/-}$ mice. To determine if a protective immune response could be generated in immunodeficient animals, we infected $CD4^{-/-}$ mice with 10^5 *C. difficile* BI17 spores after antibiotic administration and monitored them for disease. We expected that if the anti-toxin Ab response required CD4 co-receptor expression on T cells, then the $CD4^{-/-}$ mice would likely not recover from primary infection or would develop disease upon rechallenge. Surprisingly, the $CD4^{-/-}$ mice recovered from primary infection, even though they developed CDI (Fig. 4). Furthermore, upon rechallenge, these mice maintained their weight (Fig. 4A), did not develop diarrhea, and had normal colon histology (Fig. 4B), indicating that they were protected from CDI. Colonization in $CD4^{-/-}$ mice was decreased significantly (Fig. 4C).

Because IgA Ab to protein antigens can be generated in the absence of $CD4^{+}$ T cell help (18, 19), we tested if the rechallenged $CD4^{-/-}$ mice generated serum and mucosal IgA anti-toxin Abs. We found IgA anti-toxin Ab by Western blot analysis of sera (Fig. 5A), although by ELISA, the level of IgA in these mice was less than that in sera from WT C57BL/6 mice (Fig. 5B). Additionally, we detected mucosal IgA anti-toxin Ab in colon homogenates (Fig. 5A), although the level of IgA in these mice was less than that in colon

FIG 6 Western blot analysis of toxin-IgA complexes in rechallenge $CD4^{-/-}$ mouse colon and serum. Immunoprecipitated colon and serum IgAs from $CD4^{-/-}$ mice that underwent *C. difficile* rechallenge were analyzed by Western blotting for the presence of toxin, using anti-toxin Ab followed by anti-IgA Ab. Data are representative of 2 independent experiments with 4 animals per group.

homogenates of WT C57BL/6 mice (Fig. 5C). No IgG anti-toxin Ab was detected (Fig. 5A).

We tested the capacity of the systemic anti-toxin Ab in the $CD4^{-/-}$ mice to neutralize toxin-induced damage of Caco-2 cells and found significantly lower neutralization titers than those in sera from rechallenge WT mice (6.0 ± 2.7 versus 19.6 ± 5.7) (Fig. 3). Similarly, we found no neutralization capacity of mucosal Ab, leading us to hypothesize that the anti-toxin Ab binding sites were occupied. To test this, we immunoprecipitated mucosal and serum IgA and performed Western blotting to detect toxin bound to IgA. We found that toxin was bound to mucosal IgA but not to serum IgA (Fig. 6), indicating that the lack of neutralization activity in the colon homogenate was due to occupation of the Ab binding sites by antigen, i.e., toxin.

To test if immunity was long-lived, $CD4^{-/-}$ mice were rechallenged at 63 days and 135 days postinfection. We found that these mice did not develop diarrhea and did maintain their weight (Fig. 7A). By Western blot analysis, we found that mice undergoing rechallenge at 63 days (Fig. 7B) and 135 days (Fig. 7C) main-

tained their IgA anti-toxin Ab responses in the serum and mucosa, with no detectable IgG anti-toxin Ab response. We concluded that protection mediated by IgA anti-toxin Ab in $CD4^{-/-}$ mice can be long-lived.

If the IgA anti-toxin Ab generated in $CD4^{-/-}$ mice was independent of T cell help, we expected that $MHCII^{-/-}$ mice would also be protected from CDI. As with WT mice, we found that $MHCII^{-/-}$ mice developed CDI and recovered from primary infection. However, instead of being protected from CDI upon rechallenge, $MHCII^{-/-}$ mice developed diarrhea, weight loss, and severe cellular infiltration and epithelial damage in the colon, similar to mice undergoing primary infection (Fig. 8A). Furthermore, mice were colonized with BI17 *C. difficile* to a larger extent in rechallenge than in primary infection (Fig. 8B). By ELISA, no detectable anti-toxin Ab was found in sera of the $MHCII^{-/-}$ mice (Fig. 8C), and neither anti-toxin Ab nor Ab directed against cell surface molecules was found in colonic extracts (data not shown). We conclude that protection from CDI requires MHCII and is dependent on T cell help.

Protection from CDI in $pIgR^{-/-}$ mice. To determine if mucosal Ab was required for protective immunity to CDI, we infected polymeric immunoglobulin receptor knockout ($pIgR^{-/-}$) mice with BI17 *C. difficile* and rechallenged mice after they recovered. After rechallenge, the $pIgR^{-/-}$ mice maintained their weight (Fig. 9A), did not develop diarrhea, and had normal colon histology (Fig. 9B), indicating that the $pIgR^{-/-}$ mice developed protective immunity against *C. difficile*. Although the mice were actively colonized with *C. difficile* (Fig. 9C), colonization in the $pIgR^{-/-}$ mice was decreased nearly 100-fold. As expected,

FIG 7 Long-lived immunity in $CD4^{-/-}$ mice. (A) Percentages of initial body weight 0 to 10 days after primary infection and rechallenge after 63 days and 135 days. Western blots are shown for DEAE-enriched toxin (toxin) and nontoxic *C. difficile* supernatant control (NT) probed with colon homogenates (21 d.p.r.) and sera (10 d.p.r.) from mice undergoing rechallenge after 63 days (B) or 135 days (C), followed by anti-IgA or protein A/G. Data are representative of 2 independent experiments with 4 mice per group. *, $P < 0.05$ (Student's *t* test).

FIG 8 Protection of $MHCII^{-/-}$ mice rechallenge with *C. difficile*. (A) H&E-stained colon sections (7 μ m) at 3 d.p.i. and 3 d.p.r. Magnification, $\times 100$. (B) Numbers of *C. difficile* CFU/g of spores in feces at 10 d.p.i. and 10 d.p.r. (C) ELISA of sera (1:40) from $MHCII^{-/-}$ and WT C57BL/6 mice, tested on toxin-coated microtiter plates and with data normalized to the preimmune control serum. Data are representative of 3 independent experiments with 4 mice per group. *, $P < 0.05$ (Mann-Whitney test).

minimal mucosal IgA Ab was detected (Fig. 10A), indicating that, as expected, protection was not mediated by mucosal IgA Ab. By ELISA, we found that the rechallenge $pIgR^{-/-}$ mice generated a robust serum IgA response, more than rechallenge WT mice (Fig. 10B). The $pIgR^{-/-}$ mice also developed IgG anti-toxin Ab, and although we did not quantitate the levels of IgG and IgA Abs, it appears that the level of IgG anti-toxin Ab was significantly less than that of IgA anti-toxin Ab (Fig. 10B). To determine if the serum anti-toxin Ab was capable of neutralizing toxin, we performed a neutralization assay on Caco-2 cells and found a neutralization titer of nearly 1,000 (977 ± 212) (Fig. 3), compared to that of ~ 20 for WT mice. Additionally, we tested the neutralization capacity of intestinal washings and found none (data not shown). These data suggest that the serum anti-toxin Ab is highly neutralizing and is likely responsible for the protective immunity of $pIgR^{-/-}$ mice.

DISCUSSION

CDI is on the rise, especially in hospitals, in large part because of increased use of antimicrobials. Standard antibiotic treatment for CDI is often ineffective, and no vaccines are available (1). To facilitate development of an effective vaccine, we characterized the nature of protective immune responses in mice after infection with *C. difficile* and identified toxin as the sole target of the immune response. We found that WT C57BL/6 mice generated protective immunity to the epidemic BI17 *C. difficile* strain and that this response included IgG and IgA serum anti-toxin Abs as well as mucosal IgA anti-toxin Ab. Although mucosal Ab may be protective, it is not required, as $pIgR^{-/-}$ mice were protected from CDI upon rechallenge with *C. difficile*. In the absence of $CD4^{+}$ T cells,

mice were also protected from CDI, and they generated exclusively IgA anti-toxin Ab; however, the generation of anti-toxin Ab was dependent on T cell help, as $MHCII^{-/-}$ mice did not develop protective immunity to CDI.

As expected, *C. difficile* was not cleared from the intestine by anti-toxin Ab (14). Persistent colonization of mice with *C. difficile* has been demonstrated (20). To eliminate the possibility that protection from CDI upon rechallenge was due to a chronic inflammatory response caused by persistent *C. difficile*, we cleared the mice of *C. difficile* prior to rechallenge. Mice were treated with vancomycin for 1 week, cohoused with naive mice 4 weeks prior to rechallenge, and shown to be cleared of *C. difficile* colonization. Upon rechallenge with *C. difficile*, we found that these mice were still protected, suggesting that protection was not attributed to persistent *C. difficile* colonization.

While colonization levels following *C. difficile* rechallenge in WT mice did not decrease compared to those after the initial challenge, the levels were considerably decreased in rechallenge $CD4^{-/-}$ and $pIgR^{-/-}$ mice. This decreased level of colonization could be the result of *C. difficile* clearance by Ab directed against *C. difficile* surface antigen, but this is not likely to be the case, because the only Ab detected was directed against toxin. Although we cannot rule out the possibility that anti-toxin Ab affects the level of *C. difficile* colonization in immunodeficient mice, we think that the decrease in CFU is more likely due to differences in the intestinal microbiota that occur as a result of the mice being immunocompromised (21).

The major protective Ab described for *C. difficile* is anti-toxin Ab (7–13, 17, 22–26). In both hamsters and mice, passive transfer of IgG anti-toxin mediates protection from CDI-associated mor-

FIG 9 Protection of *pIgR*^{-/-} mice rechallenge with *C. difficile*. (A) Percentages of initial body weight 0 to 10 days after primary infection and rechallenge. (B) H&E-stained colon sections (7 μ m) at 3 d.p.i. and 3 d.p.r. Magnification, $\times 100$. (C) Numbers of *C. difficile* CFU/g of spores in feces at 10 d.p.i. and 10 d.p.r. Data are representative of 3 independent experiments with 4 to 7 mice per group. Student's *t* test and the Mann-Whitney test were used for analysis. *, $P < 0.05$; **, $P < 0.01$.

tality and disease, although protection is dependent on the amount of anti-toxin Ab administered (9, 27). In humans, passive immunization of CDI patients with IgG anti-toxin A and B MAb resulted in decreased recurrent CDI (10), and IgG anti-toxin immunity actively acquired during CDI appears to decrease CDI recurrence (8, 26), indicating that serum IgG anti-toxin Ab can be protective. In our study, WT and *pIgR*^{-/-} mice generated serum IgG and IgA anti-toxin Abs and were protected from CDI. Furthermore, both serum IgG and IgA antibodies neutralized toxin, consistent with the idea that serum Ab can be protective. In addition, since *pIgR*^{-/-} mice were protected and do not have mucosal

Ab, we conclude that although mucosal Ab may contribute to protection, it is not required.

IgG Ab responses to protein antigens are generally dependent on CD4⁺ T cell help. T cell-dependent responses are compromised in immunodeficient individuals such as HIV⁺ patients, and these patients are at increased risk for CDI (28–30). We therefore tested if a protective immune response could be generated in *CD4*^{-/-} T cell-deficient mice. *CD4*^{-/-} mice infected with *C. difficile* generated a protective immune response that was exclusively IgA anti-toxin Ab. In contrast, WT mice generated both IgG and IgA anti-toxin Abs. The IgA anti-toxin Ab in the *CD4*^{-/-} mice was

FIG 10 Quantitation by ELISA of fecal IgA and anti-toxin Ab generated in rechallenge *pIgR*^{-/-} mice. (A) Total mucosal IgA in fecal pellets from WT and *pIgR*^{-/-} mice at 3 d.p.r. Bars represent means and SEM. (B) Anti-toxin Ab in serially diluted sera (10 d.p.r.) from *pIgR*^{-/-} (□, IgA; ○, IgG), WT (■, IgA; ●, IgG), and preimmune (▼, IgA; ▲, IgG) mice. Microtiter plates were coated with toxin. Data are representative of 4 to 7 animals per group from 3 independent experiments.

found in both serum and the mucosa. Whereas Johnson et al. demonstrated that most of the toxin neutralizing activity came from serum IgA in humans (23), we found that neutralization activity of serum IgA in $CD4^{-/-}$ mice that recovered from CDI was either minimal or undetectable, suggesting that these mice were not protected from CDI by serum anti-toxin Ab. We found that only mucosal anti-toxin Ab, not serum anti-toxin Ab, was bound to toxin, suggesting that $CD4^{-/-}$ mice were protected by mucosal IgA anti-toxin Ab. Our data are supported by the work of Kelly et al., who showed that mucosal IgA anti-toxin Ab from CDI patients had toxin neutralizing activity (25). Furthermore, mucosal anti-toxin Ab was required in hamsters for efficient protection after toxoid immunizations (11).

On one hand, the generation of a protective immune response to *C. difficile* in the absence of $CD4^{+}$ T cell help surprised us; on the other hand, the generation of IgA Ab in $CD4^{-/-}$ mice did not, because Macpherson et al. demonstrated that IgA can be generated in the mucosa, independent of T cell help (18), and anti-toxin Ab in the $CD4^{-/-}$ mice was likely derived from the submucosa, because serum IgA anti-toxin Ab is dimeric. Protective intestinal IgA Ab has previously been found in $\alpha\beta/\gamma\delta$ $TCR^{-/-}$ mice as well as in mice depleted of $CD4^{+}$ T cells (31). However, we found that the IgA anti-toxin Ab required the expression of MHCII, suggesting that a population of $CD4^{+}$ T cells in the $CD4^{-/-}$ mice may be required to provide help to B cells. These data are supported by several studies. Sha and Compans showed that a $CD4^{+}$ $CD8^{-}$ T cell population could provide help to B cells in response to influenza virus infection (32), and Yao et al. identified a dendritic cell population that had the capacity to provide $CD4^{+}$ T cell-like help to B cells (33).

Immunity mediated exclusively by IgA anti-toxin Ab provides an alternative method of protection, particularly since it can be generated in $CD4^{-/-}$ T cell-deficient mice. Vaccines promoting IgA Ab responses to *C. difficile* toxin may be effective at protecting individuals impaired in IgG anti-toxin Ab responses. Serum IgA generated in $CD4^{-/-}$ mice only weakly neutralizes toxin, and most IgA anti-toxin Ab is found primarily in the mucosa, where there is minimal IgG. The mechanism by which IgA anti-toxin Ab can mediate protection differs from that of IgG anti-toxin Ab. Mucosal IgA Ab is dimeric and, as such, may be more efficient at neutralizing toxin at the site of infection. IgA may also mediate protection by binding toxin that has gained access to the lamina propria through damaged epithelium (although undetectable histologically), followed by pIgR-mediated transcytosis to the lumen, where the IgA-toxin complex may be cleared from the host (34, 35). Finally, IgA may mediate intracellular neutralization of toxin via pIgR-mediated transport, thereby limiting damage to the epithelium and preventing an inflammatory response, as has been demonstrated for lipopolysaccharide and rotavirus (36, 37).

C. difficile is a pathogen of the colonic mucosa, and since $CD4^{-/-}$ mice generated a mucosal IgA anti-toxin Ab that mediates protection, we expected that mucosal Ab would be required for protection from CDI upon rechallenge. Surprisingly, $pIgR^{-/-}$ mice were protected from CDI upon rechallenge. These mice had considerably elevated levels of serum IgA and IgG anti-toxin Abs compared to WT mice, with almost no mucosal IgA. We propose that protection is mediated by systemic IgA and/or IgG anti-toxin Ab. In these mice, elevated serum anti-toxin Ab may be elicited during primary infection due to a deficiency of mucosal Ab. Mucosal IgA limits both the adherence of bacteria to the epithe-

lium and the production of antimicrobial and proinflammatory molecules (37, 38). A deficiency in mucosal IgA may increase susceptibility of the epithelial barrier to *C. difficile*-induced damage, and such epithelial damage may allow the toxin to become systemic in $pIgR^{-/-}$ mice. Increased levels of systemic toxin correlate with an increased severity of CDI in animal models (39). Systemic toxin, especially the toxin A receptor binding domain, may act as an adjuvant (40), leading to a robust inflammatory response and the generation of serum anti-toxin Ab.

Our data suggest that the serum anti-toxin Ab generated in $pIgR^{-/-}$ mice mediates protection from CDI; however, we cannot rule out the possibility that small amounts of mucosal Ab may also contribute to protection. Although transcytosis of dimeric IgA is deficient in these mice, IgA is detectable in the lumen, albeit at significantly reduced levels compared to those in WT mice. The presence of IgA in the lumen is likely due to asialoglycoprotein expression on hepatocytes, which can transport serum IgA into bile (41). Additionally, $pIgR^{-/-}$ mice are deficient in secretory IgA, and the epithelial barrier may be compromised to a larger extent than that in WT mice after primary infection and rechallenge. If the barrier is compromised, we suggest that Ab produced in the lamina propria crosses into the mucosa, independent of pIgR, resulting in the observed increased levels of anti-toxin Ab in sera of $pIgR^{-/-}$ mice to elicit a protective effect in the mucosa. Neutralization assays for fecal Ab to address this possibility have remained unsuccessful, likely due to the low abundance of Ab itself or to toxin occupation of Ab binding sites, as we found in mucosal Ab of the $CD4^{-/-}$ mice.

Taking together our studies in WT, $CD4^{-/-}$, and $pIgR^{-/-}$ mice, the only detectable Ab generated in these mice was anti-toxin Ab. This finding indicates that anti-toxin Ab is the key protective Ab, as suggested by others (7–13, 17, 22–26). Our data demonstrate that pIgR-mediated transport of mucosal Ab is not required for protection from CDI; however, when the immune system is compromised, as in $CD4^{-/-}$ T cell-deficient mice, mucosal IgA anti-toxin Ab likely mediates protection. Our studies underscore the importance of differing vaccine strategies that are dependent on the immune competency of the host. If an individual is healthy and has the capacity to generate both $CD4^{+}$ T cell-dependent and $CD4^{+}$ T cell-independent anti-toxin Ab responses, then serum anti-toxin Ab may be sufficient for protection, and a mucosal immune response may not be required. However, if an individual is immunodeficient, mucosal IgA anti-toxin Ab may be required for protection. We suggest that the IgA anti-toxin Ab response remains an attractive candidate for protection in immunocompromised individuals, such as those impaired in generating strong IgG Ab responses.

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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. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J, Wilcox MH. 2010. Clinical practice guidelines for Clostridium difficile infection in adults: update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). *Infect. Control Hosp. Epidemiol.* 31:431–455. <http://dx.doi.org/10.1086/651706>.

3. 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>.
4. Savidge TC, Pan WH, Newman P, O'Brien M, Anton PM, Pothoulakis C. 2003. Clostridium difficile toxin B is an inflammatory enterotoxin in human intestine. *Gastroenterology* 125:413–420. [http://dx.doi.org/10.1016/S0016-5085\(03\)00902-8](http://dx.doi.org/10.1016/S0016-5085(03)00902-8).
5. Kelly CP. 2008. Clostridium difficile—more difficult than ever. *N. Engl. J. Med.* 359:1932–1940. <http://dx.doi.org/10.1056/NEJMr0707500>.
6. Khoruts A, Sadowsky MJ. 2011. Therapeutic transplantation of the distal gut microbiota. *Mucosal Immunol.* 4:4–7. <http://dx.doi.org/10.1038/mi.2010.79>.
7. Kyne L, Warny M, Qamar A, Kelly CP. 2000. Asymptomatic carriage of Clostridium difficile and serum levels of IgG antibody against toxin A. *N. Engl. J. Med.* 342:390–397. <http://dx.doi.org/10.1056/NEJM200002103420604>.
8. Leav BA, Blair B, Leney M, Knauber M, Reilly C, Lowy I, Gerding DN, Kelly CP, Katchar K, Baxter R, Ambrosino D, Molrine D. 2010. Serum anti-toxin B antibody correlates with protection from recurrent Clostridium difficile infection (CDI). *Vaccine* 28:965–969. <http://dx.doi.org/10.1016/j.vaccine.2009.10.144>.
9. Babcock GJ, Broering TJ, Hernandez HJ, Mandell RB, Donahue K, Boatright N, Stack AM, Lowy I, Graziano R, Molrine D, Ambrosino DM, Thomas WD, Jr. 2006. Human monoclonal antibodies directed against toxins A and B prevent Clostridium difficile-induced mortality in hamsters. *Infect. Immun.* 74:6339–6347. <http://dx.doi.org/10.1128/IAI.00982-06>.
10. Lowy I, Molrine DC, Leav BA, Blair BM, Baxter R, Gerding DN, Nichol G, Thomas WD, Jr, Leney M, Sloan S, Hay CA, Ambrosino DM. 2010. Treatment with monoclonal antibodies against Clostridium difficile toxins. *N. Engl. J. Med.* 362:197–205. <http://dx.doi.org/10.1056/NEJMoa0907635>.
11. Torres JF, Lysterly DM, Hill JE, Monath TP. 1995. Evaluation of formalin-inactivated Clostridium difficile vaccines administered by parenteral and mucosal routes of immunization in hamsters. *Infect. Immun.* 63:4619–4627.
12. Sougioultzis S, Kyne L, Drudy D, Keates S, Maroo S, Pothoulakis C, Giannasca PJ, Lee CK, Warny M, Monath TP, Kelly CP. 2005. Clostridium difficile toxoid vaccine in recurrent C. difficile-associated diarrhea. *Gastroenterology* 128:764–770. <http://dx.doi.org/10.1053/j.gastro.2004.11.004>.
13. Aboudola S, Kotloff KL, Kyne L, Warny M, Kelly EC, Sougioultzis S, Giannasca PJ, Monath TP, Kelly CP. 2003. Clostridium difficile vaccine and serum immunoglobulin G antibody response to toxin A. *Infect. Immun.* 71:1608–1610. <http://dx.doi.org/10.1128/IAI.71.3.1608-1610.2003>.
14. Siddiqui F, O'Connor JR, Nagaro K, Cheknis A, Sambol SP, Vedantam G, Gerding DN, Johnson S. 2012. Vaccination with parenteral toxoid B protects hamsters against lethal challenge with toxin A-negative, toxin B-positive Clostridium difficile but does not prevent colonization. *J. Infect. Dis.* 205:128–133. <http://dx.doi.org/10.1093/infdis/jir688>.
15. Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknis A, Gerding DN, Kelly CP. 2008. A mouse model of Clostridium difficile-associated disease. *Gastroenterology* 135:1984–1992. <http://dx.doi.org/10.1053/j.gastro.2008.09.002>.
16. Wilson KH, Kennedy MJ, Fekety FR. 1982. Use of sodium taurocholate to enhance spore recovery on a medium selective for Clostridium difficile. *J. Clin. Microbiol.* 15:443–446.
17. Ghose C, Kalsy A, Sheikh A, Rollenhagen J, John M, Young J, Rollins SM, Qadri F, Calderwood SB, Kelly CP, Ryan ET. 2007. Transcutaneous immunization with Clostridium difficile toxoid A induces systemic and mucosal immune responses and toxin A-neutralizing antibodies in mice. *Infect. Immun.* 75:2826–2832. <http://dx.doi.org/10.1128/IAI.00127-07>.
18. Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288:2222–2226. <http://dx.doi.org/10.1126/science.288.5474.2222>.
19. Kaminski DA, Stavnezer J. 2006. Enhanced IgA class switching in marginal zone and B1 B cells relative to follicular/B2 B cells. *J. Immunol.* 177:6025–6029.
20. 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>.
21. Ubeda C, Lipuma L, Gobourne A, Viale A, Leiner I, Equinda M, Khanin R, Pamer EG. 2012. Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. *J. Exp. Med.* 209:1445–1456. <http://dx.doi.org/10.1084/jem.20120504>.
22. Johnson S, Gerding DN, Janoff EN. 1992. Systemic and mucosal antibody responses to toxin A in patients infected with Clostridium difficile. *J. Infect. Dis.* 166:1287–1294. <http://dx.doi.org/10.1093/infdis/166.6.1287>.
23. Johnson S, Sypura WD, Gerding DN, Ewing SL, Janoff EN. 1995. Selective neutralization of a bacterial enterotoxin by serum immunoglobulin A in response to mucosal disease. *Infect. Immun.* 63:3166–3173.
24. Katchar K, Taylor CP, Tummala S, Chen X, Sheikh J, Kelly CP. 2007. Association between IgG2 and IgG3 subclass responses to toxin A and recurrent Clostridium difficile-associated disease. *Clin. Gastroenterol. Hepatol.* 5:707–713. <http://dx.doi.org/10.1016/j.cgh.2007.02.025>.
25. Kelly CP, Pothoulakis C, Orellana J, LaMont JT. 1992. Human colonic aspirates containing immunoglobulin A antibody to Clostridium difficile toxin A inhibit toxin A-receptor binding. *Gastroenterology* 102:35–40.
26. Kyne L, Warny M, Qamar A, Kelly CP. 2001. Association between antibody response to toxin A and protection against recurrent Clostridium difficile diarrhoea. *Lancet* 357:189–193. [http://dx.doi.org/10.1016/S0140-6736\(00\)03592-3](http://dx.doi.org/10.1016/S0140-6736(00)03592-3).
27. Sun X, Wang H, Zhang Y, Chen K, Davis B, Feng H. 2011. Mouse relapse model of Clostridium difficile infection. *Infect. Immun.* 79:2856–2864. <http://dx.doi.org/10.1128/IAI.01336-10>.
28. Sanchez TH, Brooks JT, Sullivan PS, Juhasz M, Mintz E, Dworkin MS, Jones JL. 2005. Bacterial diarrhea in persons with HIV infection, United States, 1992–2002. *Clin. Infect. Dis.* 41:1621–1627. <http://dx.doi.org/10.1086/498027>.
29. Pulvirenti JJ, Gerding DN, Nathan C, Hafiz I, Mehra T, Marsh D, Kocka F, Rice T, Fischer SA, Segreti J, Weinstein RA. 2002. Difference in the incidence of Clostridium difficile among patients infected with human immunodeficiency virus admitted to a public hospital and a private hospital. *Infect. Control Hosp. Epidemiol.* 23:641–647. <http://dx.doi.org/10.1086/501987>.
30. Mody LR, Smith SM, Dever LL. 2001. Clostridium difficile-associated diarrhea in a VA medical center: clustering of cases, association with antibiotic usage, and impact on HIV-infected patients. *Infect. Control Hosp. Epidemiol.* 22:42–45. <http://dx.doi.org/10.1086/501825>.
31. Franco MA, Greenberg HB. 1997. Immunity to rotavirus in T cell deficient mice. *Virology* 238:169–179. <http://dx.doi.org/10.1006/viro.1997.8843>.
32. Sha Z, Compans RW. 2000. Induction of CD4(+) T-cell-independent immunoglobulin responses by inactivated influenza virus. *J. Virol.* 74:4999–5005. <http://dx.doi.org/10.1128/JVI.74.11.4999-5005.2000>.
33. Yao Q, Zhang R, Guo L, Li M, Chen C. 2004. Th cell-independent immune responses to chimeric hemagglutinin/simian human immunodeficiency virus-like particles vaccine. *J. Immunol.* 173:1951–1958.
34. Kaetzel CS, Robinson JK, Chintalacharuvu KR, Vaerman JP, Lamm ME. 1991. The polymeric immunoglobulin receptor (secretory component) mediates transport of immune complexes across epithelial cells: a local defense function for IgA. *Proc. Natl. Acad. Sci. U. S. A.* 88:8796–8800. <http://dx.doi.org/10.1073/pnas.88.19.8796>.
35. Robinson JK, Blanchard TG, Levine AD, Emancipator SN, Lamm ME. 2001. A mucosal IgA-mediated excretory immune system in vivo. *J. Immunol.* 166:3688–3692.
36. Fernandez MI, Pedron T, Tournibize R, Olivo-Marin JC, Sansonetti PJ, Phalipon A. 2003. Anti-inflammatory role for intracellular dimeric immunoglobulin A by neutralization of lipopolysaccharide in epithelial cells. *Immunity* 18:739–749. [http://dx.doi.org/10.1016/S1074-7613\(03\)00122-5](http://dx.doi.org/10.1016/S1074-7613(03)00122-5).
37. Corthesy B, Benureau Y, Perrier C, Fourgeux C, Parez N, Greenberg H, Schwartz-Cornil I. 2006. Rotavirus anti-VP6 secretory immunoglobulin A contributes to protection via intracellular neutralization but not via immune exclusion. *J. Virol.* 80:10692–10699. <http://dx.doi.org/10.1128/JVI.00927-06>.
38. Mantis NJ, Rol N, Corthesy B. 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol.* 4:603–611. <http://dx.doi.org/10.1038/mi.2011.41>.

39. Steele J, Chen K, Sun X, Zhang Y, Wang H, Tzipori S, Feng H. 2012. Systemic dissemination of *Clostridium difficile* toxins A and B is associated with severe, fatal disease in animal models. *J. Infect. Dis.* 205:384–391. <http://dx.doi.org/10.1093/infdis/jir748>.
40. Brun P, Scarpa M, Grillo A, Palu G, Mengoli C, Zecconi A, Spigaglia P, Mastrantonio P, Castagliuolo I. 2008. *Clostridium difficile* TxAC314 and SLP-36kDa enhance the immune response toward a co-administered antigen. *J. Med. Microbiol.* 57:725–731. <http://dx.doi.org/10.1099/jmm.0.47736-0>.
41. Phillips JO, Russell MW, Brown TA, Mestecky J. 1984. Selective hepatobiliary transport of human polymeric IgA in mice. *Mol. Immunol.* 21: 907–914. [http://dx.doi.org/10.1016/0161-5890\(84\)90147-0](http://dx.doi.org/10.1016/0161-5890(84)90147-0).