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Contribution of Adenosine A_{2B} Receptors in *Clostridium difficile* Intoxication and Infection

Cirle A. Warren,[a] Yuesheng Li,[a] Gina M. Calabrese,[a] Rosemayre S. Freire,[b] Snjezana Zaja-Milatovic,[a] Edward van Opstal,[a] Robert A. Figler,[c] Joel Linden,[d] and Richard L. Guerrant[a]

Department of Medicine, University of Virginia, Charlottesville, Virginia, USA[a]; Federal University of Ceara, Fortaleza, Brazil[b]; Hemoshear, LLC, Charlottesville, Virginia, USA[c]; and La Jolla Institute for Allergy and Immunology, La Jolla, California, USA[d]

***Clostridium difficile* toxins A (TcdA) and B (TcdB) induce a pronounced systemic and intestinal inflammatory response. A_{2B} adenosine receptors (A_{2B}ARs) are the predominant adenosine receptors in the intestinal epithelium. We investigated whether A_{2B}ARs are upregulated in human intestinal cells by TcdA or TcdB and whether blockade of A_{2B}ARs can ameliorate *C. difficile* TcdA-induced enteritis and alter the outcome of *C. difficile* infection (CDI). Adenosine receptor subtype (A_1, A_{2A}, A_{2B}, and A_3) mRNAs were assayed in HCT-8 cells. Ileal loops from wild-type rabbits and mice and $A_{2B}AR^{-/-}$ mice were treated with TcdA, with or without the selective A_{2B}AR antagonist ATL692 or PSB1115. A murine model of CDI was used to determine the effect of A_{2B}AR deletion or blockade with the orally available agent ATL801, on clinical outcome, histopathology and intestinal interleukin-6 (IL-6) expression from infection. TcdA and TcdB upregulated A_{2B}AR gene expression in HCT-8 cells. ATL692 decreased TcdA-induced secretion and epithelial injury in rabbit ileum. Deletion of A_{2B}ARs reduced secretion and histopathology in TcdA-challenged mouse ileum. Deletion or blockade of A_{2B}ARs reduced histopathology, IL-6 expression, weight loss, diarrhea, and mortality in *C. difficile*-infected mice. A_{2B}ARs mediate *C. difficile* toxin-induced enteritis and disease. Inhibition of A_{2B}AR activation may be a potential strategy to limit morbidity and mortality from CDI.**

Adenosine receptors—A_1, A_{2A}, A_{2B}, and A_3—are G protein-coupled receptors expressed in a wide variety of tissues. A_{2B} adenosine receptors (A_{2B}ARs) are the predominant adenosine receptors in intestinal epithelial cells and are increased in the presence of inflammation (36). A_{2B}AR mRNA had been shown to be upregulated in the presence of colitis in both murine and human tissues (23). Adenosine, through its activity on A_{2B}AR, mediates chloride secretion (4, 23, 36) and secretion of inflammatory cytokines, interleukin-6 (IL-6), and keratinocyte-derived chemokine (KC) (33). In murine models of inflammatory bowel disease (IBD), the effects of blocking or deleting A_{2B}ARs have been inconsistent. Reduced tissue levels of inflammatory mediators, clinical disease activity score, and histopathology were reported by Kolachala et al. (24, 25), while increased pathology was reported by Frick et al. (13).

Clostridium difficile infection (CDI) induces a pronounced systemic and intestinal inflammatory response. Intestinal disease is induced by large clostridial toxins A (TcdA) and B (TcdB) (39). These toxins glucosylate the small G protein family of Rho, Rac, and Cdc42 leading to actin depolymerization, cytoskeleton disruption, and intestinal barrier dysfunction (20, 21). Although the exact mechanisms involved are still unclear, infiltration of inflammatory cells and secretion of proinflammatory mediators occur (32). Inflammatory diarrhea ensues, and a systemic inflammatory response is observed in severe cases. Severe infection presents as pseudomembranous colitis, toxic megacolon, severe sepsis, or septic shock. Although primarily considered antibiotic-precipitated disease, treatment of CDI still relies on antimicrobial agents active against *C. difficile*. Unfortunately, antimicrobial therapy is associated with recurrent disease in 20% of the initial cases, with increasing recurrence with increasing numbers of prior CDIs (22). Treatment of severe disease and recurrences is challenging.

This study aims to determine whether A_{2B}ARs are involved in *C. difficile* toxin-induced intestinal injury, secretion, and inflammation. Furthermore, we examine the effects of inhibition of A_{2B}AR activity during CDI in the mouse model of the disease.

(This study was partially presented at the 48th Annual Meeting of the Infectious Disease Society of America, Vancouver, Canada, 21 to 24 October 2010.)

MATERIALS AND METHODS

Adenosine receptor subtype assay. Adenosine receptor subtype (A_1, A_{2A}, A_{2B}, and A_3) mRNAs were assayed by quantitative PCR (qPCR) in a human colonic cell line, HCT-8, with or without TcdA or TcdB. (Toxins were kindly provided by David Lyerly.) HCT-8 cells were grown in filtered RPMI 1640 medium in the presence of 10% fetal bovine serum, 1 mM sodium pyruvate, and 0.1 U of Pen Strep (penicillin-streptomycin; Gibco catalog no. 15140) in 5% CO_2 at 37°C. A 96-well plate was seeded with trypsin-EDTA-dissociated cells in 200 μl of the medium. Upon 80% confluence, the cells were treated with TcdA (0.01, 0.1, 1, 10, and 100 ng/ml) and were incubated from 0 to 4 h. The Invitrogen Fast SYBR green cells-to-CT one-step kit was used under the manufacturer's instructions. For qPCR, 2 μl cDNA sample was mixed with 5 μl Fast SYBR green PCR master mix, 0.2 μl each of 10

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Address correspondence to Cirle A. Warren, ca6t@virginia.edu.
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TABLE 1 List of primer sequences for reverse transcription-qPCR analyses

Gene type	Accession no.	Locus positions	Primer	Sequence
A_1AR	NM_000674	530–675	Forward	GCGGTGAAGGTGAAC
			Reverse	AGGCAGGTGTGGAAG
A_{2A}AR	NM_000675	1340–1460	Forward	AGTTCCGCCAGACCTTCC
			Reverse	ACCTGCTCTCCGTCACTG
A_{2B}AR	NM_000676	704–824	Forward	GGTCATTGCTGTCCTCTG
			Reverse	TTCATTCGTGGTTCCATCC
A_3AR	NM_000677	621–737	Forward	AGGGTAGGAATGAGCAAGTTG
			Reverse	CAGGTGAGCCAGCAAGATC
18S rRNA	NR_003286	1373–1561	Forward	CCGATAACGAACGAGACTCTGG
			Reverse	TAGGGTAGGCACACGCTGAGCC

μM forward and reverse primers (Table 1), and 2.6 μl DNase-free distilled water (dH_2O) in a 96-well plate for DNA amplification performed with the CFX96 real-time PCR detection system (Bio-Rad). The PCR parameters were sequentially set for 3 stages: 1 cycle for 20 s at 95.0°C, 40 cycles for 3 s at 95.0°C and 30 s at 60.0°C, and 1 cycle of a hold at 25.0°C. The relative gene expression was determined using 18S rRNA as the housekeeping gene.

Radioligand binding assay of A_{2B}AR antagonists. Radioligand binding assays were performed with ATL692, ATL801, and PSB1115 as described previously (37). Recombinant mouse ARs were stably expressed in HEK-293 cells (A_{2A}AR, A_{2B}AR, and A_3AR), or CHO-K1 cells (A_1AR) or transiently expressed along with G protein subunits by baculoviral infection of Sf9 cells (A_{2A}AR, high-affinity assay). The radioligands were ^{125}I-ABA for A_1AR and A_3AR, ^{125}I-ZM241385 for A_{2A}AR, ^{125}I-ABOPX for A_{2B}AR, or ^{125}I-APE for the A_{2A}AR/high-affinity assay, and radioligand was counted in a Wallac Wizard 1470 gamma counter (Perkin Elmer, Boston, MA). Nonselective binding was measured with the nonselective agonist NECA. Each assay was performed at least in triplicate. Competition binding curves ($n = 3$ to 4) were constructed and 50% inhibitory concentrations (IC_{50}s) were calculated using a 4-parameter logistic fit (PRISM 5.0; GraphPad Software, San Diego, CA). K_i values were calculated from IC_{50}s (10).

Rabbit ileal loop model. All animal experiment protocols were approved by the Center for Comparative Medicine of the University of Virginia and Animal Care and Use Committee. New Zealand White rabbits (Burlington) weighing approximately 2 kg were used in the rabbit ileal loop model as described previously (1, 2). Control loops were injected with either phosphate-buffered saline (PBS) (1 ml/loop) alone or TcdA (10 to 20 μg/loop dissolved in 1 ml of PBS). A_{2B}ARs in rabbit ileal loops, with or without TcdA (10 μg), were blocked with either ATL692 (0.5 or 5 μM; Dogwood Pharmaceuticals) or PSB1115 (5 μM; Tocris Bioscience), which are highly and moderately A_{2B}AR selective at mouse and human receptors, respectively (3). In one experiment, ileal loops were treated with TcdA (10 μg) alone, PBS alone, and ATL692 (5 μM) alone (no TcdA challenge). Ileal loops were harvested after 4 h of incubation and assessed for intraluminal secretion (expressed as the ratio of the volume of fluid to the length of the loop). A small section of the ileum was snap-frozen for the later myeloperoxidase (MPO) assay. Ileal tissues were fixed with 10% formalin overnight. Fixed tissues were sent in 70% ethanol (EtOH) for further processing and staining with hematoxylin-eosin (H&E) by the UVA Research Histology Core. Each slide was read blindly by at least 2 investigators (C.A.W. and G.M.C.). A grading scale, previously formulated and published (1, 2), was used to grade each slide according to mucosal disruption, cellularity, and vascular congestion.

Murine ileal loop model. To confirm results from the rabbit experiments and determine the effects of deletion of A_{2B}AR, mouse ileal loop experiments were also performed. C57BL/6 wild-type (WT) and $A_{2B}AR^{-/-}$ mice, weighing 23 to 25 g each, were used for the mouse ileal loop model as previously described (8). Ileal loops were injected with either PBS (100 μl/loop) alone or TcdA (50 μg/loop dissolved in 100 μl of PBS). The ligated ileal loops were then returned to the abdomen, and the abdominal wall was sutured closed. After 4 h of incubation, intraluminal fluid and histopathology were assessed in the harvested ileal specimens as described above. In addition, ileal weight was measured and the weight/length (W/L) ratio was reported in milligrams per centimeter.

MPO assay. Neutrophilic infiltration was estimated by measuring myeloperoxidase (MPO) activity. Rabbit ileal tissue (50 mg) was homogenized in 1 ml hexadecyltrimethylammonium bromide (Sigma) buffer (1 mg/50 ml) for 15 s, placed in a −70°C freezer for 10 min, and again homogenized. The homogenate was centrifuged at 4,500 rpm for 20 min at 4°C. An aliquot of 7 μl from each sample was pipetted into a 96-well plate in duplicate. Two hundred microliters of *o*-dianisidine dihydrochloride (Sigma) and 1% hydrogen peroxide were then added. Absorbance was read immediately (time zero, basal level) and at 1 min using an enzyme-linked immunosorbent assay (ELISA) reader at 450 nm. The results were reported as MPO units per mg tissue. One unit of activity was defined as that degrading 1 μmol of hydrogen peroxide per min at 22°C.

Preparation of *C. difficile* inocula. *C. difficile* VPI10463 was purchased from the American Type Culture Collection (Manassas, VA). This strain was maintained in anaerobic chopped meat broth (CMB; Anaerobe System) and incubated for 24 h at 37°C. After incubation, the culture was centrifuged (10,000 × *g*, 4°C, 10 min) and pelleted. The pellet was washed 2 additional times with brain heart infusion (BHI) prior to resuspension in fresh reduced CMB and quantified by spectrophotometer. An optical density (OD) reading of 1.0 was calculated to be equivalent to 5×10^7 CFU *C. difficile*/ml. The final concentration of *C. difficile* in CFU/ml was validated by counting the bacteria using a hemocytometer under a light microscope. All visualized cells were rod shaped, with the minority containing spores.

Murine model of *C. difficile* infection. The infection model is a modification of the published protocol by Chen et al. (9). This protocol has been approved by the Center for Comparative Medicine at the University of Virginia. From 6 to 4 days prior to infection, mice were given an antibiotic cocktail containing vancomycin (0.0045 mg/g), colistin (0.0042 mg/g), gentamicin (0.0035 mg/g), and metronidazole (0.0215 mg/g) in drinking water. One day prior to infection, clindamycin (32 mg/kg) was injected subcutaneously. Infection was performed with VPI10463 at an inoculum of 10^5 cells administered by oral gavage. For knockout infection studies, $A_{2B}AR^{-/-}$ mice (originally from Katya Ravid and bred in-house) with age-matched (6- to 8-week-old) and sex-matched (male or female) wild-type littermates, were used. Mice were divided into the following groups: uninfected $A_{2B}AR^{-/-}$, infected $A_{2B}AR^{-/-}$, uninfected A_{2B}AR WT littermates, and infected A_{2B}AR WT littermates. For the wild-type treatment studies, C57BL/6 (The Jackson Laboratory) male 8-week-old mice were used. The mice were divided into the following groups: uninfected wild-type (WT), infected WT, and infected WT treated with A_{2B}AR.

Treated mice were given either A_{2B}AR antagonist ATL801 (10 mg/kg/day) or vehicle by chow given daily for 5 days, beginning 1 day postinfection. A clinical scoring system was developed on the basis of weight loss, diarrhea, activity level, and appearance of eyes and hair (with each parameter scored from 0 to 3, where 0 is considered normal and 3 as the worst, with a maximum score of 20). Mice judged moribund by the clinical score (score of ≥14) on any day and all surviving mice at the end of the experiment were sacrificed by cervical dislocation under sedation (ketamine-xylazine). Stool specimens were collected daily. Diarrhea was scored as follows: 1 for soft or color change (yellow), 2 for wet tail or mucoid, and 3 for liquid or no stool (ileus). Mice were observed for either 1 week (knockout studies) or 2 weeks (wild-type treatment studies) after infection. Upon euthanasia, cecal and colonic tissues were fixed in 10% zinc formalin overnight and then placed in 10% ethanol before being sent for paraffin embedding and H&E staining at the UVA Research Histology Core. Histopathologic scoring was performed blindly (C.A.W.) as we have previously described (31).

IHC. Immunohistochemistry (IHC) was performed on 5-μm formalin-fixed, paraffin-embedded (FFPE) sections of cecum and colon. After deparaffinization through xylene and gradients of EtOH, tissue slides were retrieved in IHC-Tek proteinase solution (catalog no. IW-1101) for 20 min at 37°C. The slides were then processed with the primary antibody anti-IL-6 (1:200 rabbit polyclonal ab6672; Abcam, Cambridge, United Kingdom) diluted in antibody diluent (catalog no. IW-1000 or IW-1001) to reduce background and nonspecific staining for 60 min at room temperature. A serum blocking step was not needed. The anti-rabbit (Dako, Carpinteria, CA) secondary antibody was applied for 30 min. IL-6 was detected by using a high-sensitivity peroxidase visualization kit from Dako (Carpinteria, CA) and 3,3′-diaminobenzidine (DAB) chromogen (Vector, Burlingame, CA). For counterstaining, Mayer's hematoxylin was used (30 s). Slides were washed and rehydrated through gradients of EtOH and xylene. Staining was performed at the Tissue Research Core Facility at the University of Virginia Medical School. Images were taken using an Olympus DP71 microscope and Microsuite Pathology Edition software. IL-6-stained tissue sections were scored blindly (C.A.W. and S.Z.M.) based on the presence and intensity of staining in the epithelial cells, lamina propria, and submucosa. Each section was scored from 0 (absence of staining) to 3 (most intense staining), and the sum of all scores was considered the final IHC score of the tissue section. The maximum score was 9.

Toxin A/B ELISA. TcdA or TcdB was detected using a Toxin A/B II ELISA kit (Tech Lab, Blacksburg, VA). Each stool sample was weighed, and the amount of diluent per sample was normalized to provide the same mass stool/diluent ratio for each sample. The diluent-sample mixtures were homogenized by grinding and vortexing, and 1:10, 1:100, and 1:1,000 serial dilutions of the sample were made. One hundred fifty microliters of the 1:1,000 dilution of each sample was added to a precoated well provided in the kit. The rest of the procedure was performed as recommended by the manufacturer. The absorbance was read at 450 nm on a Bio Tek Gen5 plate reader (Bio Tek Instruments, Highland Park, VT).

Quantification of *C. difficile* in stool. Genomic DNA was extracted from the stool or cecal samples using a modified protocol for the QIAamp DNA stool minikit (Qiagen). All qPCRs were performed on the Bio-Rad iCycler using primers to detect the toxin B (*tcdB*) gene (forward, GGAG AGTCATCCAACTTATATG; reverse, CCACCAATTTCTTTTAATGC AG). qPCRs, in a final volume of 20 μl, consisted of 10 μl QuantiFast SYBR green Supermix (Qiagen), 1 μl each of forward and reverse primers, 4 μl sterile water, and 4 μl sample DNA. PCR assays were performed in duplicate under the following cycling conditions: 3 min at 95°C, 10 s at 95°C, and 30 s at 57°C for 40 repeats and with a melt curve between 65°C and 95°C of 5 s at 0.5°C intervals.

Toxin assay of *C. difficile* VPI10463. ATL801 (stock in dimethyl sulfoxide [DMSO]) was diluted into BHI at final concentrations of 1, 8, and 64 μg/ml using 24-well plates. *C. difficile* (VPI10463; 5.5 log CFU) was added to each well and incubated in an anaerobic chamber for a total of 24 h. The presence of toxin was assessed in supernatants from each well using the TcdA/B II kit from TechLab according to the manufacturer's instructions as described above.

Statistical analysis. Results of measurements of gene expression, secretion and inflammation, histopathology, percentage of weight change, clinical score, and diarrhea were expressed as means ± standard errors of the mean (SEM), as generated by GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). The differences between experimental groups were compared using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. Student's *t* test was performed to analyze differences between 2 groups. To compare experimental groups across a time period, two-way ANOVA with Bonferroni's correction was performed. Survival curves were subjected to the log-rank (Mantel-Cox) test. Statistical significance was set at $P \leq 0.05$. The mortality of wild-type C57BL/6 mice (The Jackson Laboratory) infected with 10^5 VPI10463 cells was expected to be 80 to 100% in dose-ranging studies. To detect a 50% difference between control untreated and treated infected mice, a sample size of 6 to 11 per group was calculated to be sufficient to attain a power of 80% with a significance level of 5%.

RESULTS

TcdA or TcdB upregulates A_{2B}AR gene expression in human ileocolonic cells. To determine whether *C. difficile* toxins affect expression of adenosine receptors, quantitative real-time PCR was performed on intoxicated HCT-8 cells. In the absence of either TcdA or TcdB, the mean A_{2B}AR mRNA expression was 2- to 4-fold higher than that of A_{2A}AR and ≥20-fold higher than those of A_1AR and A_3AR transcripts in HCT8 cells at baseline. A_{2B}AR mRNA increased by over 50-fold after 4 h of exposure to 0.1 ng/ml TcdA and to a lesser extent by TcdB (Fig. 1). A_{2A}AR mRNA was also induced, to a lesser extent, while A_1AR and A_3AR were not induced. Of note, we have previously shown that both toxins induce apoptosis in HCT-8 cells (11), and thus, the decrease in adenosine receptor expression at higher toxin doses may be explained by cell loss. However, even at the highest dose of toxins (100 ng/ml at 4 h of incubation), A_{2B}AR mRNA levels were still high relative to those of the other adenosine receptor subtypes. These results demonstrate that intestinal epithelial cells upregulate A_{2B}AR mRNA expression in response to *C. difficile* toxins.

The A_{2B}AR antagonists ATL692 and PSB1115 decrease TcdA-induced secretion or epithelial injury in rabbit ileum. To test whether inhibition of A_{2B}ARs affects toxin-induced intestinal secretion and histopathology, rabbit ileal loops were treated with or without the selective A_{2B}AR blocker ATL692 or PSB1115. Ileal loops challenged with TcdA had markedly increased secretion compared with PBS control loops ($P < 0.0001$) (Fig. 2). ATL692 (0.5 and 5 μM) significantly reduced TcdA-induced secretion (by 39% and 61%, with *P* not significant and $P < 0.001$, respectively). PSB1115 (5 μM) also significantly decreased intestinal secretion by 56% ($P < 0.001$). Upon microscopy, intestinal tissues from TcdA-challenged mice had pronounced mucosal disruption, cellularity, and vascular congestion. By blinded histopathologic scoring, ATL692 (0.5 and 5 μM) significantly reduced TcdA-induced epithelial injury (by 24% and 44%, with $P = 0.004$ and $P = 0.001$, respectively) in rabbits. In contrast, PSB1115 (5 μM) did not alter TcdA's effect on histopathology.

In an experiment in which loops were treated with only PBS and ATL692 (no TcdA challenge), intestinal secretion and histopathology scores were not different between the 2 treatment groups ($n = 5$ per group) (see Fig. S1 in the supplemental material). As expected, loops treated with TcdA alone had significantly

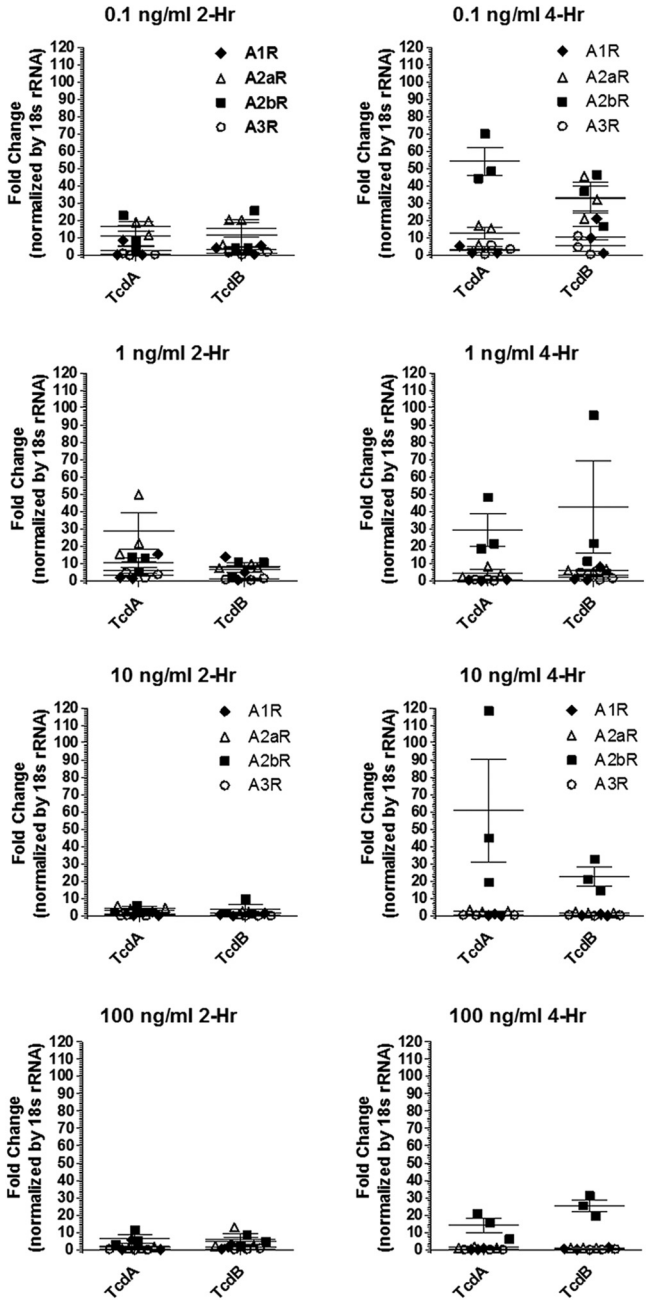

FIG 1 Adenosine receptor (A_1, A_{2A}, A_{2B}, and A_3) mRNA expression in HCT-8 cells treated with *C. difficile* toxin A (TcdA) or B (TcdB). Assays were performed 2 and 4 h postintoxication with either TcdA or TcdB at concentrations from 0.1 to 100 μg/ml. Each treatment had 3 replicates per time point.

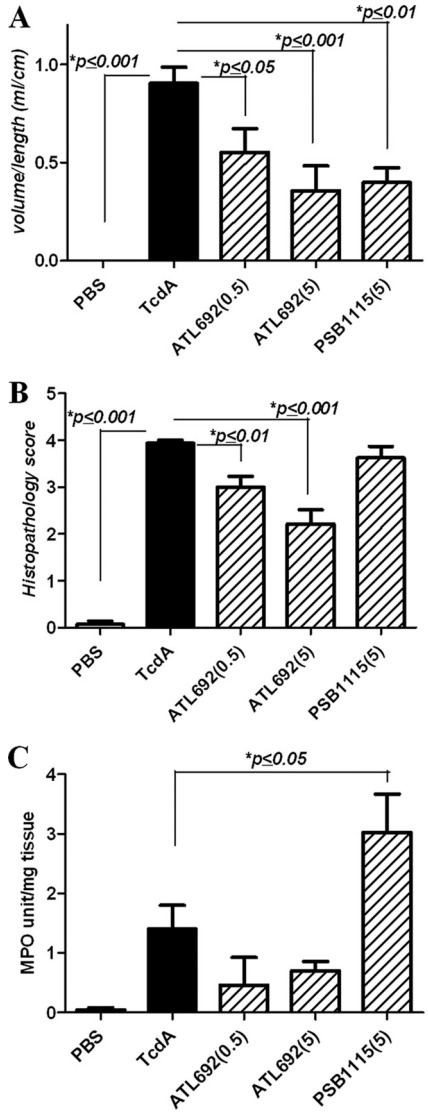

FIG 2 Effects of A_{2B}AR inhibition in *C. difficile* toxin A (TcdA)-stimulated rabbit ligated ileal loops. Ileal loops were treated with TcdA (10 μg/loop) with or without the A_{2B}AR antagonist ATL692 (0.5 or 5 μM) or PSB1115 (5 μM) and incubated for 4 h. (A) Intestinal secretion measured by the volume of fluid in the lumen over the length of the ileal segment ($P < 0.001$ by ANOVA with Bonferroni's multiple comparison test). (B) Ileal histopathology scores ($P < 0.001$ by ANOVA with Bonferroni's multiple comparison test). (C) Myeloperoxidase (MPO) activity ($P < 0.0001$ by ANOVA with Bonferroni's multiple comparison test). $n = 7$ or 8/group).

elevated secretion and damage (histopathology) compared to either of the unchallenged groups.

In addition to histopathology, we assessed MPO activity as a measure of intestinal tissue inflammation. MPO activity was reduced in ATL692-treated ileal tissues by at least 50%. PSB1115-treated tissues, which had reduced secretion but displayed increased histopathology, had the most elevated MPO activity (116% increase) compared with TcdA control loops, possibly due to the relatively less selectivity by this agent. Results of radioligand binding assays are presented in Table 2. ATL692 was more selective for mouse A_{2B}ARs than PSB1115 and ATL801. Binding affinities to A_1ARs and A_3ARs were uniformly poor for all 3 A_{2B}AR antagonists assayed.

Deletion of A_{2B}AR protects against TcdA-induced enteritis in mice. We next examined whether the deletion of the A_{2B}ARs

TABLE 2 Binding affinity of A_{2B}AR antagonists to mouse adenosine receptors

A_{2B}AR antagonist	K_i (nM) for:			
	A_1	A_{2A}	A_{2B}	A_3
ATL692	3,810	8,365	5	>10,000
PSB1115	2,210	7,700	332	>10,000
ATL801	7,550	8,340	187	>10,000

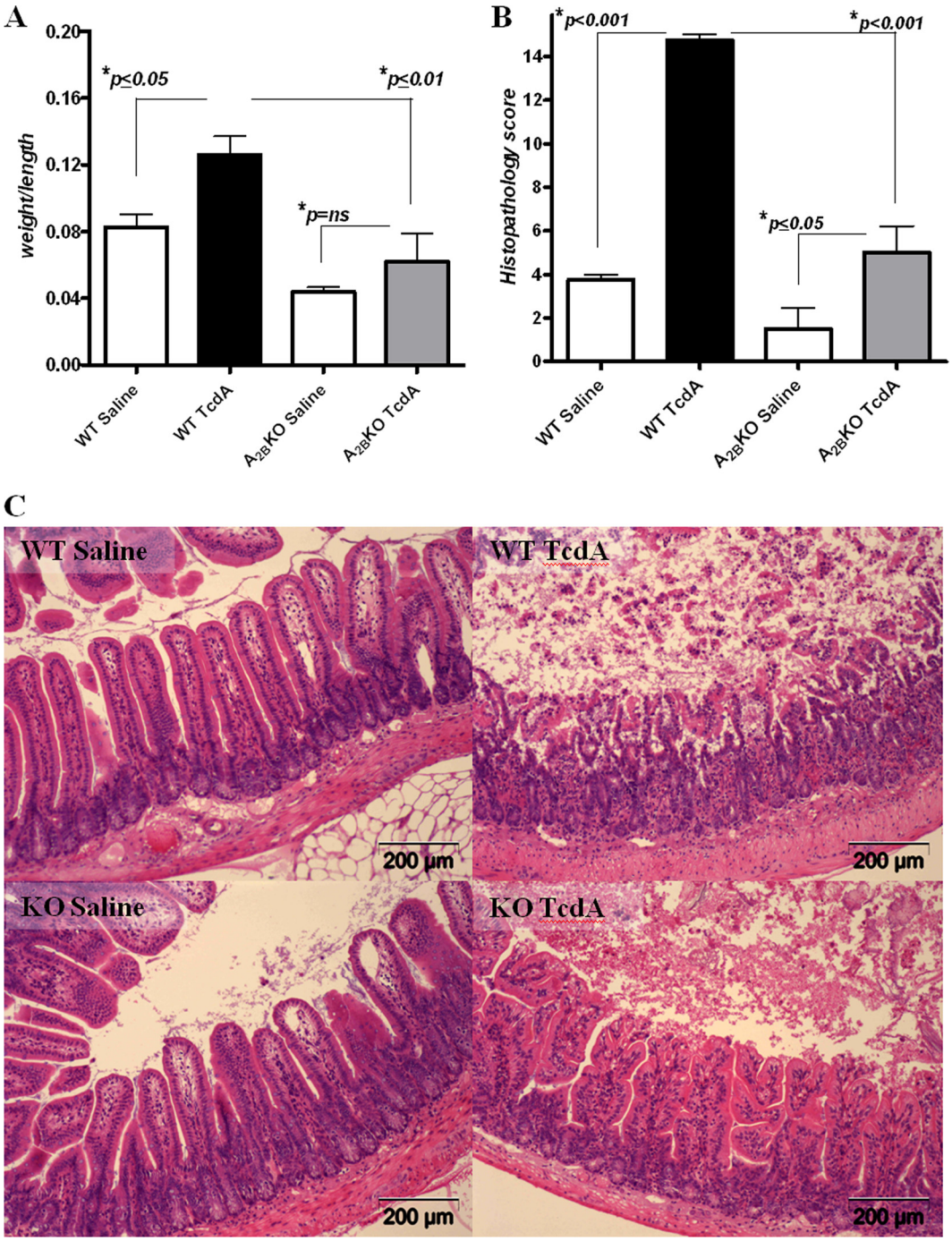

FIG 3 Effects of *C. difficile* toxin A (TcdA) in C57BL/6 wild-type (WT) and A_{2B}AR knockout (KO) mouse ligated ileal loops. Ileal loops (1 loop/mouse, 4 mice/group) were treated with TcdA (50 μg/loop) and incubated for 4 h. (A) Intestinal secretion measured by weight over the length of the ileal loop ($P = 0.0006$ by ANOVA with Bonferroni's multiple comparison test). (B) Ileal histopathology scores ($P < 0.0001$ by ANOVA with Bonferroni's multiple comparison test). (C) Representative H&E-stained tissue sections of ileal loops from WT saline-treated, WT TcdA-treated, A_{2B}AR KO saline-treated, and A_{2B}AR KO TcdA-treated mice.

abrogates TcdA effects in ileal loops. As expected, intestinal secretion and histopathology scores were significantly increased in ligated ileal loops from wild-type (WT) mice as observed in rabbits. $A_{2B}AR^{-/-}$ mice challenged with TcdA had less secretion (by 52%; $P < 0.01$) and a lower histopathology score (by 67%; $P < 0.001$) than WT TcdA control mice (Fig. 3A and B). $A_{2B}AR^{-/-}$ mice treated with saline alone also displayed decreased secretion (by 45%), but this was not a statistically significant difference compared with saline-treated WT mice. Representative ileal tissues are shown in Fig. 3C.

Deletion of the A_{2B}AR protects mice from CDI-induced morbidity and mortality. To investigate the relevance of the A_{2B}AR in

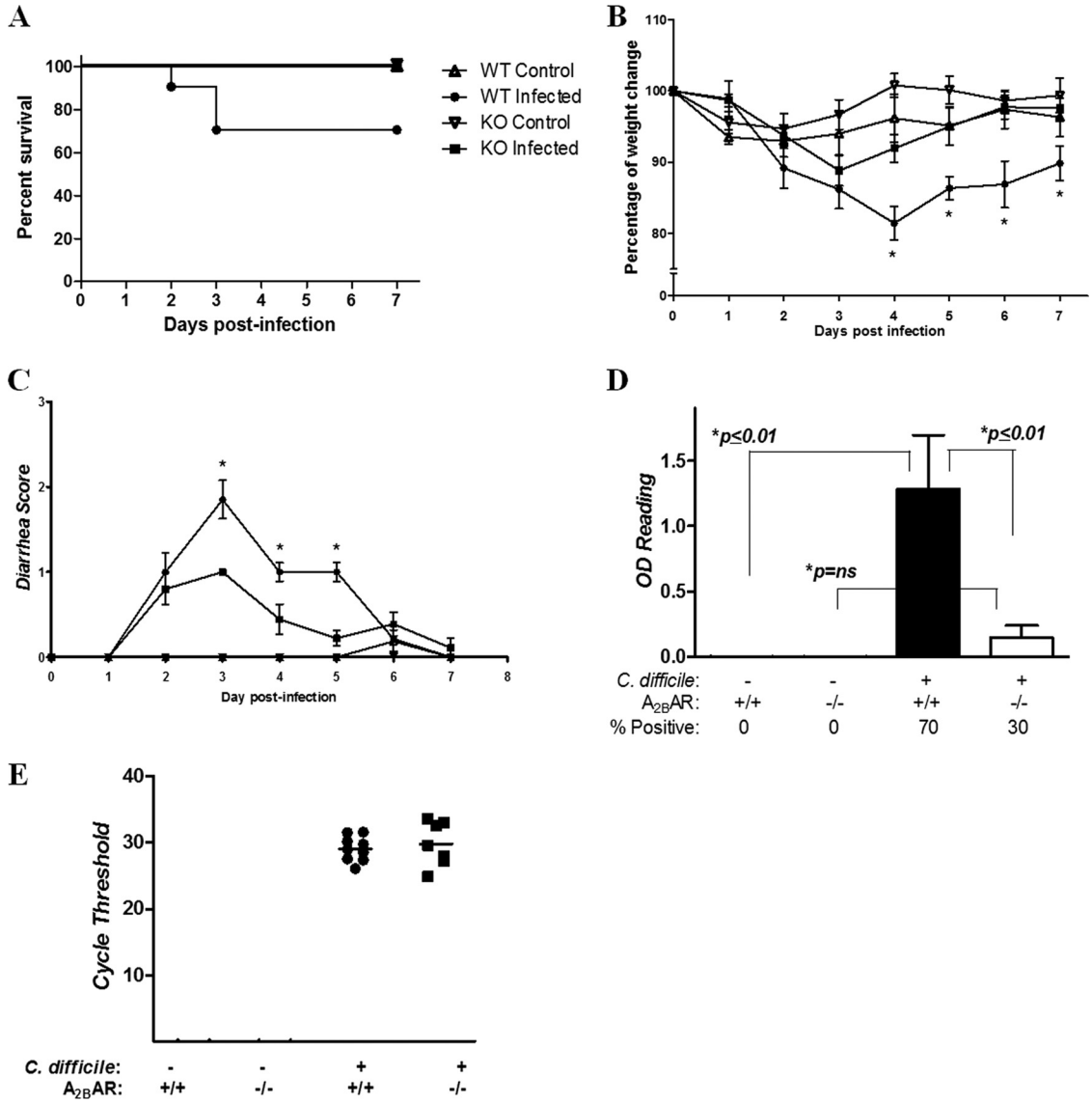

FIG 4 Clinical course of $A_{2B}AR$ knockout (KO) and littermate C57BL/6 wild-type (WT) mice infected with *C. difficile* VPI 10463. $n = 7$ to 9 in the control group, and $n = 10$/group in the infected groups. (A) Survival curve ($P = 0.067$ for WT infected versus KO infected, by Mantel-Cox test). (B) Percentage of weight change from day of inoculation (day 0). For statistical analyses, the latest weights of dead mice were carried through the end of observation (*, $P < 0.05$ on days 4 to 7 for WT infected versus KO infected, by two-way ANOVA with Bonferroni's correction). (C) Diarrhea scores. For statistical analyses, the latest scores of dead mice were carried through the end of observation. *, $P < 0.01$ for WT infected versus KO infected on days 3 to 5 by two-way ANOVA with Bonferroni's correction). (D) *C. difficile* toxin positivity in cecal contents by ELISA ($P < 0.0001$ by ANOVA with Bonferroni's multiple comparison test). (E) Clostridial shedding, detected by PCR of the *C. difficile* TcdB gene (*tcdB*) in available fecal specimens from infected KO and WT mice. The lower the cycle threshold value, the higher the degree of clostridial shedding. Uninfected controls did not have detectable *tcdB.* Histopathology is presented in Fig. 5.

the development of disease from *C. difficile*, we infected $A_{2B}AR^{-/-}$ and littermate WT mice (bred in-house) with VPI10463. Of 10 $A_{2B}AR$ WT mice, 3 (30%) died within 2 to 3 days postinfection (Fig. 4). Diarrhea and weight loss occurred in $A_{2B}AR^{-/-}$ mice to a lesser extent than in controls, and no $A_{2B}AR^{-/-}$ mice died from the disease. The surviving infected WT mice had marked weight loss compared to infected $A_{2B}AR^{-/-}$ mice, and starting on day 2, the latter displayed weight loss that was reversed by day 6. Fecal *C. difficile* toxin levels were higher in WT than $A_{2B}AR^{-/-}$ mice, although there was no difference observed in clostridial shedding. Histopathology scores in $A_{2B}AR^{-/-}$ mice were lower, suggesting a greater ability to control infection and reduced toxin-induced intestinal tissue injury in the absence of $A_{2B}AR$ activation (Fig. 5).

Treatment by $A_{2B}AR$ blockade increases survival in mice with CDI. To test whether $A_{2B}AR$ blockade alters the outcome of infection, we treated infected mice (The Jackson Laboratory) with either the $A_{2B}AR$ antagonist ATL801 or vehicle starting 1 day after inoculation. ATL801 was used in the mouse model of infection because this compound has better oral availability than ATL692. Moreover, ATL801 has also been shown previously to decrease disease and histopathology scores in the mouse models of inflammatory bowel disease (24). While infected mice receiving vehicle alone developed severe disease, infected mice treated with ATL801 developed only mild diarrhea and disease that peaked at day 3 and was followed by progressive improvement (Fig. 6). All untreated infected mice died by day 4, while only 2 of 8 (25%) of the

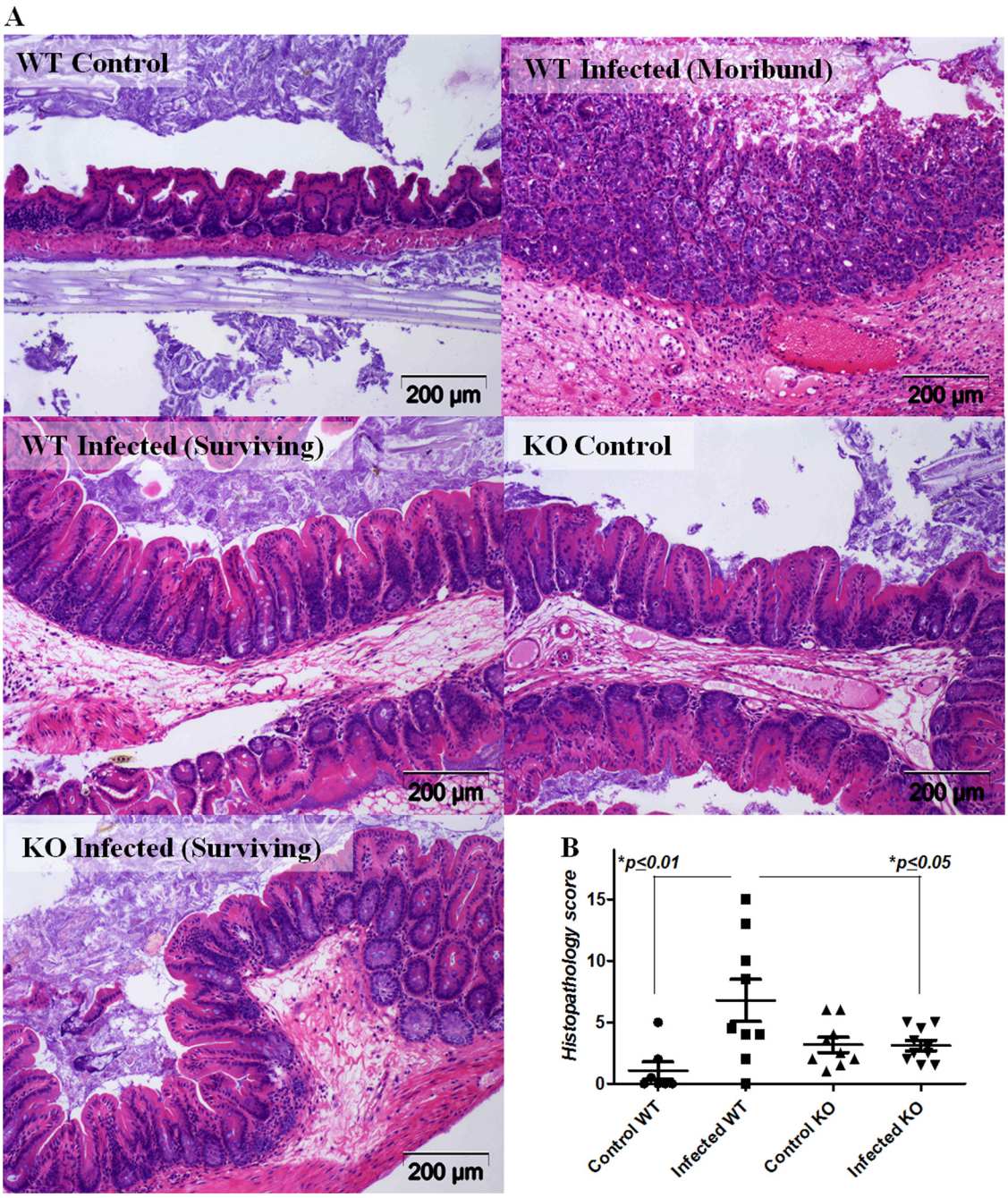

FIG 5 Histopathology in *C. difficile* (VPI 10463)-infected A_{2B}AR knockout (KO) and littermate C57BL/6 wild-type (WT) mice. (A) Representative H&E-stained tissue sections from cecal specimens obtained during sacrifice of moribund mice or of surviving mice at the end of the observation period. (B) Cecal histopathology scores ($P = 0.0047$ by ANOVA with Bonferroni's multiple comparison test). $n = 7$ to 9 in the control group, and $n = 10$/group in infected groups. Survival, clinical and diarrhea scores, *C. difficile* shedding, and toxin burden are presented in Fig. 4.

ATL801-treated mice succumbed to infection (with deaths occurring on days 4 and 6) indicating that A_{2B}AR blockade attenuates severe disease and death. Similar to what was observed in infected $A_{2B}AR^{-/-}$ mice, only a few ATL801-treated infected mice were toxin positive at sacrifice (2 weeks after infection) despite there being no significant differences in fecal clostridial shedding between infected controls and treated infected mice. Given that these mice developed clinical disease, it is possible that treatment contributed to amelioration of symptoms and recovery rather than prevention of infection.

Of note, $A_{2B}AR^{-/-}$ and littermate WT mice (Fig. 4) were bred in-house. We have noted that mice bred in-house are less susceptible to severe infection than mice directly bred by The Jackson Laboratory, such as those used for the treatment studies with ATL801 (Fig. 6). Moreover, a higher degree of bacterial shedding in infected mice (The Jackson Laboratory) was asso-

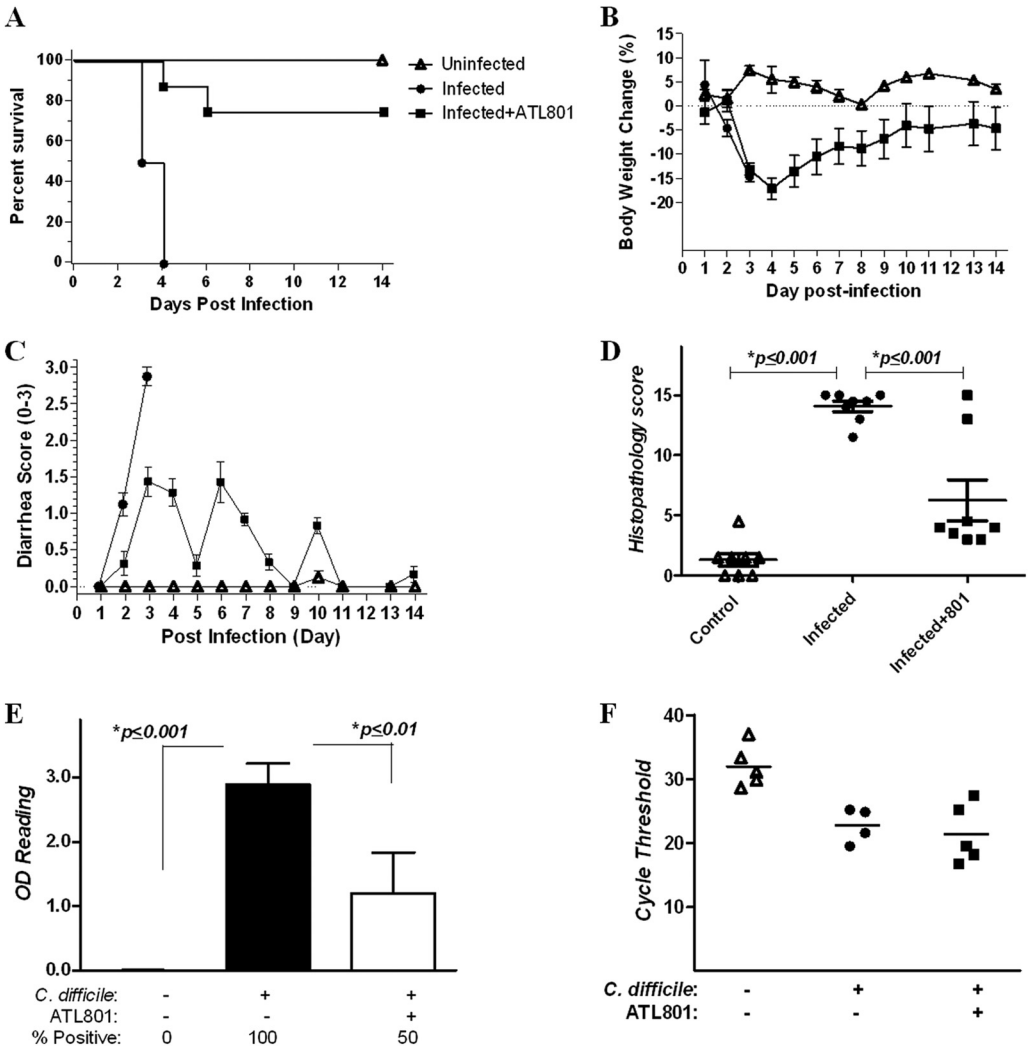

FIG 6 Effects of A_{2B}AR inhibition on *C. difficile* infection in mice. C57BL/6 wild-type mice (8 mice/group) were infected with *C. difficile* VPI 10463 (10^5 CFU) and treated with the A_{2B}AR antagonist ATL801 (10 mg/kg/day for 5 days) 24 h after infection. (A) Survival curve ($P < 0.0001$, by Mantel-Cox test). (B) Percentage of weight change from day of inoculation (day 0). No significant differences between untreated infected versus ATL801-treated mice were observed when weights of dead mice where carried through the end of observation for statistical analysis. (C) Diarrhea scores. For statistical analyses, the latest scores of dead mice were carried through the end of observation. (The only significant difference was the diarrhea score at day 5 for untreated infected versus ATL801-treated infected mice [$P < 0.05$ by two-way ANOVA with Bonferroni's correction[.) (D) Cecal histopathology scores ($P < 0.0001$ by ANOVA with Bonferroni's multiple comparison test). (E) *C. difficile* toxin positivity in cecal contents by ELISA ($P < 0.0001$ by ANOVA with Bonferroni's multiple comparison test). (F) Clostridial shedding, detected by PCR of the *C. difficile* TcdB gene (*tcdB*) in available fecal specimens from uninfected and infected mice. The lower the cycle threshold value, the higher the degree of clostridial shedding.

ciated with increased contamination or colonization of uninfected controls housed in the same sash, an observation not noted in mice bred in-house (Fig. 4E and 6F). Differences in microbiota among mice housed in different environments may explain the variation in susceptibility to infection.

***C. difficile* toxin production *in vitro* is not affected by A_{2B}AR blockade.** Because TcdA/B levels were decreased in stools from mice treated with ATL801, we asked whether the A_{2B}AR blocker alters clostridial toxin production *in vitro*. ATL801, at various doses, did not affect toxin levels in pure *C. difficile* culture supernatant, indicating that ATL801 does not have any direct activity against clostridial toxin production.

Intestinal tissue IL-6 expression is augmented during CDI and depressed with A_{2B}AR blockade or deletion. IL-6 secretion in intestinal epithelial cells has been shown by others to be mediated by activation of A_{2B}AR by apical or basolateral adenosine (33). Therefore, IL-6 production in both wild-type and $A_{2B}AR^{-/-}$ mice during infection was measured by IHC. As shown in Fig. 7A, cecal tissues from infected C57BL/6 mice had greater IL-6 immunoreactivity in the mucosa as well as in the submucosa than tissues from uninfected mice. Infected mice treated with the A_{2B}AR antagonist ATL801 had significantly less IL-6 immunoreactivity in intestinal tissues than untreated infected mice. In studies with $A_{2B}AR^{-/-}$ mice, although there were no significant differences in total IHC scores between all groups, wild-type littermates exhibited enhanced IL-6 expression in the enterocytes and submucosa during infection compared to infected $A_{2B}AR^{-/-}$ mice (Fig. 7B), suggesting possible compartmentalization of the A_{2B}AR effect. Overall, these findings provide evidence that *C. difficile*-induced

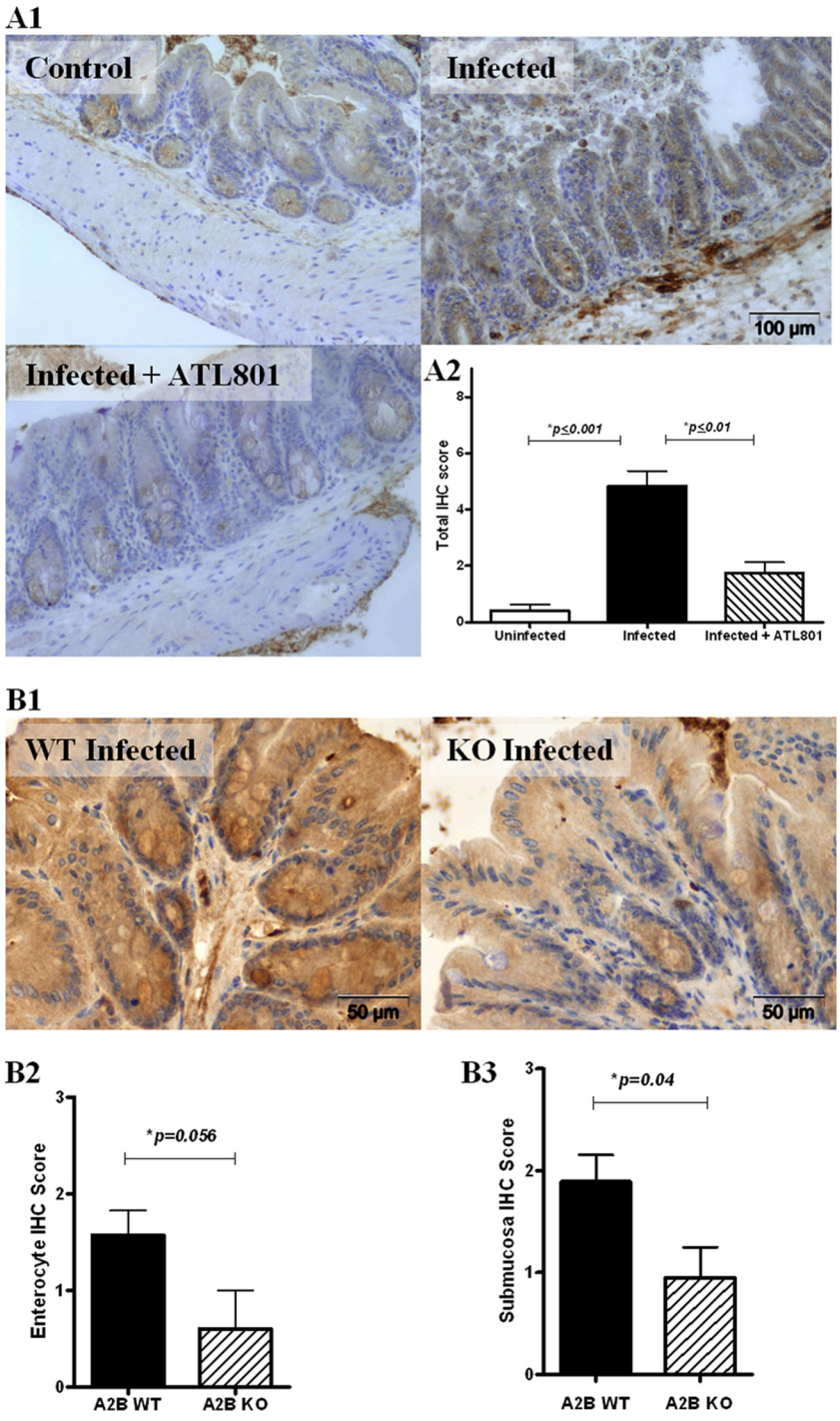

FIG 7 IL-6 intestinal tissue expression during *C. difficile* infection in mice. (A1) IL-6 immunohistochemistry (IHC) in uninfected, infected, and ATL801-treated infected C57BL/6 mice. (A2) Mean IHC scores were from combined enterocyte, lamina propria, and submucosa scores from 3 representative mice per group ($P < 0.0007$ by ANOVA with Bonferroni's multiple comparison test). (B1) IL-6 IHC in infected wild-type (WT; $n = 7$) littermate and infected A_{2B}AR knockout (KO; $n = 5$) littermate. (B2) Enterocyte IHC scores (unpaired two-tailed *t* test). (B3) Submucosa IHC scores (unpaired two-tailed *t* test).

disease may be mediated by IL-6 secreted as a consequence of A_{2B}AR activation.

DISCUSSION

Clostridium difficile infection is characterized by intense intestinal inflammation and injury and, in severe cases, a systemic inflammatory response. We have demonstrated that intestinal epithelial A_{2B}AR expression is upregulated by TcdA or TcdB and that A_{2B}AR deletion or blockade ameliorates *C. difficile* toxin-induced intestinal epithelial injury, secretion, and inflammation. Furthermore, A_{2B}AR deletion or blockade improves outcome from infection in the mouse model.

Although adenosine has been recognized to be produced during inflammatory conditions, expression of adenosine receptors had never been studied during CDI. The A_{2B}AR is the predominant adenosine receptor subtype expressed in the cecum, colon, and intestinal epithelial cells (34, 35). In the study, we found that upregulation of A_{2B}ARs occurs in TcdA- or TcdB-challenged human intestinal epithelial cells. Others have shown that both A_{2A}AR and A_{2B}AR are expressed during dextran sodium sulfate (DSS)-induced colitis (13), while A_{2B}AR is selectively induced in ischemia-reperfusion injury in mice (17). A_{2A}AR mRNA is not as prominently expressed as A_{2B}AR in epithelial cells; A_{2A}AR may be more localized in immune rather than epithelial cells in the intestinal tract (12, 18). Although it was previously shown that A_{2A}AR agonists decreased intestinal secretion and epithelial injury in TcdA-induced enteritis in both rabbit and mouse ileal loop models (8, 41), we recently found that A_{2A}AR activation provides only a modest benefit against CDI in the murine model (C. A. Warren et al., personal communication). Limited benefit may occur because during infection, the initial insult from the bacteria is localized in the intestinal epithelium where A_{2B}ARs, and not A_{2A}ARs, predominate.

How the *C. difficile* toxins affected adenosine receptor expressions was unclear. Tumor necrosis factor alpha (TNF-α), a cytokine that is expressed during colitis, has been shown to upregulate A_{2B}AR transcript and protein (23). TcdA and TcdB are known to cause apoptosis (5, 7, 14). Adenosine released or generated from adenine nucleotides as a consequence of cell death could bind stimulatory G protein (Gs)-coupled A_{2B}ARs to stimulate cyclic AMP (cAMP) generation, thereby stimulating CREB-mediated release of cytokines, such as IL-6 (33). IL-8 also is increased in response to TcdA or TcdB stimulation of intestinal epithelial cell lines (19, 29), but whether this cytokine, or IL-6, also upregulates A_{2B}ARs is unclear. Moreover, protein kinase A activates the apical cystic fibrosis conductance regulator, causing net Cl secretion (15, 36), which may explain increased ileal secretion in TcdA-challenged tissues. Indeed, in addition to increasing intestinal permeability by its effect on epithelial tight junctions, TcdA has been shown to increase Cl secretion in guinea pig ileum (30).

TcdA causes mucosal disruption and increased intestinal secretion in both rabbit and mouse ileal loops (27, 38, 40). Treatment with anti-inflammatory agents, such as COX-2 inhibitors (1), other nonsteroidal anti-inflammatory drugs (NSAIDs) (28), or angiotensin II subtype I receptor blockers (2), ameliorates epithelial injury and intestinal fluid accumulation in the ileal lumen. In the present study, we show that reduction of inflammation by A_{2B}AR blockade also controls the inflammatory response and prevents toxin-induced intestinal epithelial damage and secretion. Interestingly, another A_{2B}AR inhibitor, PSB1115, blocked secretion but unlike A_{2B}AR deletion did not alter the histopathology score and even increased myeloperoxidase activity, suggesting differences in the selectivity of these compounds and possible off-target effects by PSB1115. Our findings on blockade or deletion of A_{2B}AR in both TcdA-induced enteritis and *C. difficile* infection in mice further confirm the role of A_{2B}AR in mediating epithelial injury and secretion. These findings mirror what others have found in murine models of colitis in which $A_{2B}AR^{-/-}$ mice were reported to be less susceptible to chemicals (DSS or 2,4,6-trinitrobenzene sulfonic acid [TNBS]) or infection with *Salmonella enterica* serovar Typhimurium (given to streptomycin-pretreated mice) than WT controls (25). However, in the latter study, $A_{2B}AR^{-/-}$ mice were observed to be more susceptible to overwhelming sepsis and death from exposure to live *S.* Typhimurium cells (given without pretreatment with streptomycin). In contrast to the invasive nature of *S.* Typhimurium, disease from *C. difficile* is primarily mediated by its toxins (39). Although a systemic inflammatory reaction is observed in CDI, bacteremia is rarely observed (26). Control of toxin-induced intestinal inflammation locally by inhibition or deletion of A_{2B}AR appears to prevent progression of disease and death. Indeed, when WT mice (from The Jackson Laboratory and which are highly susceptible to severe infection) were infected but treated with ATL801, protection was conferred to 75% of the mice. While fecal toxin levels in ATL801-treated mice were reduced, we showed that the A_{2B}AR antagonist itself did not alter clostridial toxin production *in vitro*. Since fecal toxin also decreased in $A_{2B}AR^{-/-}$ infected mice, this effect appears to be indirect. We speculate that modulation of the epithelial inflammatory response has beneficial effects to the intestinal microbiota, which in concert with the host, is critical for the control of *C. difficile* growth and possibly secretion of toxin (6). Indeed, despite being bred in the same facility (and therefore presumably sharing the same microbiome) and infected with the same amount of *C. difficile*, $A_{2B}AR^{-/-}$ mice were still less susceptible to severe disease and had lower toxin levels than their littermate wild-type controls.

Interestingly, other investigators have demonstrated more of an anti-inflammatory and protective role for A_{2B}AR receptors in ischemia-reperfusion injury (16, 17) and murine colitis (13). Differences in experimental protocols, animal strains, and environmental conditions have been proposed as possible reasons for the discrepancies in the findings (13). In addition, it is possible that the A_{2B}ARs may actually have different effects, depending on the cell types involved and the localization and type of tissue insult. Global A_{2B}AR deletion may also produce changes in the development of epithelial and immune cells. Studies using targeted gene deletions in immune and epithelial cells and the use of more selective pharmacologic agents will be critical to elucidate the roles of the different adenosine receptors in various cells in various disease states.

In conclusion, we have shown evidence that A_{2B}ARs are upregulated by *C. difficile* toxin in intestinal epithelial cells. Blockade or deletion of A_{2B}ARs decreased toxin-induced intestinal tissue injury and secretion and improved outcomes in the murine model of infection. A_{2B}AR inhibition may potentially limit damage from toxigenic *C. difficile* infection.

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