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Journal

The Journal of Infectious Diseases, 230(3)

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Publication Date

2024-09-23

DOI

10.1093/infdis/jiae021

Peer reviewed

The Journal of Infectious Diseases

MAJOR ARTICLE

Loratadine as an Anti-inflammatory Agent Against *Clostridium difficile* Toxin B

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(See the Editorial Commentary by Villafuerte Gálvez and Kelly on pages 527–8.)

Background. *Clostridium difficile* infection (CDI) is a debilitating nosocomial infection. *C. difficile* produces toxins A and B, which cause inflammation. Existing therapies have issues with recurrence, cost, and safety. We aim to discover a safe, effective, and economical nonmicrobiological therapeutic approach against CDI.

Methods. We included human primary peripheral blood mononuclear cells (PBMCs), fresh human colonic explants, and humanized HuCD34-NCG mice. Toxin A^+B^+ VPI 10463 and A^-B^+ ribotype 017 *C. difficile* strains were used. We used single-cell RNA profiling and high-throughput screening to find actionable toxin B–dependent pathways in PBMCs.

Results. Histamine 1 receptor–related drugs were found among the hit compounds that reversed toxin-mediated macrophage inflammatory protein (MIP) 1α expression in PBMCs. We identified loratadine as the safest representative antihistamine for therapeutic development. Loratadine inhibited toxin B–induced MIP-1α secretion in fresh human colonic tissues. Oral loratadine (10 mg/kg/d) maintained survival, inhibited intestinal CCl3 messenger RNA expression, and prevented vancomycin-associated recurrence in the VPI 10463–infected mice and ribotype 017-infected hamsters. Splenocytes from loratadine-treated mice conferred anti-inflammatory effects to the VPI 10463–infected T/B-cell–deficient $Rag^{-/-}$ mice. Oral loratadine suppressed human MIP-1α expression in monocytes/macrophages in toxin B–expressing ribotype 017-infected humanized HuCD34-NCG mice.

Conclusions. Loratadine may be repurposed to optimize existing therapies against CDI.

Keywords. histamine receptor; cytokine; splenocytes.

Clostridium difficile infection (CDI) is a common nosocomial infection among patients who received prolonged antibiotic treatment. *C. difficile* bacterium produces toxin A, toxin B, and binary toxin. CDI symptoms include severe diarrhea, fever, abdominal pain, loss of appetite, and nausea (Centers for Disease Control and Prevention) [1]. As CDI infection rates soar, the disease burden and associated morbidity and mortality rates continue to rise [2]. *C. difficile* was the most frequently reported pathogen, resulting in 12.1% of all healthcare-associated infections [3]. CDI affected nearly half a million people in 2011 and quadrupled the cost of hospitalizations [4]. Old age, hospitalization, immunocompromised status, and previous *C. difficile* exposure are risk factors for CDI [5].

About 1 in 6 CDI cases will recur within 2–8 weeks. Metronidazole and vancomycin are associated with high relapse rates; while fidaxomicin can reduce recurrence, it is expensive and cost-ineffective [6]. Anti–toxin B antibody (bezlotoxumab) can prevent recurrent CDI with a modest, sustained cure rate (64%) [7]. Some CDI cases are not responsive to medications and eventually require surgical resection [8].

Fecal microbiota transplantation has a 90% success rate, but the US Food and Drug Administration (FDA) has not approved the treatment owing to adverse effects [9]. On the other hand, a phase 3 PUNCH CD3 clinical trial showed that a single dose of REBYOTA, a live fecal microbiota biotherapeutic product from Ferring Pharmaceuticals, demonstrated superiority over placebo for reducing CDI recurrence after standard-of-care antibiotic treatment [10]. REBYOTA was approved by the FDA in November 2022. Similarly, a phase 3 ECOSPOR III clinical trial showed that oral administration of SER-109, an orally active therapeutic with fecal microbiota spores, live-brpk in capsules, from Seres Therapeutics, reduced the risk of recurrent CDI [11]. Ser-109 was later renamed VOWST and approved by the FDA in April 2023.

Received 19 July 2023; editorial decision 09 December 2023; accepted 17 January 2024; published online 19 January 2024

Presented in part: Loratadine and Genistein are Effective Repurposed Candidate Drugs for Treating Clostridium Difficile Infection, Digestive DiseaseWeek meeting, 23 May 2021, virtual meeting.

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The Journal of Infectious Diseases® 2024;230:545–57

https://doi.org/10.1093/infdis/jiae021

Small-molecule experimental drugs showed mixed results in preclinical models. For example, niclosamide inhibits endosomes and primary CDI but cannot prevent weight loss during relapse in mice [12]. Ebselen targets toxin B cysteine protease domain [13], while VB-82252 inhibits toxin B–mediated uridine diphosphate-glucose hydrolysis [14], but they failed to protect the infected hamsters [13, 15]. Ceragenin CSA-13 can treat CDI in mice by modifying intestinal microbiota and metabolites [16]. However, it is toxic to host cells at high concentrations [17].

Exposure to toxins A and B leads to cytokine secretion [18, 19]. We used single-cell RNA profiling and found that a CDI-dependent proinflammatory cytokine is secreted from toxin B–treated peripheral blood mononuclear cells (PBMCs). Therefore, we used high-throughput screening to discover drugs that prevent toxin-mediated proinflammatory cytokine release. The hits reflected toxin-mediated pathogenic pathways. We found an FDA-approved drug for modulating a toxin-mediated pathogenic pathway. Our study also included validations with clinically relevant human colonic tissues, primary cells, and immunologically humanized mice.

METHODS

Chemical information is shown in Supplementary Table 1. PBMCs from healthy male and female donors (70025; Stemcell Technologies) were suspended in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum and 1% penicillin-streptomycin. The baseline characteristics of the blood donors are shown in Supplementary Table 1.

RESULTS

C. difficile Toxin B Affected Macrophage Inflammatory Protein 1α Expression in Monocytes

Our previous report identified macrophage inflammatory protein (MIP) 1α as a proinflammatory mediator in CDI among human and mouse colons [19]. Immune cells are the probable source of MIP-1α in CDI. We therefore used 10× genomics single-cell fixed RNA profiling to determine the changes in messenger RNA (mRNA) expression in specific cell subpopulations in PBMCs.

Toxin B is more clinically significant than toxin A in mediating pathogenic effects in CDI [7]. The exposure to toxin B caused a different mRNA expression pattern in healthy donor–derived PBMCs (Figure 1*A*). We identified CD14-expressing classic monocytes in cluster 2 in both phosphate buffered saline–treated and toxin B–treated PBMCs (Figure 1*B*). IRAK2/3, CXCL1, and GPR84 in cluster 7 are monocyte markers (Figure 1*B*). Interestingly, toxin B induced mRNA expression of CCL3 (gene name of MIP-1α) and other proinflammatory cytokines—such as interleukin 1β (IL-1β), interleukin 6, and tumor necrosis factor—in cluster 7, suggesting an intermediate monocyte phenotype (Figure 1*B*).

High-Throughput Drug Screening Identified the Involvement of Histamine 1 Receptor in Toxin B–Mediated MIP-α Expression in PBMCs

As the single-cell RNA profiling identified PBMCs as a source of toxin B–dependent CCL3/MIP-1α (Figure 1*B*), we used drug screening to find anti-inflammatory drugs against toxin B–mediated MIP-1α secretion in PBMCs. Of 2621 FDA-approved drugs, 111 (4%) showed inhibition of toxin B–mediated MIP-1α secretion in PBMCs by ≥1 standard deviation. STITCH chemical association analysis showed that some are linked to histamine, serotonin, and estrogen receptors and SRC (Figure 2*A*). The relevant hit drugs are shown in Figure 2*B*. Modulation of the serotonin pathway is unlikely useful for treating CDI because it affects nervous system functions. As the Human Protein Atlas shows the positive expression of histamine H_1 receptor (HRH_1) protein in the human colon, HRH_1 antagonism may be a promising anti-inflammatory approach for CDI.

Loratadine as a Safe Histamine 1 Receptor Antagonist

The median age of patients *C. difficile* in the United States is 74 years [20]. Acrivastine is an FDA-approved antihistamine, but it is not recommended for patients above the age of 65 years. Five of 6 antihistamines in the hit list (Figure 2*B*) are uncommon for treating allergies owing to adverse effects and interference with other receptors. Histamine H_2 receptor (HRH_2) antagonists are contraindicated due to an elevated risk of CDI [21]. Many first-generation H_1 receptor antagonists cross the blood-brain barrier and cause sedation [22]. However, this adverse effect can be circumvented by using the specific second-generation H_1 blocker loratadine [23]. Loratadine is approved for patients aged ≥2 years. It has a more extended history of clinical uses than other second-generation H_1 blockers, such as desloratadine and fexofenadine. Its bioavailability, pharmacokinetic-pharmacodynamic, and absorption, distribution, metabolism, excretion, and toxicity data are available [24, 25].

Non–life-threatening somnolence, tachycardia, and headache had been reported with overdoses (40–180 mg) in adults (loratadine product information). No deaths occurred in mice at 5000 mg/kg (oral) (PubChem). Oral loratadine (median lethal dose, >5000 mg/kg) is safer than desloratadine (median lethal dose, 353 mg/kg) in mice (Claritin and Clarinex product information). Loratadine is FDA pregnancy category B, safer than desloratadine and fexofenadine at class C [26]. Loratadine is less likely to cause drowsiness than cetirizine (Drugs.com). Thus, we chose over-the-counter loratadine for further studies.

Loratadine Inhibited Toxin-Mediated MIP-1α Expression in Immune Cells via SRC and NF-kB Inhibition

Loratadine reduced toxin B–induced MIP-1α secretion in fresh human colonic explants (Figure 3*A*). Starting at 30 μmol/L, loratadine inhibited toxin B–induced MIP-1α secretion in

primary human PBMCs (Figure 3*B*). Toxin A modestly induced MIP-1α secretion in fresh human explants and PBMCs unaffected by loratadine treatment (Figure 3*A* and 3*B*). Loratadine is metabolized to desloratadine [27], an approved second-generation nonsedating H_1 blocker (Clarinex). Similarly, desloratadine also inhibited toxin A– and toxin B–mediated MIP-1α secretion in PBMCs (Figure 3*B*). However, other second-generation antihistamine/HRH_1 antagonists (cetirizine and fexofenadine) and HRH_4 receptor antagonist JNJ7777120 failed to inhibit toxin A– and B–mediated MIP-1α secretion in PBMCs (Figure 3*C*).

Loratadine inhibits endotoxin-induced cytokine production in macrophages via SRC binding and NF-κB inhibition [28]. Single-cell fixed RNA profiling showed SRC activation in toxin B–treated monocyte clusters 2 and 7 (Figure 1*B*). We therefore used an NF-κB phosphorylation protein array to discover the protein target of loratadine. Toxin B increased NF-κB phosphorylation in human PBMCs, which was reduced by 30 μmol/L loratadine (Figure 3*D* and 3*E*). Pretreatment with an SRC family activator or NF-κB activator reversed the anti-inflammatory effect of loratadine in toxin B–exposed PBMCs (Figure 3*F*). Thus, the anti-inflammatory effect of loratadine against toxin B in PBMCs was mediated by SRC and NF-κB inhibition.

Oral Loratadine Administration Was Safe for Mice and Reached the Colon

A previous study used loratadine at 10 mg/kg/d for 6–7 days to treat atopic dermatitis in mice [29]. We therefore used the same dosage dose to treat CDI in animals. Based on the blood chemistry panel data, repeated treatment of the normal C57BL/6J mice with a 4-fold amount of loratadine (40 mg/kg/d) via oral gavage for 7 days did not cause a toxic response in the liver and kidney functions (Supplementary Figure 1A).

Consistent with findings of a previous study [30], oral loratadine treatment (10 mg/kg given once) in normal mice resulted in the presence of loratadine and its metabolites (desloratadine and 3-hydroxydesloratadine) in the fecal samples collected at 6 hours later (Supplementary Figure 1*B* and 1*C*). These results indicated that the colons of the loratadine-treated mice had exposure to loratadine.

Loratadine Inhibited CDI in Mice via Anti-inflammatory Effects in Splenocyte-derived Immune Cells

To determine the protective effect of loratadine against CDI in vivo, we started administering loratadine to the infected mice 24 hours after infection (Figure 4*A*). The mice were infected with toxin A^+B^+ reference strain VPI 10463 [31]. Oral loratadine treatment prevented death in mice with primary CDI (Figure 4*B*). The body weights of all groups fluctuated within a narrow range throughout the 10-day observation period (Figure 4*B*). Loratadine treatment did not affect the colonic mucosal structure in normal mice (Figure 4*C*).

As expected, CDI colitis caused colonic mucosal injury on day 3 after infection, as reflected by the histology scores (Figure 4*C*). Our previous studies indicated that MIP-1α is crucial in mediating CDI disease activity [19, 32]. CDI increased colonic mRNA expression of CCl3 in the infected mice (Figure 4*D*). Oral loratadine treatment ameliorated CDI colitis with reduced colonic injury and CCl3 mRNA expression (Figure 4*C* and 4*D*).

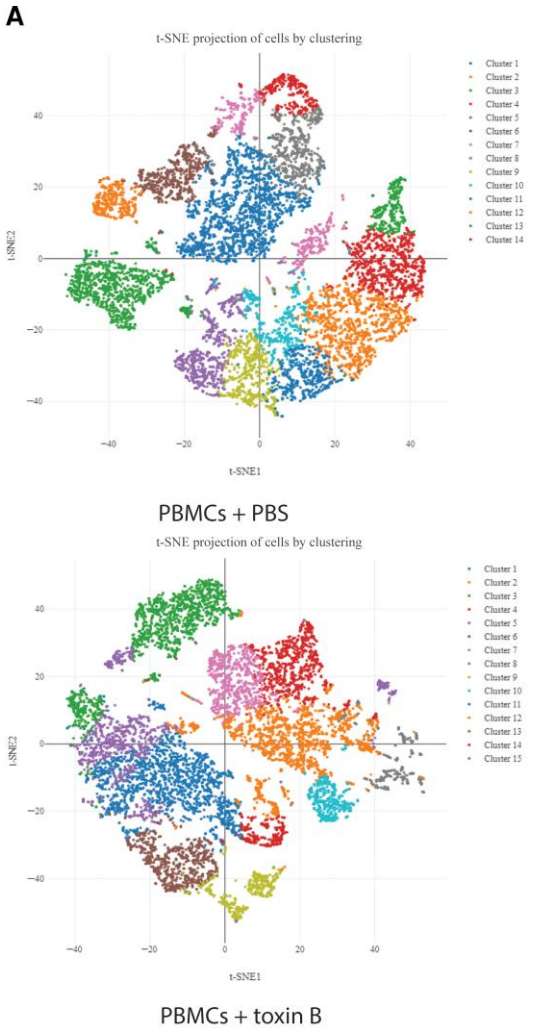
Multicolor flow cytometric immunophenotyping indicated that loratadine treatment restored the CD8 memory T-cell count in the colonic intraepithelial lymphocytes of the infected mice (Figure 4*E*). As T cells are derived from the spleen, we isolated splenocytes from loratadine-treated *C. difficile*–infected mice and transplanted them to T-cell deficient $Rag^{-/-}$ mice via intraperitoneal injection (Figure 4*A*). Splenocytes from the loratadine-treated donors successfully prevented CDI deaths in mice without affecting body weight (Figure 4*B*). The loratadine-conditioned splenocytes also protected colonic mucosal integrity and reduced colonic CCl3 mRNA expression in the infected recipient $Rag^{-/-}$ mice (Figure 4*C* and 4*D*). Thus, the protective effect of loratadine was mediated by splenocytes.

Oral loratadine treatment did not affect fecal toxin levels in the infected mice (Figure 4*F*). Although loratadine affected beta diversity in the fecal microbiome of the infected mice, it did not affect fecal alpha diversity and relative abundance (including *C. difficile*) in the infected mice (Supplementary Figure 2*A* and 2*C*).

As the intestinal memory CD8 T cells may protect against reinfection [33], we evaluated the long-term effect of loratadine in vancomycin-associated CDI recurrence (Supplementary Figure 2*D*). Consistent with a previous study [34], vancomycin initially protected the mice but showed mortality after tapering (Supplementary Figure 2*E*). Loratadine treatment prevented recurrent CDI-associated mortality in the vancomycin-treated mice (Supplementary Figure 2*E*). Body weights of mice fluctuated within a narrow range regardless of vancomycin or loratadine treatment (Supplementary Figure 2*F*).

Loratadine Exerted Toxin B–Specific Anti-inflammatory Affects in Human Immune Cells in Humanized Mice.

Toxin B is a main pathogenic factor in patients with CDI [7]. We established the first CDI colitis model with immunologically humanized HuCD34-NCG mice to study the toxin B–dependent human immune cell responses (Figure 5*A*). The HuCD34-NCG mice infected with hypervirulent toxin A^-B^+ *C. difficile* ribotype 017 showed mild weight loss without death (Figure 5*B*). The infected mice also had moderate colonic mucosal injury, as reflected by an increased histology score (Figure 5*C* and 5*D*). Oral loratadine treatment partially reversed the weight loss and colonic mucosal damage (Figure 5*B*–5*D*). As in the regular mice (Figure 4*E*), loratadine increased the proportion of human naive CD8 T cells in the



B

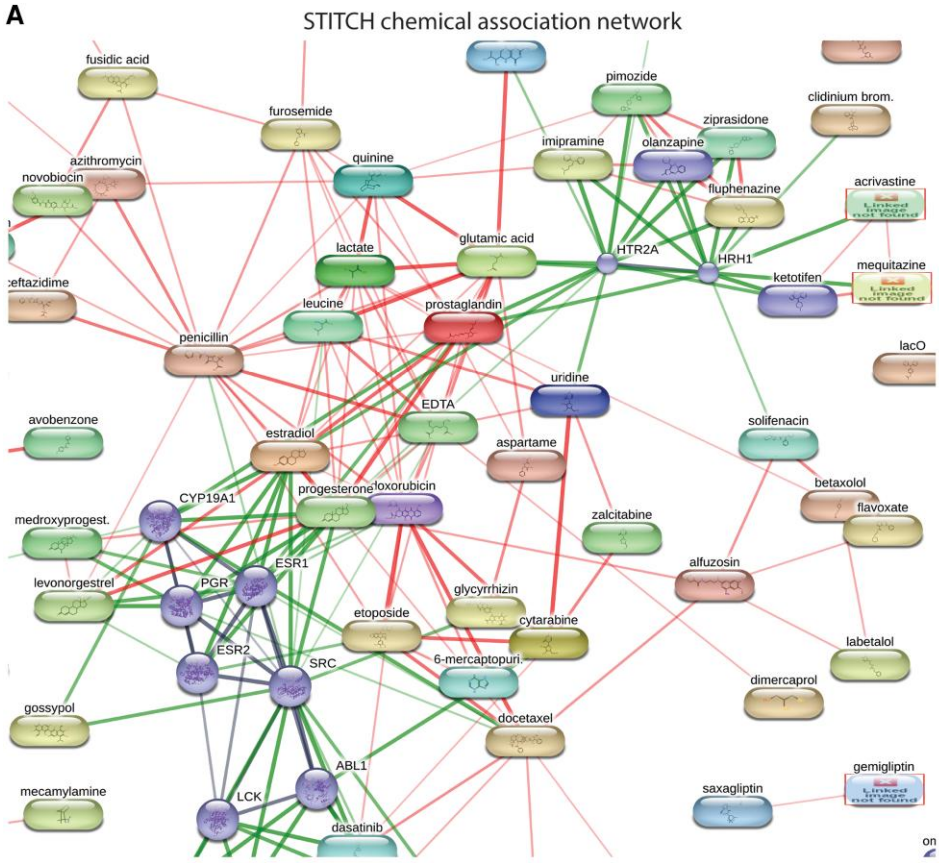
Cluster 2 classic monocytes Control	Cluster 2 classic monocytes Toxin B		CCL3-expressing intermediate monocyte cluster 7 Toxin B	
ACTN1	ACSL1	LHFPL2	ACOD1	ILR1
ANPEP	ADGRE1	LILRA5	ACSL1	IL24
APBB3	AIF1	LILRB2	AQP9	IL6
APPOBEC3A	ALCAM	LMNA	C1orf122	INHBA
C5AR1	APOBEC3A	LRRC25	CCL20	IRAK2
CCR1	APOBR	LYZ	CCL3	IRAK3
CD14	ATF3	MAFB	CCL3L1	ITGB8
CD93	C1orf122	MAP3K7CL	CCL4L2	LAIR1
CLEC5A	C5AR1	MARCKS	CCL4L2	LHFPL2
CSF3R	CALCRL	MGLL	CCL7	MAFB
CXCL3	CASP5	MMP14	CCL8	MARCKS
CXCL5	CCDC50	MS4A7	CCRL2	MMP14
CXCL8	CCL2	MYOF	CSF3R	MMP19
DMXL2	CCL22	NIBAN2	CXCL1	NLRP3
FCAR	CCL4	OLR1	CXCL2	PHLDA1
FCGRT	CCL7	P2RX7	CXCL3	PLAUR
FPR1	CCL8	PAPSS2	CXCL5	PPBP
FPR2	CD14	PECAM1	CXCL8	PTGS2
HPSE	CD86	PID1	DDX60L	PTX3
LRP1	CFP	PLEKHO2	DMXL2	RIN2
LRRK2	CSF2RA	PLIN3	DUSP6	S100A12
MPEG1	CSF3R	PLXNB2	EREG	SERPINB2
MXC1	CST3	PTX3	F3	SLC39A8
NFXL1	CTSB	RHOB	FCAR	SLC7A7
NRP1	CXCL16	RHOC	GJB2	SRC
OSCAR	CYP1B1	RIN2	GPR84	THRS1
S100A12	DMXL2	S100A10	HBEGF	TNF
S100A8	EMP1	S100A12	IER3	TNFAIP6
S100A9	ENG	S100A4	IL19	TNIP3
SERPINB2	FGL2	S100A8	IL1A	VCAN
SUL2	FPR1	S100A9	IL1B	
TGFBI	GBP1	SEMA6B		
THBS1	GNB4	SERPINB1		
TLR2	HCAR3	SLC16A10		
TMEM170B	HCK	SLC39A8		
TREM 1	IDO1	SC44A1		
VCAN	IER2	SLCA7		
	IFI30	SLC8A1		
	IL19	SRC		
	IL24	THBD		
	IL3RA	THBS1		
	IRAK3	TNFAIP2		
	ITGB8	TNFAIP6		
	KLF4	TNIP3		
	LAIR1	VCAN		
	LGALS1	WARS		

Figure 1. Single-cell fixed RNA profiling discovered toxin B–dependent gene signature in human peripheral blood mononuclear cells (PBMCs). *A*, t-Distributed stochastic neighbor embedding (t-SNE) projection of cells by clustering. The PBMCs were treated with either phosphate-buffered saline (PBS) or toxin B (0.1 μg/mL) for 6 hours. PBMCs are heterogeneous. Toxin B changed the gene expression signature in multiple clusters of cells, as determined by 10x genomics single-cell fixed RNA profiling. *B*, Differential expression analysis shows the highly expressed genes in a specific cluster relative to the rest of the sample. The $\log_2$ fold change is an estimate of the $\log_2$ ratio of expression in a cluster to that in all other cells. A value of 1.0 indicates a 2-fold greater expression in the cluster of interest. Significantly up-regulated genes are shown in the tables. Cluster 2 in PBS-treated and toxin B–treated cells showed a CD14 monocyte marker. Overall, the gene signature suggested that cluster 2 might be classic monocytes. Cluster 7 in toxin B–treated cells expressed CCL3 (macrophage inflammatory protein [MIP] 1α) and monocyte-relevant markers (CXCL1, IRAK2/3, and GPR84), suggesting them as intermediate monocytes. The single-cell fixed RNA profiling used pooled PBMCs from 3 male and 3 female donors.

blood and spleen of humanized mice (Figure 5*E*). Flow cytometry also detected a reduced proportion of human MIP-1α^{hi} cells in human CD14^{hi}-expressing cells in colonic intraepithelial lymphocytes (Figure 5*F*), suggesting reduced human MIP-1α expression in colonic monocytes and macrophages.

Loratadine Inhibited CDI in Hamsters Infected With Toxin B–Expressing *C. difficile*

Since loratadine inhibited toxin B–mediated MIP-1α secretion in human colonic explants and PBMCs (Figure 3*A* and 3*B*), we further evaluated whether loratadine ameliorates toxin B–dependent cecitis in hamsters infected with a hypervirulent toxin A$^-$B$^+$ ribotype 017 *C. difficile* strain by an established method (Figure 6*A*) [18, 32]. Consistent with our group's previous studies [18, 32], all hamsters infected with ribotype 017 survived with insignificant changes in body weight (Figure 6*B*). However, on day 3 after infection, the infected hamsters presented with signs of cecal inflammation, such as neutrophil infiltration and thickened submucosal layer, as reflected by increased histology score (Figure 6*C* and 6*D*). Oral loratadine treatment partially reduced cecal neutrophil infiltration and submucosa thickness (Figure 6*C*), as reflected by a lowered histology score (Figure 6*D*). Loratadine also significantly reduced cecal MIP-1α levels without affecting circulating MIP-1α levels in the infected hamsters (Figure 6*E*).



B MIP-1α-dependent targets in PBMCs (FDA-approved drug panel):
Histamine receptor
Serotonin receptor
Estrogen receptors
SRC

Hit drugs	Targets
Avobenzone	Apoptosis; estrogen receptor/ESR
Bosutinib	Autophagy; Bcr-Abl; Src
Acrivastine	Histamine 1 receptor (short-acting)
Lafutidine	Histamine 2 receptor
Mequitazine	Histamine 1 receptor, anticholinergic
Fluphenazine decanoate	Histamine 1 receptor, dopamine receptor
Ziprasidone	Histamine 1 receptor, serotonin receptor; dopamine receptor
Mecamylamine (hydrochloride)	Histamine receptor; nAChR
Mosapride	Serotonin receptor
Imipramine (hydrochloride)	Serotonin transporter

Figure 2. High-throughput screening identified histamine 1 receptor antagonists that prevented toxin B–mediated macrophage inflammatory protein (MIP) 1α secretion in peripheral blood mononuclear cells (PBMCs). *A*, STITCH analysis of all chemicals in the hit list. Some hits are associated with histamine 1 receptor (HRH_1), serotonin receptor (HTR2A), estrogen receptors (ESR1 and ESR2), and SRC. Abbreviation: EDTA, ethylenediaminetetraacetic acid. *B*, Target-grouped hit list for Food and Drug Administration (FDA)–approved drugs that inhibited MIP-1α secretion in toxin B–treated PBMCs.

As in the infected mice (Figure 4*F*), oral loratadine treatment did not affect cecal toxin B levels in infected hamsters (Figure 6*F*). Shotgun metagenome sequencing showed that loratadine did not influence the hamsters' cecal alpha and beta diversities or the relative abundance of *C. difficile* and other dominant bacterial species (Supplementary Figure 3*A*–3*C*).

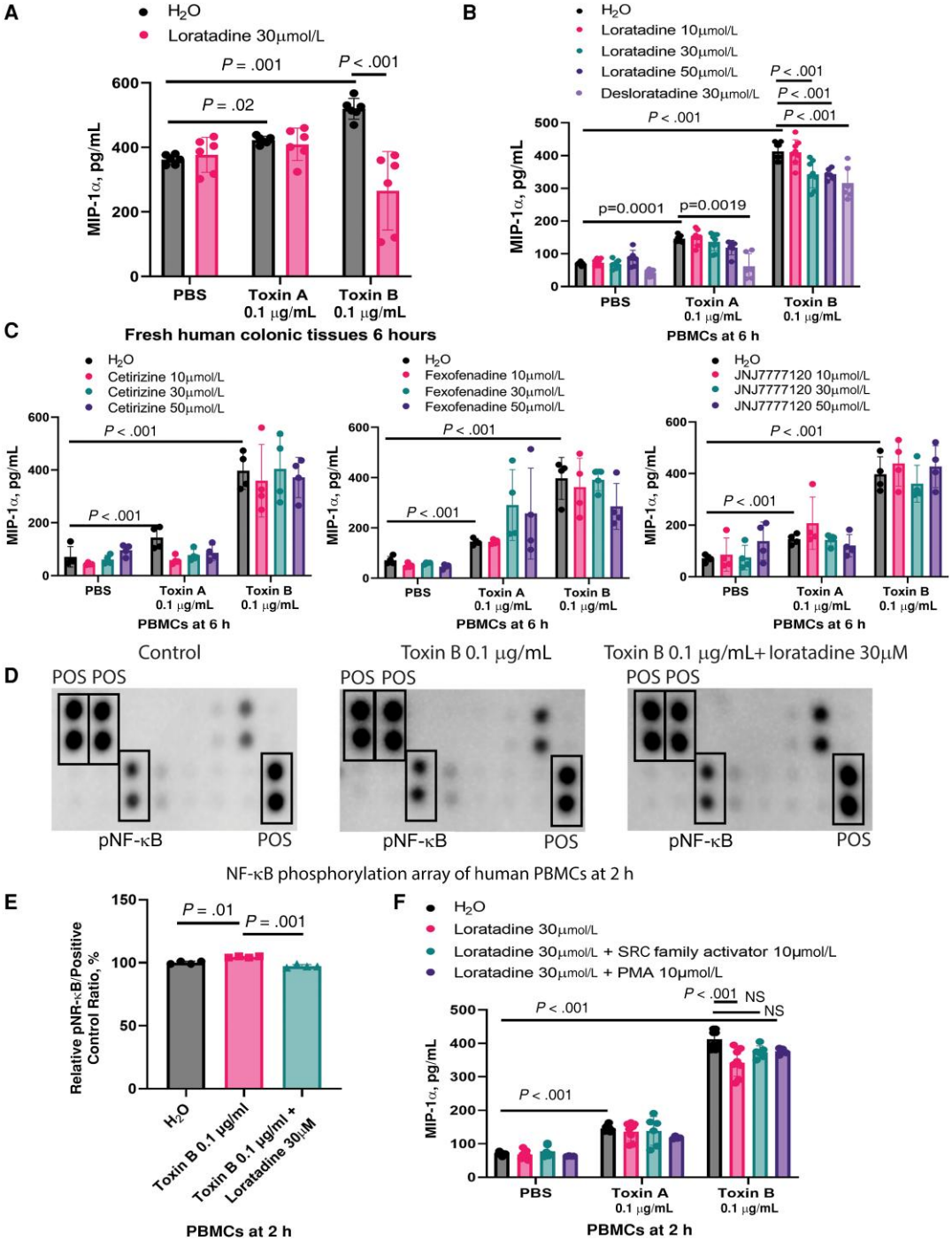


Figure 3. Loratadine inhibited toxin B–mediated macrophage inflammatory protein (MIP) 1α secretion in peripheral blood mononuclear cells (PBMCs). *A*, MIP-1α enzyme-linked iimmunosorbent assay (ELISA). Fresh human colonic tissues from 3 male and 3 female patients were pretreated with loratadine for 30 minutes, followed by toxins for 6 hours. Loratadine diminished MIP-1α secretion. Abbreviation: PBS, phosphate-buffered saline. *B*, MIP-1α ELISA. PBMCs from 3 male and 3 female donors were pretreated with loratadine and desloratadine for 30 minutes, followed by toxins for 6 hours. Loratadine (30 μmol/L) inhibited toxin B–mediated MIP-1α secretion. Desloratadine inhibited MIP-1α secretion induced by both toxins. Results were pooled from 3 experiments. *C*, MIP-1α ELISA. PBMCs from 2 male and 2 female donors were pretreated with histamine H_1 receptor antagonists (cetirizine, fexofenadine) and HRH_4 receptor antagonist JNJ7777120 for 30 minutes, followed by toxins for 6 hours. Cetirizine, fexofenadine, and JNJ7777120 had no anti-inflammatory effect against either toxin. Results were pooled from 2 experiments. *D, E*, NF-κB phosphorylation array data. PBMCs from 2 donors were pretreated with loratadine for 30 minutes, followed by toxin B for 2 hours. The cells were centrifuged and lysed by the array lysis buffer. The phosphorylation protein signals were captured and analyzed as described in Materials and Methods section. Abbreviation: pNF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; POS, positive control. Results were pooled from 2 experiments. *F*, PBMCs from 3 male and 3 healthy female donors were pretreated with SRC family activator or PMA for 30 minutes and then loratadine for 30 minutes, followed by toxins for 6 hours. The inhibitory effect of loratadine against toxin B–mediated MIP-1α secretion was reversed by the SRC family activator and PMA (NF-κB activator). Results were pooled from 3 experiments. *A–F*, Data represent means with standard deviations; 1-way analysis of variance was used. Abbreviations: NS, not significant; PMA, phorbol 12-myristate 13-acetate.

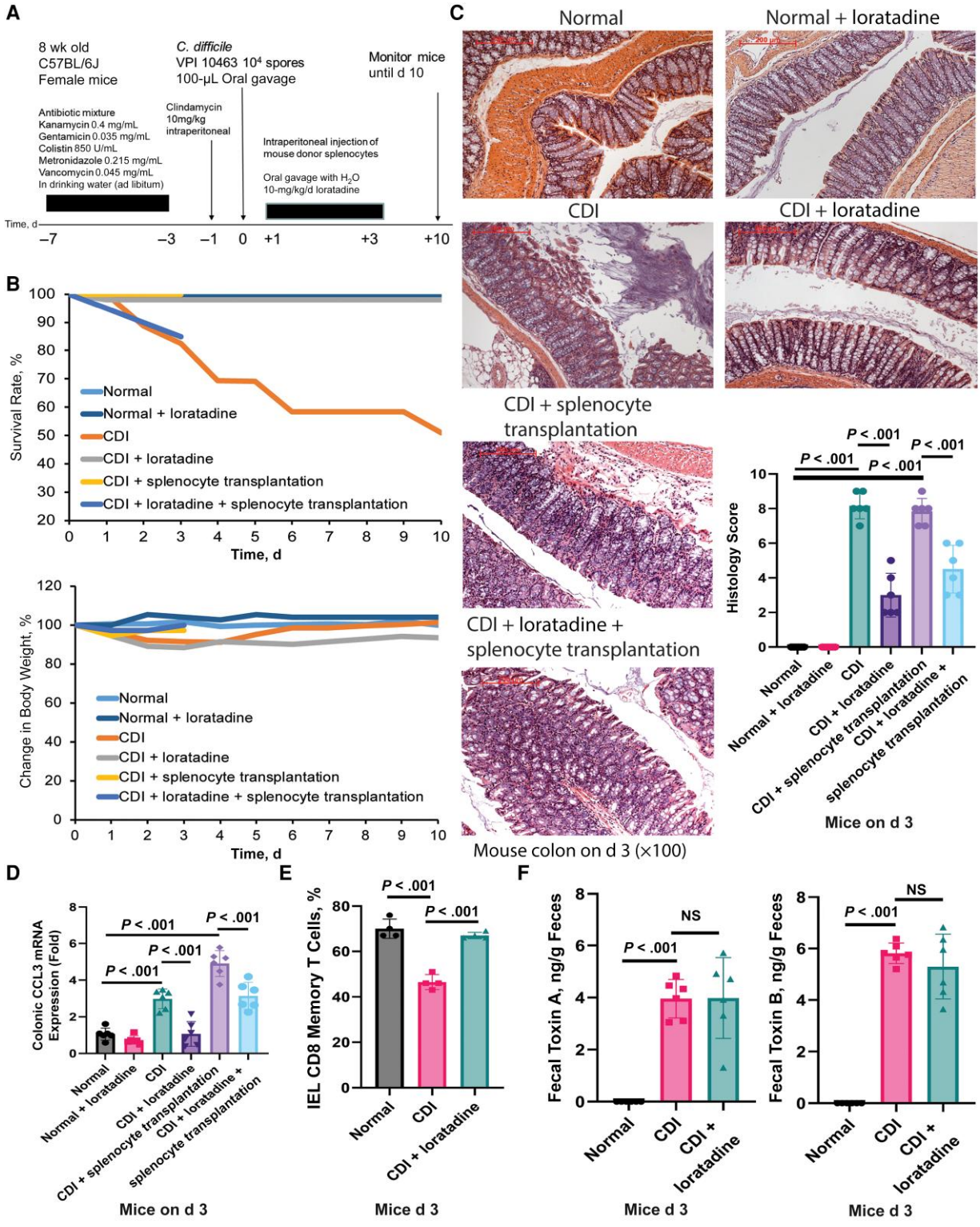


Figure 4. Oral loratadine treatment ameliorated *Clostridium difficile* infection (CDI) colitis in mice. *A*, Experimental plan of mouse primary CDI. C57BL6/J mice were infected with *C. difficile* VPI 10463. *B*, Survival rate and change in body weight. *C*, Hematoxylin-eosin–stained images of colonic tissues and colonic histology score. *D*, Colonic CCl3 messenger RNA (mRNA) expression. Both oral loratadine treatment and splenocyte transplantation of loratadine-treated mice ameliorated colitis and reduced colonic CCl3 mRNA expression in the infected mice (6 per group). *E*, Flow cytometry–based immunophenotyping of whole blood and intraepithelial lymphocyte (IEL) compartments in mice with CDI on day 3 after infection. Only statistically significant data were shown. Loratadine treatment enhanced CD8 memory T cells in the infected mice (4 mice per group). *F*, Fecal toxin A and toxin B levels in mice infected with *C. difficile* VPI 10463 (day 3; 6 mice per group. Abbreviation: NS, not significant. *D–F*, Data represent means with standard deviations; 1-way analysis of variance was used.

Again, oral loratadine treatment exerted anti-inflammatory effects in the infected hamsters without modifying the cecal microbiome.

Loratadine is Not Antibacterial Against *C. difficile*

Oral loratadine treatment did not affect the fecal relative abundance of *C. difficile* in mice and the cecal relative abundance of *C. difficile* in hamsters (Supplementary Figures 2*C* and 3*C*). Loratadine does not have antibacterial effects against *C. difficile* (Supplementary Figure 4).

DISCUSSION

This is the first study to our knowledge use single-cell fixed RNA profiling and drug screening to discover a new toxin B–dependent pathway in human immune cells. This is also the first report to describe the anti-inflammatory effect of HRH_1 antagonist loratadine in *C. difficile* toxin B–treated human immune cells. This anti-inflammatory effect conferred protection against CDI in vivo.

High-throughput drug screening to discover new therapeutic drugs against *C. difficile* pathogenesis was attempted by several groups using a variety of assays. For example, Pal and Seleem developed a high-throughput screen of the AnalytiCon MEGx library of 1000 compounds to discover natural compounds that inhibit *C. difficile* growth and toxin production [35]. Six natural compounds were identified as having good antibacterial effects against *C. difficile* with some inhibition of toxin production. These 6 compounds had no toxic effect on human colonic epithelial Caco2 cells. A compound (NP-003875) was found to confer 100% survival protection against primary CDI in mice up to 6 days after infection. Structural analysis of NP-003875 showed a similarity to mithramycin and chromomycin A2, which are known to possess antibacterial functions.

Tam et al used a toxin B–dependent cell rounding assay with image analysis [12]. Microsource's Spectrum library with 2320 compounds and Sigma's LOPAC library with 1280 compounds were screened. Sixty hit compounds reversed toxin B–mediated cell rounding of human lung IMR90 fibroblasts. Niclosamide was the best performing of several anthelmintic drugs on the hit list. Niclosamide ethanolamine (NEN) was then chosen for the in vivo study. It protects epithelial barrier function against toxin B. Although NEN does not kill *C. difficile*, it conferred 100% survival protection in the infected mice up to 16 days after infection, with a minimal impact on intestinal microbiota.

We performed a similar high-content screening to find drugs against toxins' cytopathic effect (Supplementary Figure 5*A*). We found that 60 of 4480 drugs (1.34%) reversed cell rounding in the *C. difficile*–conditioned media exposed HEK293 cells (Supplementary Figure 5*B*). STITCH chemical association analysis showed that some are linked to histamine and serotonin receptors (Supplementary Figure 5*C*). In the hit list, clemizole, homochlorcyclizine, and ziprasidone are HRH_1 antagonists. Loratadine diminished toxin-induced actin cytoskeletal disruption in toxin A^+B^+ VPI 10463–conditioned human colonic CCD-18Co fibroblasts (Supplementary Figure 6), a key event in cell rounding. Consistent with our finding, loratadine showed 57% inhibition of cell rounding in the hit list used by Tam et al [12].

Cell rounding typically occurs before cell death. However, loratadine did not affect the toxin-mediated apoptosis in human colonic epithelial cells (Supplementary Figure 7). Apoptosis in intestinal epithelial cells may be a defense tactic to restrict CDI [36]. The lack of direct antiapoptotic effects does not affect loratadine's protective effects in CDI. For the same reason, the cell rounding assay in drug screening may be inadequate to identify drugs that are protective against *C. difficile* toxins.

An HRH_1 antagonist, lodoxamide reversed toxin A–mediated leukocyte adherence and emigration and albumin leakage in rat mesenteric venules [37]. Antihistamines may mediate anti-inflammatory effects against both toxins in animals. Desloratadine, but not loratadine, inhibited toxin A–mediated MIP-1α release in PBMCs (Figure 3*B*). We speculate that loratadine and desloratadine are similarly effective against CDI in real-world situations, as toxin B is a main pathogenic factor in CDI. No drug interactions between loratadine/desloratadine and vancomycin were found (Drugs.com and DrugBank).

The binding affinities of several popular antihistamines to HRH_1 are as follows: desloratadine > cetirizine > loratadine > fexofenadine [38]. The lack of an anti-inflammatory effect of cetirizine against toxin B cannot be explained by its high binding affinity to HRH_1. The similar anti-inflammatory effect of loratadine and desloratadine against toxin B–mediated MIP-1α expression was not correlated with their different binding affinities to HRH_1 (Figure 3*B*). Thus, the binding affinity to HRH_1 is irrelevant to the anti-inflammatory effects of antihistamines against toxin B.

No report in the literature suggested the involvement of histamine in the toxin B–mediated inflammatory processes. Toxin A induces tumor necrosis factor α secretion without histamine release from rat mast cells [39]. Thus, the anti-inflammatory effect of loratadine should be independent of histamine. Loratadine binds not only to HRH_1 but also to other intracellular proteins. SRC is associated with the toxin B pathway (Figure 2*B*). Loratadine, but not other antihistamines (ketotifen and cetirizine), directly binds to SRC protein to form a complex in the whole lysates of HEK293 cells [28]. This explains why loratadine, but not cetirizine, inhibited toxin B–mediated MIP-1α expression in PBMCs (Figure 3*C*).

We used SuperPred to perform target prediction of compounds based on machine learning models (https://prediction.charite.de/index.php). Loratadine (98.58%), desloratadine (85.25%), cetirizine (97.13%), fexofenadine (98.71%), and JNJ7777120

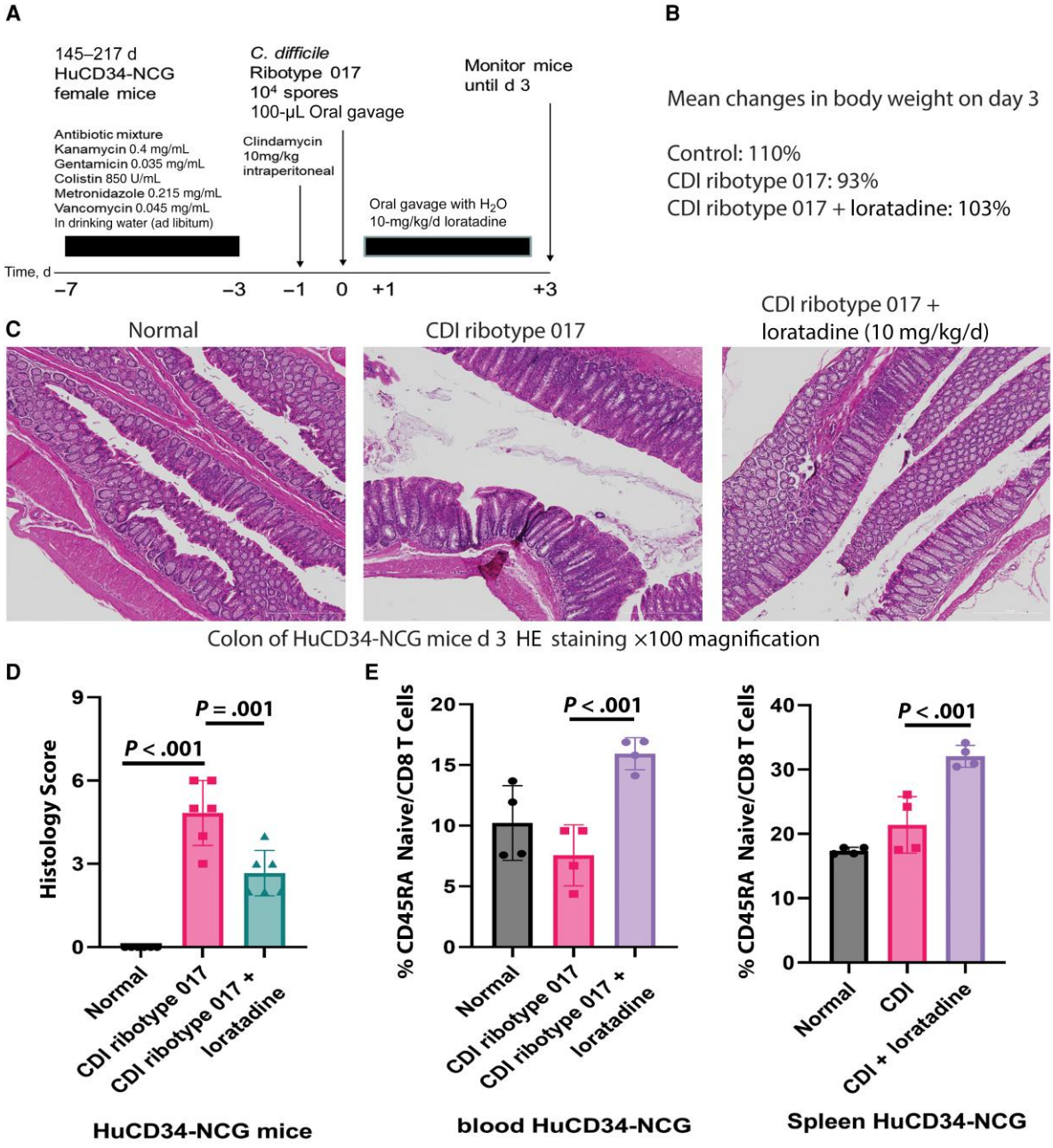


Figure 5. Oral loratadine increased circulating human naive CD8 T cells and reduced colonic human macrophage inflammatory protein (MIP) 1α–expressing monocytes in immunologically humanized HuCD34-NCG mice infected with toxin B–expressing *Clostridium difficile* ribotype 017. *A*, Experimental plan of mouse primary *C. difficile* infection (CDI). The HuCD34-NCG mice were infected with *C. difficile* ribotype 017 (2 mice per group). *B*, Body weight. *C*, Hematoxylin-eosin (HE)–stained images of colonic tissues. Multiple regions were evaluated. *D*, Colonic histology score. Data represent means with standard deviations (SDs); 1-way analysis of variance (ANOVA) was used. *E*, Flow cytometry of blood and spleen cells. Cells were labeled with human CD45RA and CD8A antibodies. Loratadine significantly increased the proportion of CD45RA^{hi} naive cells among human CD8 T cells. Data represent means with SDs; 1-way ANOVA was used. *F*, Flow cytometry of pooled colonic intraepithelial lymphocyte (IEL) cells. Cells were pooled from 2 mice per group. Cells were labeled with human MIP-1α and CD14 antibodies. Loratadine reduced the proportion of MIP-1α^{hi} cells in human CD14^{hi} monocytes and macrophages.

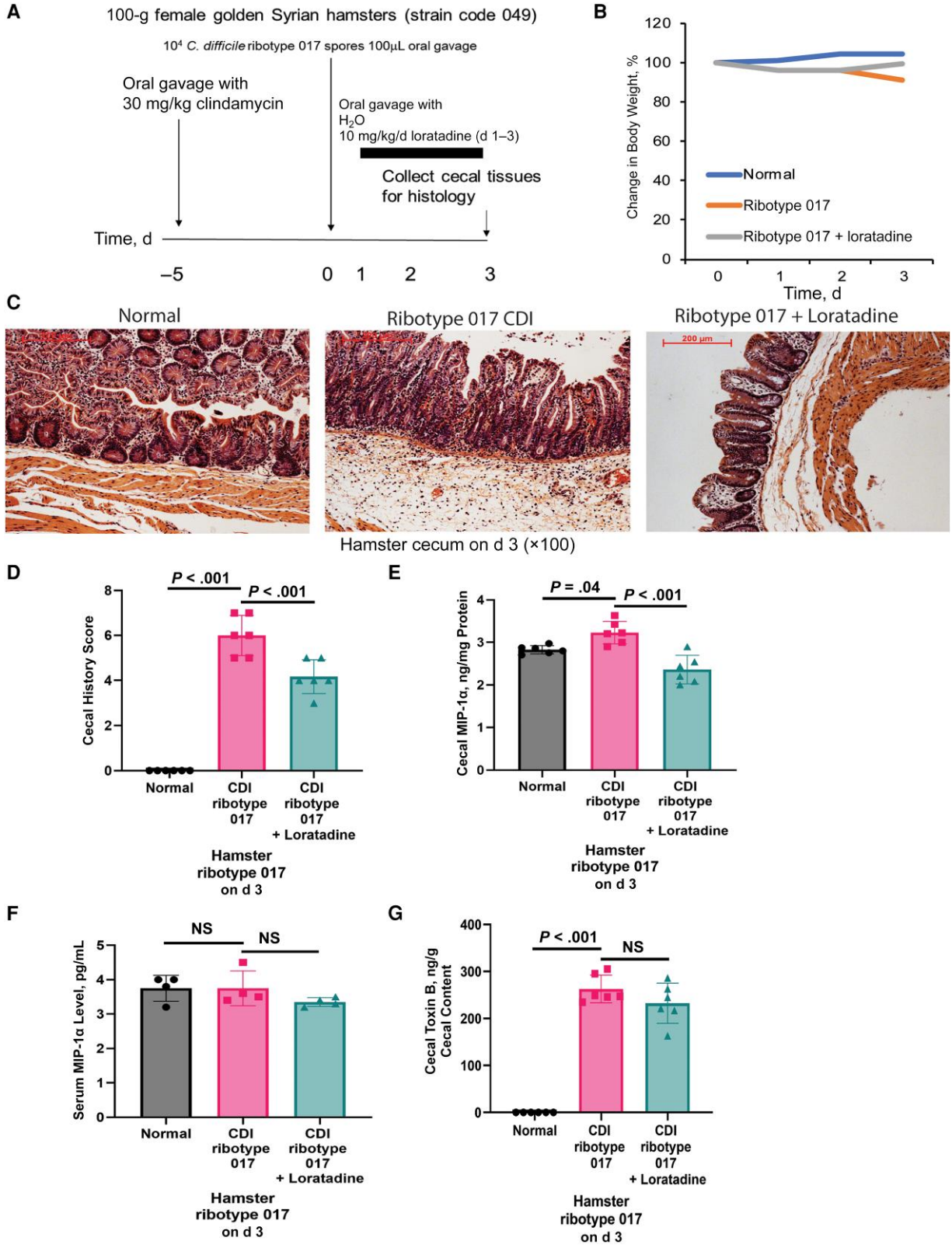


Figure 6. Oral loratadine reduced *Clostridium difficile* infection (CDI) severity in infected hamsters. *A*, Experimental plan of hamster primary CDI. Hamsters were infected with hypervirulent *C. difficile* ribotype 017, which expresses toxin B only. All infected hamsters survived until day 3. *B*, Changes in body weight in primary CDI. *C*, Hematoxylin-eosin staining images of cecal tissues. *D*, Cecal histology scores. Abbreviation: NS, not significant. *E*, Cecal toxin B levels in hamsters infected with *C. difficile* ribotype 017 (day 3; 6 hamsters per group. *F*, Cecal and serum macrophage inflammatory protein (MIP) 1α levels in hamsters. CDI caused cecitis with increased cecal histology scores and cecal MIP-1α levels, reduced by loratadine (6 hamsters per group). *D–F*, Data represent means with standard deviations; 1-way analysis of variance was used.

(96.23%) were predicted to have a high probability of interacting with NF-κB p105 subunit. On the other hand, the drug-target interaction network inference engine based on supervised analysis (https://www.genome.jp/tools-bin/dinies) predicted that loratadine, but not cetirizine and fexofenadine, may interact with the NF-κB p105 subunit with a predicted probability of 0.4428. Loratadine has a relatively higher chance of NF-κB interaction than other second-generation antihistamines.

In our previous study [19], neutralization of MIP-1α ameliorates CDI colitis with restoration of Slc26a3 and reduction of IL-1β expression in the colons of the infected mice. SLC26a3 regulates fluid transport in colonic epithelial cells, and its down-regulation leads to diarrhea [40]. IL-1β is another proinflammatory cytokine in the toxin B–treated monocyte cluster 7 (Figure 1*B*). Therefore, the loratadine-mediated MIP-1α inhibition mediates therapeutic effects via multiple downstream mechanisms. Similarly, loratadine treatment also showed anti-inflammatory effects in mouse models of gastritis, hepatitis, peritonitis, and ulcerative colitis [28].

In addition to antihistamines, serotonin receptor 2A (HTR2A) antagonists inhibited toxin-mediated pathogenesis (Figure 2). A noradrenergic and serotonergic antidepressant, mirtazapine, and a selective serotonin reuptake inhibitor (SSRI), fluoxetine, but not many other SSRIs, are associated with increased CDI risks [41]. As many SSRIs act with different mechanisms of action, the effects of drug screening with cells cannot address the diverse mechanisms of CDI in a system (Figure 2). Comorbid conditions in patients with CDI (such as cancer, depression, and immunological disorders) can further complicate the risk factor calculation of HTR2A-related drug use.

The association of the female sex hormone estrogen with CDI has been reported previously. Postmenopausal women have an increased risk of CDI-related hospitalization [42]. Our group demonstrated that estrogen depletion increased susceptibility to CDI in female mice [32]. As estrogen-related chemicals showed anti-inflammatory effects in toxin B–treated PBMCs (Figure 2*A*), a single estradiol injection delayed the onset of death in *C. difficile*–infected female mice [32]. Repeated oral estrogenlike soy isoflavone genistein treatment prevented death in the infected female mice and hamsters [32]. The estrogen-based intervention may be feasible among postmenopausal female patients with CDI.

Oral loratadine reaches peak plasma concentration in 1–2 hours. As vancomycin takes several days to stop diarrhea, loratadine may relieve CDI symptoms acutely and accelerate recovery. In hamsters infected with hypervirulent toxin A^+B^+ ribotype 027, oral loratadine treatment showed moderate survival protection (CDI survival rate, 50% with loratadine vs 20% with phosphate buffered saline) on day 3 after infection. In real-world situations, loratadine may be suitable for optimizing existing therapies rather than for stand-alone use. Although loratadine does not affect the gut microbiome in *C. difficile*–infected mice and hamsters (Supplementary Figures 2 and 3), it prevented vancomycin-associated CDI recurrence in mice (Supplementary Figure 2*E* and 2*F*). Loratadine may prevent vancomycin-associated recurrence in patients with CDI.

Some patients with CDI have inflammatory bowel disease (IBD) [43]. Patients with ulcerative colitis have a higher risk of CDI than those with Crohn's disease and those without IBD [44]. Ketotifen, an HRH_1 antagonist, partially reduced endoscopic injury in pediatric patients with ulcerative colitis [45]. Ketotifen also reduced 5-aminosalicylate intolerance among patients with IBD [46]. Thus, loratadine may be effective in patients with both CDI and IBD.

In summary, PBMCs, mainly monocytes, are the source of CDI-dependent proinflammatory cytokine MIP-1α. Histamine 1 receptor pathway is involved in the toxin B–mediated MIP-1α expression. The anti-inflammatory loratadine may be repurposed to optimize existing therapeutic approaches. A graphic summary is shown in Supplementary Figure 8.

Supplementary Data

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

Notes

Author contributions. Y. X., A. C. E., B. N., A. B., and L. F. performed the experiments. S. I. performed protein array and flow cytometry experiments. H. F. produced the *C. difficile* toxins. H. W. K. contributed to the conception and design, supervised the entire study, and wrote the manuscript. M. S. supervised and sponsored Y. X. and critically reviewed the manuscript. All authors have read, assisted with editing, and approved the final version of this manuscript. H. W. K. is the guarantor of this article.

Disclaimer. The funders were not involved in the study design, data collection, analysis and interpretation, or manuscript writing.

Data availability. We may share additional unpublished data from the study; please contact H. W. K.

Financial support. This work was supported by the National Institutes of Health (grants R01-DK128142 and R21-AI137663), the Crohn's & Colitis Foundation (grants 623027 and 1042884 to H. W. K.), and the China Scholarship Council (grant 202008210019 to Y. X.) H. W. K. paid the service charges for Prism, Endnote, Grammarly, and Biorender.

Potential conflicts of interest. All authors: No reported conflicts.

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

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