REVIEW

Exploring the epidemiology, pathogenesis, and immunotherapeutic advancements in *Clostridium difficile* infection

Lamei Wang,[1,2,3] Christina Lee,[2,3] Javier A. Villafuerte Gálvez,[2] Ciaran P. Kelly,[2] Qianyun Lin,[2] Junhu Yao,[1] Xinhua Chen,[2,*] and Yangchuan Cao[1,2,*]
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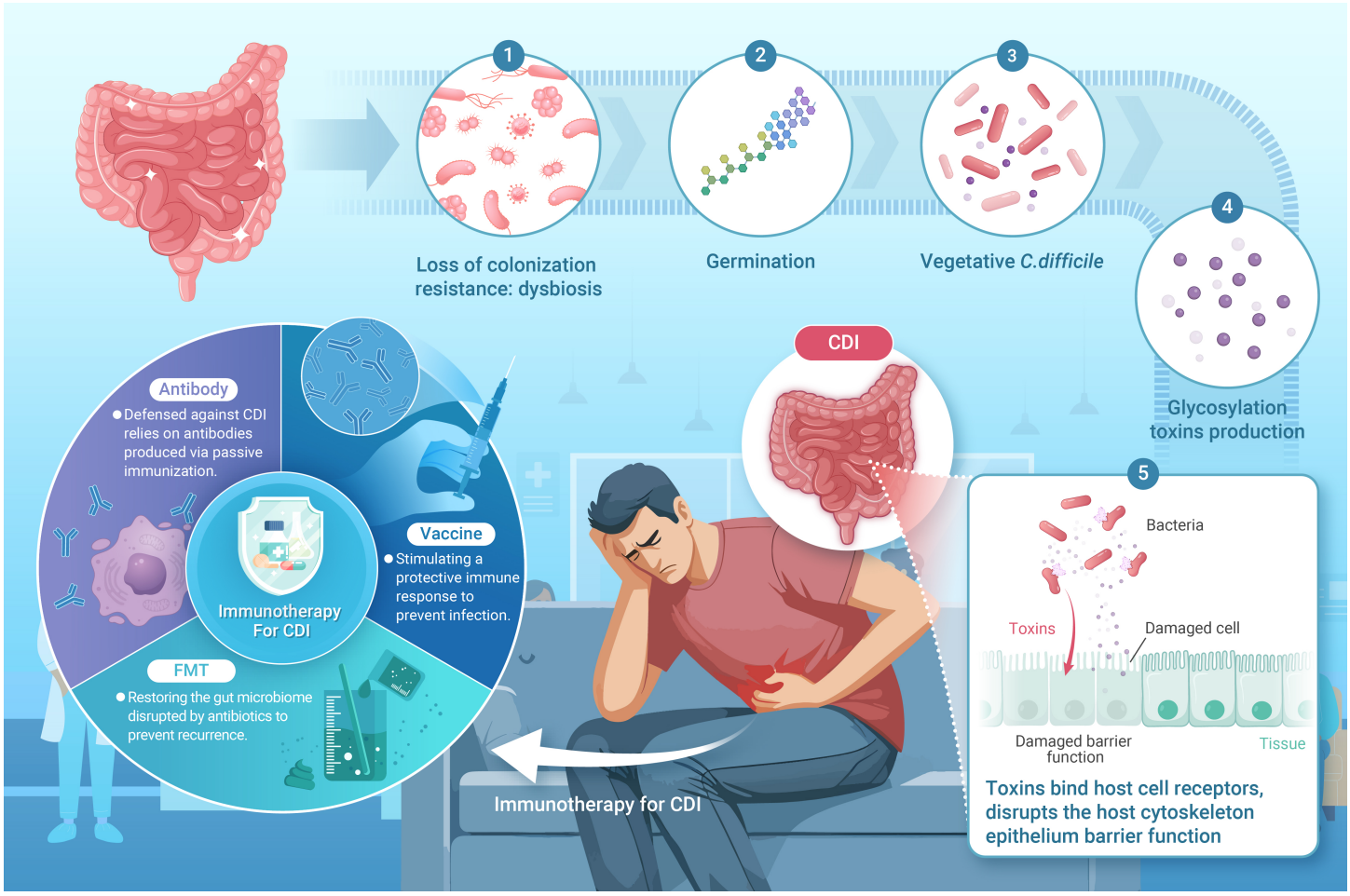
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Impact of *C. difficile*: *C. difficile* is a leading cause of severe gastrointestinal disease with significant complications.
- Traditional antibiotics disrupt the gut microbiota, increasing the risk of recurrent *C. difficile* infection (CDI).
- Emerging immunotherapies aim to reduce CDI recurrence.
- Long-term immunity strategies aim to promote lasting immunity, improving outcomes and preventing recurrent CDI.

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Clostridium difficile (*C. difficile*) is a gram-positive, spore-forming bacillus that thrives in anaerobic conditions. It serves as the primary causative agent for various diseases globally, including nosocomial antibiotic-associated diarrhea and pseudomembranous colitis, potentially leading to fatal outcomes. The production of up to three toxins by this bacterium is considered its primary virulence mechanism in *C. difficile* infection (CDI). These toxins initiate inflammation, cause tissue damage, and result in diarrhea. Conventional antibiotic treatments for CDI not only substantially reduce intestinal microbiota but also increase CDI recurrence rates. Immunotherapy has emerged as a promising approach for combating CDI, offering a novel strategy to target this challenging pathogen. Various immunotherapeutic modalities, such as monoclonal antibodies targeting specific *C. difficile* toxins, fecal microbiota transplantation (FMT) to restore microbial balance, and vaccines to stimulate protective immune responses, have shown potential in preclinical and clinical studies. This review examines the current landscape of immunotherapy for CDI, highlighting significant advancements, challenges, and future directions in utilizing the immune system to address this substantial healthcare burden.

INTRODUCTION

C. difficile infection (CDI)

Clostridium difficile (*C. difficile*) is a Gram-positive, spore-forming, obligate anaerobic bacterium. While naturally present in the gut flora of a small percentage of healthy adults, it can lead to severe infections when the normal balance of gut microbiota is disrupted, particularly after antibiotic treatment. *C. difficile* is the causative agent of *C. difficile* infection (CDI), a gastrointestinal condition that can range from mild diarrhea to life-threatening colitis.[1] The virulence of *C. difficile* is due to its ability to produce two primary toxins: Toxin A (enterotoxin): causes fluid secretion and inflammation in the intestines, leading to diarrhea; Toxin B (cytotoxin): disrupts the cytoskeleton of intestinal cells, leading to cell death and further damage to the intestinal lining.[2] *C. difficile* is primarily transmitted through the fecal-oral route. Its spores can contaminate surfaces, hands, medical equipment, and food, making it highly transmissible, particularly in healthcare settings.[3] The main risk factor for CDI is recent antibiotic use, particularly broad-spectrum antibiotics, which kill beneficial gut bacteria. Other risk factors include prolonged hospital stays, advanced age, immunosuppression, and the use of proton pump inhibitors.[4] The infection is primarily characterized by watery diarrhea, abdominal pain, fever, nausea, and loss of appetite. In severe cases, it can lead to more serious complications such as toxic megacolon, sepsis, and even death. Standard treatments include antibiotics such as metronidazole, vancomycin, and fidaxomicin. However, CDI is known for high recurrence rates, which can occur in up to 50% of cases.[5]

Epidemiology

CDI is a major cause of morbidity and mortality worldwide. In the United States, *C. difficile* is responsible for nearly 500,000 infections and about 30,000 deaths annually, making it one of the most common hospital-acquired infections in developed countries.[6,7] This results in substantial healthcare costs, including expenses related to treatment, management of recurrent infections, and extended hospital stays. A 2017 report estimated the national burden of CDI in the U.S. at 462,100 cases, with annual healthcare expenditures ranging from $1 billion to over $4 billion.[8,9] In Europe, the 2016-2017 European Point Prevalence Study reported approximately 124,000 CDI cases annually, making *C. difficile* the sixth most prevalent microorganism causing healthcare-associated infections. The burden of CDI varies by region, with North America and Europe reporting higher incidences due to better diagnostic practices and more frequent antibiotic use. However, CDI is also increasing in developing countries as global antibiotic use rises. In Japan, CDI incidence between 2006 and 2017 ranged from 0.8 to 4.71 cases per 10,000 individuals, with prevalence fluctuating between 0.3 and 5.5 per 1,000 patients. Recurrence rates in Japan ranged from 3.3% to 27.3%, highlighting ongoing challenges in managing CDI.[10]

Over the past two decades, CDI incidence has risen significantly, largely due to the emergence and spread of ribotype (RT) 027, a strain previously considered rare. This novel strain, BI/NAP1/027, has been associated with more severe clinical outcomes, higher mortality, and an increased likelihood of recurrence.[11,12] Recent data show changing *C. difficile* strain distributions across Europe and Asia, reflecting the evolving epidemiology of CDI and the impact of improved diagnostic methods.[13] Outbreaks of RT018 have been reported in Germany and France, and RT018 is now the predominant strain in Italy.[13] In contrast, RT176 has become prevalent in Poland and the Czech Republic. The historically dominant strains, such as RT027 and RT001, have seen a decline in prevalence across Europe. This shift may be attributed to advancements in diagnostic accuracy, which have improved the identification of different strains. In Asia, *C. difficile* strain diversity is notable. RT-017, characterized by the $TcdA^{-}TcdB^{+}CDT^{-}$ phenotype, is predominant in Korea and China.[10,14-17] While sporadic cases of RT027 and RT078 have been reported in China, there have been no significant outbreaks or fatalities. The first case of RT027 in China was reported in Hong Kong in 2009.[18]

This review aims to explore and critically evaluate advancements and challenges in immunotherapy for treating CDI. It provides a comprehensive analysis of how immunotherapeutic approaches—such as FMT-restore microbial balance, antibodies and vaccines target *C. difficile* toxins, and stimulate protective immune responses. This review explores emerging therapies to identify innovative strategies for reducing CDI recurrence and improving patient outcomes, offering valuable insights into addressing this persistent healthcare challenge.

PATHOGENIC MECHANISMS

CDI primarily arises from the disruption of gut microbiota, often following antibiotic use, which alters the intestinal environment and facilitates *C. difficile* colonization. Once established, *C. difficile* produces two major toxins: Toxin A (TcdA) and Toxin B (TcdB). These toxins target the epithelial cells of the colon, leading to cellular disruption, increased permeability, and subsequent cell death. This epithelial damage induces a robust immune response, causing inflammation, diarrhea, and colitis. Moreover, *C. difficile* forms highly resilient spores that can persist in the environment, contributing to the risk of recurrent infections. The interplay of microbiota disruption, toxin-mediated epithelial damage, immune activation, and spore formation underlies the pathogenesis and persistence of CDI. Indeed, the pathogenesis of CDI is multifaceted, involving intricate interactions among the pathogen itself, the host immune system, and the indigenous gut microbiota.

C. difficile Colonization

C. difficile is a prevalent healthcare-associated pathogen, indicating its

transmission in such settings and the importance of infection control measures to mitigate its spread. However, recent evidence highlights the critical role of *C. difficile* colonization in predisposing patients to infection. Colonization refers to the presence of the organism without symptoms, while infection occurs when *C. difficile* toxins or toxigenic strains are detected alongside clinical manifestations of CDI. For example, in clinical, the hallmark of CDI is diarrhea and other gastrointestinal symptoms. In asymptomatic patients, individuals are classified as colonized rather than infected. CDI is confirmed by detecting *C. difficile* toxins A or B in stool samples. Although asymptomatic individuals may carry toxigenic strains, they do not produce detectable toxin levels, and thus do not develop disease. In cases of ambiguity, molecular testing (e.g., PCR), toxigenic culture and fecal mycobiota composition may be used to determine whether the *C. difficile* strain is capable of leading infection, thus differentiating between CDI patients and asymptomatic individuals.[19] Proper classification ensures the implementation of appropriate infection control strategies. Several factors contribute to *C. difficile* colonization, including:

Antibiotic use. Antibiotic exposure is a major risk factor for *C. difficile* colonization and subsequent infection, especially in hospitalized patients. While most classes of antibiotics pose some level of risk, the extent varies depending on the specific drug and its administration route.[20] Antibiotic treatment disrupts the gut microbiota, impairing colonization resistance by altering host and microbial metabolites, thereby creating a favorable environment for *C. difficile* proliferation.[21,22] This disturbance weakens the microbiota's natural ability to resist colonization by *C. difficile*.[23] A clinical study examined the risk factors for hospital-associated CDI within a large integrated health system by analyzing a retrospective cohort of adult admissions to 21 Intermountain Healthcare hospitals from 2006 to 2012. Using multivariable logistic regression, the researchers assessed the relationship between antibiotic exposure and the risk of developing CDI. The findings revealed that prior antibiotic use was the most significant risk factor, with a 12.8% increase in the odds of CDI for each day of antibiotic therapy prior to the index admission.[20]

Immunocompromised state. Immunocompromised individuals, such as those undergoing chemotherapy or living with chronic diseases, are at heightened risk for *C. difficile* colonization.[24] Patients with inflammatory bowel disease (IBD), particularly those with colonic involvement, show increased prevalence of colonization.[25] In IBD patients, CDI is associated with pronounced intestinal dysbiosis, marked by specific alterations in the gut microbiota.[26] For example, *Helicobacter hepaticus*, a common member of the mouse gut microbiota, induces pathological inflammation in the distal intestine in mice deficient in interleukin-10 (IL-10) signaling, which mimics IBD. These mice are more susceptible to *C. difficile* colonization, even without prior antibiotic treatment, and the combination of IBD and CDI results in more severe disease than colitis alone. Importantly, IBD alters the gut microbiota, increasing susceptibility to *C. difficile*. Inflammation plays a significant role in this susceptibility; mice colonized with a less-inflammatory *H. hepaticus* mutant maintain resistance to *C. difficile*.[27] These findings underscore the critical role of host immune responses and gut microbiota in determining vulnerability to CDI in the context of IBD.

Additionally, genetic factors in IBD can influence CDI risk. A study of 319 ulcerative colitis (UC) patients identified clinical risk factors for CDI, including female gender and pancolitis, while anti-TNF therapy appeared protective. Six genetic polymorphisms, particularly in the TNFRSF14 gene, were linked to a heightened risk of CDI. The presence of high-risk genetic loci significantly increased the likelihood of CDI (20% vs 1%), suggesting that host genetics play a crucial role in CDI susceptibility among UC patients.[28]

During the COVID-19 pandemic, the increased use of antibiotics and gastrointestinal symptoms in hospitalized patients heightened the risk of CDI, particularly among the elderly.[29] Furthermore, individuals living with human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS), especially those with low CD4 cell counts and advanced disease progression, also demonstrate a greater susceptibility to *C. difficile* colonization.[30]

Hospitalization or healthcare exposure. Hospitalization is a well-established risk factor for *C. difficile* colonization. Healthcare facilities serve as reservoirs for *C. difficile* spores, which can contaminate surfaces and be transmitted to susceptible patients. Prolonged hospital stays and invasive procedures further increase this risk. To explore the risk factors associated with CDI among hospitalized adults, researchers conducted a case-control study utilizing a large insurance claims database from 2001 to 2017. They identified prior healthcare exposures through inpatient, outpatient, emergency department, and prescription drug claims, assessing these across various CDI case definitions. The study found that hospitalized CDI patients had significantly more prior healthcare exposures compared to non-CDI patients. Notably, the risk of developing CDI was highest when these exposures occurred closer to the time of hospitalization, with the association weakening as the time interval increased. These results were consistent across different CDI case definitions, emphasizing the urgent need for effective infection control strategies.[31] Here are key measures for effective sporicidal cleaning in healthcare settings: First, use Environmental Protection Agency (EPA)-approved disinfectants specifically designed to target *C. difficile* spores, typically containing sodium hypochlorite (bleach) or hydrogen peroxide. Ensure that the disinfectant is applied for the recommended contact time (usually 5 to 10 minutes) as specified by the product label, to ensure complete spore eradication. Second, healthcare staff must wear appropriate personal protective equipment (PPE), including gloves and gowns, during cleaning to prevent contamination and reduce the risk of spore transmission. These practices are essential for minimizing the spread of *C. difficile* in healthcare environments.[32]

Advanced age. Elderly individuals are particularly vulnerable to *C. difficile* colonization and infection due to age-related changes in gut microbiota, immune function, and increased healthcare exposure.[33] Physiological changes, such as reduced gastrointestinal microbial diversity and alterations in genitourinary function, create an environment conducive to *C. difficile* colonization and infection in older adults.[34] A study investigating the impact of aging on immune responses during CDI used young (2-3 months) and aged (22-28 months) mice, rendered susceptible to infection with the antibiotic cefoperazone. Researchers assessed how age and *C. difficile* strain virulence influenced disease severity. The results showed that aged mice developed more severe disease, particularly when infected with a high-virulence strain, characterized by reduced neutrophil and eosinophil levels in the ceca and colons. This reduction was associated with lower levels of the neutrophil recruiter CXCL1. Despite these immune deficiencies, aged mice displayed elevated levels of circulating white blood cells, granulocytes, and IL-17A.[35] These findings indicate age-related impairments in immune cell responses, particularly neutrophils and eosinophils, and suggest that intestinal eosinophils play a crucial role in reducing CDI severity.

Intestinal flora and the metabolite. The gut microbiota plays a critical role in preventing *C. difficile* colonization through mechanisms such as nutrient competition, bile acid metabolism, and maintaining the mucosal barrier. Antibiotic-induced disruptions in the microbiota reduce microbial diversity, alter bile acid profiles, and weaken the mucosal barrier, creating conditions favorable for *C. difficile* growth.

The gut's metabolite environment is central to host-pathogen interactions during *C. difficile* colonization. Metabolites produced by bacterial metabolism, such as short-chain fatty acids (SCFAs) like acetate, propionate, and butyrate, are generated through the fermentation of dietary fibers by commensal bacteria. These SCFAs support gut health by regulating immune responses, reducing inflammation, and maintaining the epithelial barrier. Additionally, commensal bacteria produce antimicrobial peptides and other inhibitory substances that directly suppress *C. difficile* growth.[36,37] Certain metabolites further promote competitive interactions by favoring beneficial species that inhibit *C. difficile*. For example, IL-22-mediated host glycosylation fosters the growth of beneficial bacteria, such as *Phascolarctobacterium spp.*, which competes with *C. difficile* for nutrients like succinate. This interaction underscores the collaboration between the immune system and microbiota in preventing CDI.[38]

C. difficile Toxins

C. difficile, a human intestinal pathogen, produces three distinct protein toxins: TcdA, TcdB and *C. difficile* transferase toxin (CDT). These toxins play a crucial role in the pathogenesis of CDI, contributing to the disruption of intestinal function and inflammation.[2] TcdA and TcdB are glycosylating

enzymes that disrupt the actin cytoskeleton of intestinal epithelial cells (IECs), leading to cell death and inflammation. TcdA is primarily associated with enterotoxin activity, promoting fluid secretion and contributing to diarrhea, while TcdB is more cytotoxic and has a greater impact on the integrity of the intestinal barrier. CDT is a member of the binary actin ADP-ribosylating toxins. CDT induces ADP-ribosylation of actin, resulting in cytoskeletal depolymerization and the formation of microtubule-based cell protrusions. These protrusions likely enhance bacterial adherence in the human colon, facilitating colonization and contributing to the pathogenesis of CDI. CDT further complicates the disease process by altering host cell signaling pathways and enhancing the inflammatory response. The combined effects of these toxins lead to mucosal damage, colitis, and the characteristic symptoms of CDI, including severe diarrhea and abdominal pain.

Cell Entry Mechanisms of Toxins. TcdA and TcdB, as large multi-domain proteins, must traverse the intestinal epithelial barrier to exert their toxic effects on host cells. Their cell entry process involves several steps, including binding to host cell receptors, undergoing endocytosis, and translocating across cellular membranes.

Receptor binding. TcdA and TcdB are single-chain, multidomain toxins that enter host cells via receptor-mediated endocytosis, with TcdB relying on clathrin and TcdA utilizing PACSIN.[39,40] Recent CRISPR-Cas9 screenings have identified additional receptor targets for TcdA, including sulfated glycosaminoglycans (sGAGs) and the low-density lipoprotein receptor (LDLR), enhancing our understanding of TcdA's binding and internalization mechanisms.[41] For TcdB, tissue factor pathway inhibitor (TFPI) has been recognized as a receptor, while TcdB also interacts with Frizzled proteins (FZDs) and chondroitin sulfate proteoglycan 4 (CSPG4). Notably, type-3 TcdB (TcdB3) exploits FZDs to disrupt the epithelial barrier and CSPG4 to impair the intestinal subepithelial myofibroblast layer.[42-44] This dual mechanism illustrates TcdB3's sophisticated strategy for causing gastrointestinal damage during CDI. By elucidating how these toxins interact with their target receptors, researchers can design targeted interventions to block or inhibit these pathways, offering potential for more effective treatments against toxin-mediated diseases.

Endocytosis. Endocytosis is critical for the internalization of *C. difficile* toxins into host cells after receptor binding. A recent study has demonstrated the rapid endocytosis of low-density lipoprotein receptor-related protein-1 (LRP1) in both fibroblasts and Caco-2 cells following toxin binding, highlighting LRP1's key role in TcdA-mediated internalization. In contrast, another study found that gp96 did not undergo endocytosis in the presence or absence of TcdA, suggesting a selective mechanism for cellular entry that specifically involves LRP1.[45] Chandrasekaran et al. (2016) revealed that the internalization of toxin A into CaCo2 and MEF cells is independent of clathrin but instead relies on dynamin and the Fer-CIP4 homology BAR (F-BAR) domain-containing protein PACSIN2.[40] Additionally, *C. difficile* spores interact with E-cadherin, contributing to spore adherence and internalization into IECs. Notably, TcdA and TcdB also induce the opening of adherens junctions, further enhancing spore adherence to IECs.[46]

Non-receptor-mediated membrane remodeling. Non-receptor mediated membrane remodeling is essential to the pathogenesis of CDI. This process occurs when *C. difficile* toxins interact with host cell membranes, resulting in significant alterations to cellular structure and function. A primary outcome of this remodeling is the disruption of cellular integrity, which facilitates toxin entry into host cells. Once inside, the toxins gain access to the intracellular environment and exert cytotoxic effects, thereby contributing to the progression of the infection. Notably, TcdA binds and organizes bacterial DNA, activating Toll-like receptor (TLR)9, which arranges the DNA into an inflammatory, spatially periodic structure. This interaction has profound implications for cellular processes, further emphasizing the complex mechanisms underlying CDI.[47]

Translocation. Translocation occurs through pore formation, enabling N-terminal glucosyltransferase domains of toxins to traverse from endosomes into the host cells' cytosol. Once inside, these toxins target small GTPases, disrupting various cellular processes and ultimately leading to cell death and tissue damage.

Overall, receptor binding, endocytosis, non-receptor-mediated membrane remodeling, and translocation are crucial steps in understanding how toxins contribute to cytotoxicity within cells. These mechanisms highlight the intricate nature of pathogenic bacteria during infection and provide insights into potential therapeutic targets for treating infections caused by *C. difficile*.

Action Mechanisms of Toxins. *C. difficile* causes severe gastrointestinal infections primarily through the production of toxins. These toxins alter the glycosylation of Rho and Rac GTPases, which are crucial regulators of the actin cytoskeleton.[48] This disruption compromises the integrity of IECs, leading to increased mucosal permeability. Additionally, TcdA and TcdB stimulate the production of various pro-inflammatory cytokines and chemokines, including IL-1β, TNF-α, IL-8, IL-23, IL-6, macrophage inflammatory protein-1 alpha (MIP-1α), and MIP-2 (leptin).[49] IL-23, in particular, plays a crucial role in recruiting neutrophils to the site of infection, further exacerbating tissue damage. Toxins also upregulate vascular endothelial growth factor A (VEGF-A), increasing the permeability of intestinal microvascular endothelial cells and contributing to disease progression.[50] Certain *C. difficile* strains produce a binary toxin, CDT, which binds to host cell receptors and enters IECs via endocytosis. Subsequently, CDT catalyzes actin depolymerization, leading to tissue damage and disease progression.[51] The inflammatory response induced by toxins plays a key role in CDI, driving both the severity of symptoms and tissue damage.[52]

Proinflammatory pathways of toxin A and B. TcdA and TcdB, which activate the Pyrin inflammasome by targeting glycosylphosphatidylinositol (GPI)-anchored proteins on the plasma membrane-an essential pathway for triggering inflammatory responses. Disruption of these GPI-anchored proteins allows TcdA and TcdB to activate the inflammasome, leading to pyroptosis, a form of programmed cell death associated with inflammation. This mechanism contributes to the pathogenesis of CDI by promoting inflammation and tissue damage in the host.[53]

Inflammasomes, cytosolic innate immune signaling complexes, are pivotal in host defense against diverse pathogens and danger signals. Activation of inflammasome signaling is indispensable for recovering from acute CDI. Complete abrogation of the inflammasome complex in $ASC^{-/-}$ mice or inhibition of caspase-1 in vivo results in severe mortality, increased bacterial burden, and impaired neutrophil chemotaxis mediated by keratinocyte chemoattractant (KC; also known as CXCL1).[54] Moreover, *C. difficile* enterotoxins trigger activation of executioner caspases 3/7 through the intrinsic apoptosis pathway, and caspase-3/7-mediated IECs apoptosis plays a crucial role in early-stage host defense against CDI.[55]

Controversies on the role of toxin A and toxin B in CDI

Initial studies suggest that TcdA alone can induce symptoms associated with CDI, while TcdB necessitates either coexistence with TcdA or pre-existing gut mucosa damage to establish infection.[56] This was demonstrated in animal models, where intragastric administration of TcdA alone led to severe effects, suggesting its primary role in toxicity. However, subsequent research challenged this notion by showing that disruption of TcdB significantly reduced virulence, highlighting TcdB as the key driver of pathogenesis in CDI.[57] In 2003, a study confirmed toxin B's independent pathogenicity, demonstrating that *C. difficile* strains lacking toxin A but producing toxin B could still cause intestinal infections and spread in healthcare settings.[58] Further research revealed that toxin B induced a stronger inflammatory response, more extensive colonic wall damage, and higher mortality rates in animal models compared to toxin A.[59] Studies comparing $TcdA^{+}TcdB^{-}$, $TcdA^{-}TcdB^{+}$, and $TcdA^{+}TcdB^{+}$ strains found that $TcdA^{+}TcdB^{-}$ strain strains exhibit lower virulence.[57,59] However, Kuehne et al. showed in hamster models that $TcdA^{+}TcdB^{-}$, $TcdA^{+}TcdB^{+}$ and $TcdA^{-}TcdB^{+}$ strains had comparable virulence levels.[60] More recently, clinical strains with high TcdA but minimal or no TcdB expression presented a paradox regarding the individual roles of these toxins.[61] Despite this, it is widely accepted that both toxins possess pro-inflammatory, cytotoxic, and enterotoxic properties within the human colon. Understanding the contributions of both toxins to CDI pathogenesis is essential for developing effective strategies for prevention, diagnosis, and treatment.

IMMUNOTHERAPY FOR CDI

A comprehensive understanding of the *C. difficile* life cycle and the pathogenesis of CDI is crucial for developing effective prevention and treatment strategies. Recent advances have expanded our knowledge of both primary

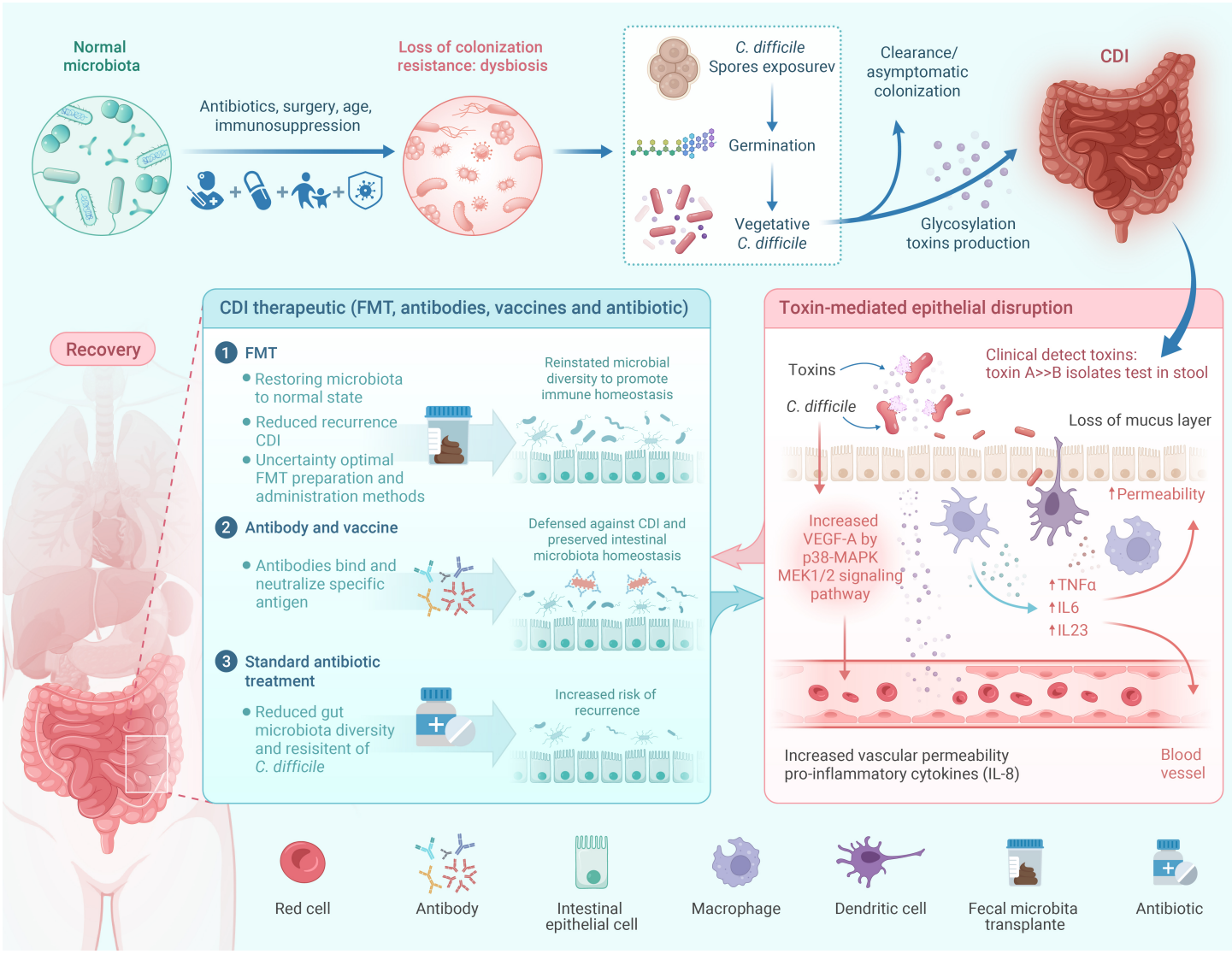

Figure 1. Pathogenesis of CDI and emerging treatment approaches for CDI *Clostridium difficile* (*C. difficile*) can colonize the colon when normal intestinal flora is disrupted due to factors such as antibiotic or surgical treatment. Following this disruption, spores germinate, leading to the emergence of *C. difficile* vegetative cells that produce toxins, ultimately resulting in CDI. In clinical settings, the concentrations of toxin A in humans significantly exceed those of toxin B, highlighting the predominant role of toxin A in CDI pathogenesis. *C. difficile* toxins initiate disease by destroying colonic epithelial cells and mucosa, disrupting the intestinal barrier, and leading to acute diarrheal illness. Several treatment approaches have emerged for CDI: 1. FMT Therapy: FMT restores microbial homeostasis and enhances colonization resistance, offering long-term protection against CDI and reducing the risk of recurrence. However, its availability is currently limited. 2. Anti-Toxin Antibodies: These antibodies, produced through passive immunization or vaccination, are released from systemic circulation into the gut lumen to prevent CDI. 3. Standard Antibiotic Treatment: While recovery often follows standard antibiotic treatment, antibiotics can further suppress gut microbiota, increasing the risk of CDI recurrence. Despite this, antibiotic therapy remains a recommended treatment approach.

and recurrent CDI, particularly in the mechanisms of pathogenesis and the development of emerging antibody-based therapies. These insights suggest new, more targeted and sustainable approaches to managing CDI. Additionally, the developments illustrated in Figure 1 underscore the importance of a multifaceted approach to effectively address CDI management.

Conventional antibiotic therapies, such as metronidazole and vancomycin, have limitations due to their broad-spectrum effects, which disrupt the gut microbiota and hinder the restoration of intestinal homeostasis, contributing to recurrent CDI. The rise of antibiotic resistance and high recurrence rates further underscore the need for alternative treatments. Immunotherapeutic strategies offer promising alternatives by harnessing the host immune system and effectively reducing recurrence without disrupting the gut microbiota. These include FMT, monoclonal antibodies, and vaccines. Immunotherapeutic strategies offer viable alternatives to conventional antibiotics for the treatment and prevention of CDI, and ongoing research holds promise for improving patient outcomes and reducing the burden of recurrent infections.

Fecal microbiota transplantation for CDI

FMT is an effective treatment for recurrent CDI. The procedure involves transferring fecal matter from a healthy donor into the gastrointestinal tract of the recipient to restore microbial diversity and balance. The introduced microbial communities outcompete *C. difficile* and stimulate the production of beneficial microbiota-derived metabolites. These metabolites promote antimicrobial activity and modulate the host immune response, thereby re-establishing intestinal homeostasis and improving patient outcomes (Figure 2).

Numerous studies have shown that FMT is highly effective in treating recurrent CDI, with success rates often exceeding 80%.[62,63] However, the precise mechanisms underlying its clinical efficacy remain unclear. Further investigation into the immune effects of FMT in CDI patients is needed to better understand the complex relationship between the microbiome and the immune system. These studies could enhance our understanding of FMT and optimize its therapeutic use in CDI management. The following section will explore potential mechanisms of FMT's clinical effects.

Modulation of Gut Immune Function. Restoration of microbial diversity is key in addressing CDI. FMT functions by reinstating microbial diversity, which is essential for immune homeostasis. It is vital that high donor microbiota diversity and enrichment in specific commensal bacteria correlate with FMT

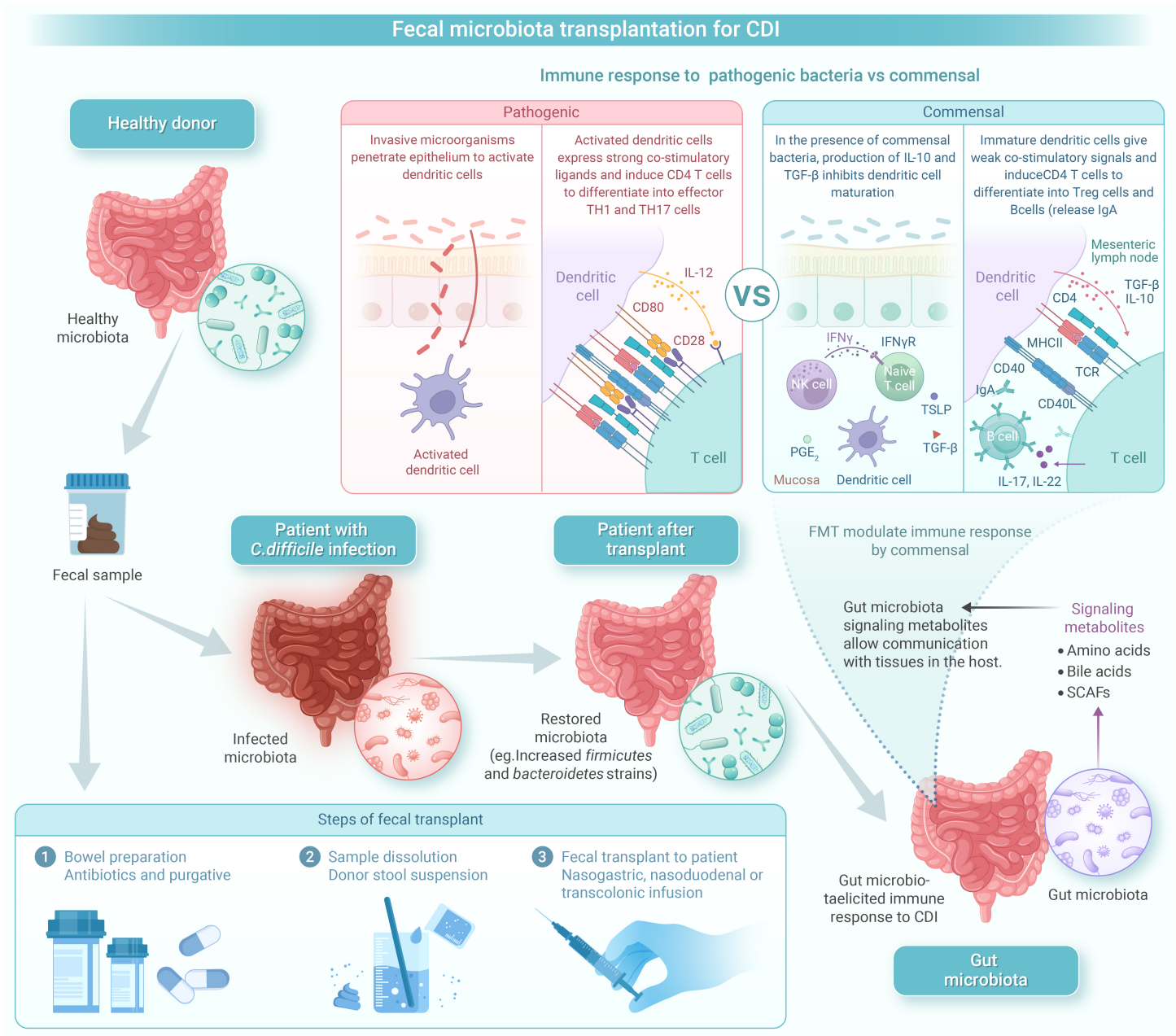

Figure 2. Fecal Microbiota transplantation (FMT) for patients with recalcitrant CDI by modulating immune response FMT restores intestinal microbial composition, thereby modulating adaptive immune responses and re-establishing microbial homeostasis in patients with CDI. The FMT process involves three main steps: 1. Bowel Preparation: Patients undergo bowel preparation with oral antibiotics, followed by a laxative to clear the intestines. 2. Sample Dissolution: Stool samples (100-150 g) are dissolved in a saline solution (0.9% NaCl) and homogenized. Larger particles are removed through filtration. Fresh fecal samples should be used within 6 hours of dissolution and at least 24 hours after the last dose of oral antibiotics. 3. FMT Procedure: The patient receives donor fecal material via capsules, naso-enteral tubes, or gastrointestinal endoscopy to restore gut microbiota homeostasis. These bacteria produce metabolites that promote the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β. In turn, these cytokines inhibit dendritic cell maturation. Immature dendritic cells provide weak co-stimulatory signals, inducing CD4 T cells to differentiate into regulatory T (Treg) cells and B cells. This process results in the release of cytokines IL-17 and IL-22 by T cells and IgA by B cells, which help counteract CDI. In contrast, in CDI patients, pathogenic microorganisms can penetrate the epithelium, activating dendritic cells to express strong co-stimulatory ligands. This activation leads to the differentiation of CD4 T cells into effector Th1 and Th17 cells, perpetuating the infection.

success in both CDI and non-CDI contexts.[64] High-throughput 16S rRNA gene sequencing studies have demonstrated significant differences in gut microbial composition before and after FMT compared to healthy donors. Post-FMT samples from patients showed an increase in *Firmicutes* and *Bacteroidetes* strains, indicative of a healthier gut composition. Notably, these compositional changes were observed within three days and coincided with symptom resolution and CDI recovery.[65,66]

FMT modifies host-microbiome interactions by altering gut microbial composition and metabolic activity, leading to enhanced immune function. Specifically, FMT promotes the production of SCFAs, such as acetate, which strengthens the gut barrier and modulates immune responses. In a mouse model of CDI, acetate alleviates symptoms by activating free fatty acid receptor 2 (FFAR2), enhancing neutrophil recruitment, inflammasome activation, and IL-1β release.[67] Additionally, acetate boosts the expression of IL-1 receptors in type 3 innate lymphoid cells (ILC3s), thereby stimulating the production of IL-22, which further enhances the host's innate immunity against CDI.[67]

Beyond SCFAs, FMT induces immune modulation by promoting the activity of regulatory T cells, increasing the production of antimicrobial peptides,[68] and elevating anti-inflammatory cytokines like IL-10 and IL-4, while simultaneously suppressing pro-inflammatory cytokines such as IL-1β and TNF-α.[69] This shift in the immune landscape is crucial in reducing inflammation and tissue damage. FMT also facilitates the accumulation of eosinophils and ILC2s, both of which contribute to immune tolerance and the maintenance of

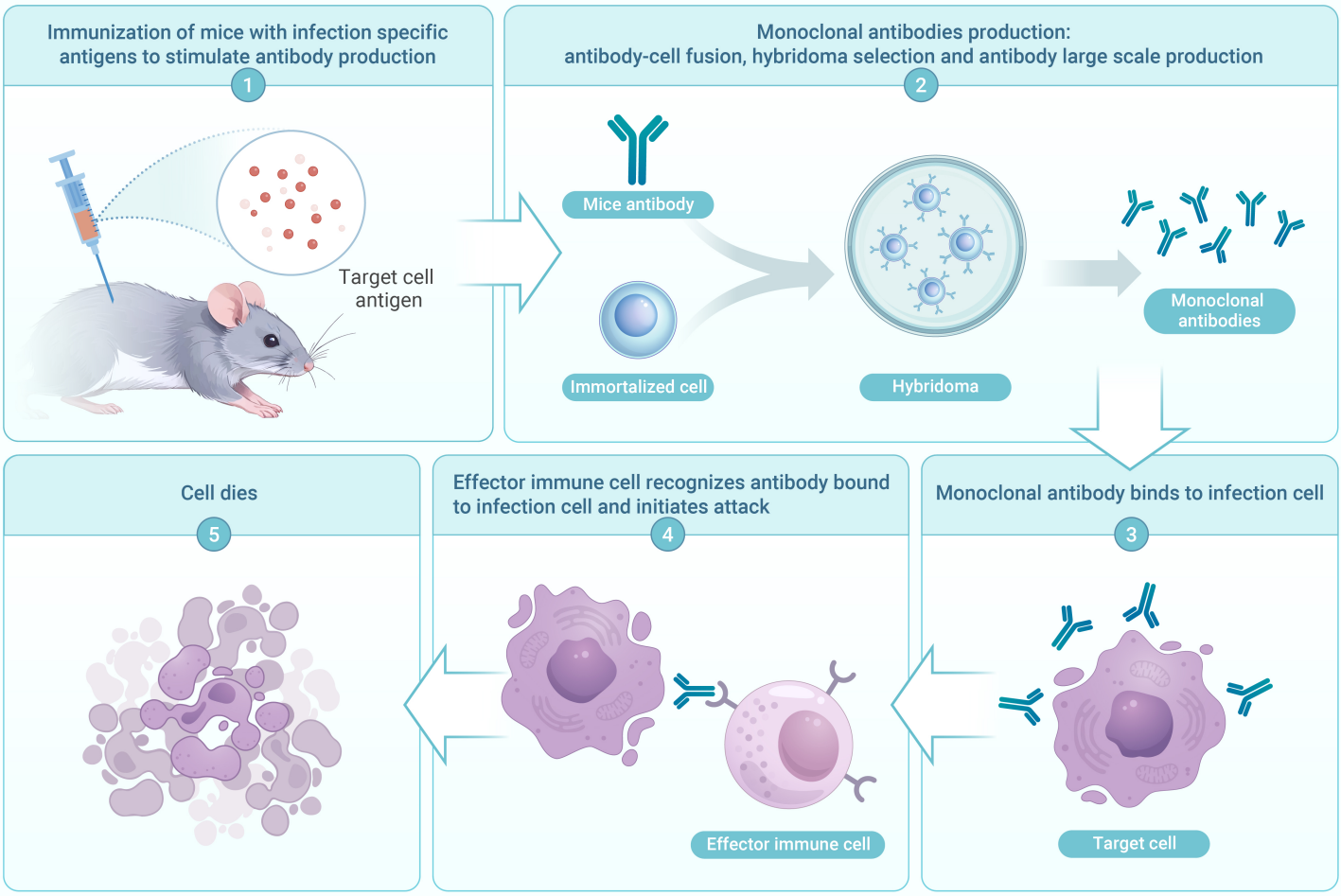

Figure 3. Schematic of monoclonal antibodies for immunotherapy Monoclonal antibodies have emerged as promising agents for immunotherapy due to their precise targeting of specific antigens. Derived from a single clone of cells, these antibodies can be engineered to recognize various targets, including tumor cells, pathogens, and immune checkpoints. The production process of monoclonal antibodies generally involves the following steps: 1. Immunization: The first step in creating a monoclonal antibody involves immunizing an animal, typically a mouse, with a specific antigen. This immunization stimulates the animal's B cells to produce antibodies that bind to the antigen. 2. Cell Fusion and Screening: After immunization, the antigen is injected into the animal to further stimulate antibody production. B cells are then fused with myeloma cells to create hybridoma cells, which are screened and cultured to identify strains capable of stably producing the desired monoclonal antibody while maintaining robust proliferation. 3. Immunomodulation: Monoclonal antibodies bind to infected or abnormal cells, triggering mechanisms that kill or inhibit the growth of these cells. 4. Mechanism of Action: Infected cells present specific antigens on their surface. When effector cells recognize these antigens, B lymphocytes produce corresponding antibodies that bind to the infected cells, initiating an immune attack. 5. Induction of Cell Apoptosis: Monoclonal antibodies activate apoptosis pathways in targeted cells, leading to their programmed cell death and enhancing the elimination of the infection.

mucosal integrity.[70-72] Specifically, FMT-induced recovery is associated with increased Th17 cell levels and the production of IL-17A and IL-22 in the large intestine's lamina propria, both of which are vital for promoting mucosal immunity.[73]Research by Buffie et al. (2015) demonstrated that FMT enhances bile acid-mediated resistance to *C. difficile* by improving mucosal integrity and bolstering immune responses.[74] Another key finding is the restoration of the microbiota-regulated cytokine IL-25, which drives the accumulation of eosinophils in the colon, offering protection against CDI.[69]

These findings highlight the role of FMT in restoring immune homeostasis and enhancing host defenses against CDI through both microbial and immune-mediated mechanisms.

Impact on systemic immune activation. Gut dysbiosis can trigger systemic immune activation, contributing to various inflammatory and autoimmune diseases. FMT has shown promise in alleviating this activation by restoring gut barrier integrity, preventing microbial product translocation into systemic circulation, and attenuating systemic inflammatory responses. Successful FMT therapy correlates with elevated levels of systemic anti-toxin IgA and IgG, which are associated with a reduced risk of recurrent CDI.[75]Furthermore, FMT increases TcdB-specific Th17 cells and specific IgG and IgA antibodies against TcdA and TcdB, supporting its protective role. This aligns with findings of reduced TcdB-specific $CD4^+$ T cells in recurrent CDI patients compared to healthy controls.[76] Both animal models and early human studies have demonstrated that successful FMT induces several immunological changes, including an increase in regulatory T cells (Tregs), which help modulate immune responses and reduce intestinal inflammation.[77] Certain bacterial species, such as *Bacteroides ovatus* from the *Bacteroidetes phylum*, stimulate significant mucosal IgA production, which restricts bacterial access to epithelial cells, promotes bacterial clearance, and regulates colonization.[78] Additionally, FMT enhances the production of the anti-inflammatory cytokine IL-10 by innate and adaptive immune cells, while reducing the production of the pro-inflammatory cytokine IL-17. Impaired antigen presentation by macrophages, monocytes, and dendritic cells further contributes to this immunomodulatory effect.[79]

These findings underscore the critical role of FMT in managing recurrent CDI. Furthermore, the potential benefits of FMT in reducing disease activity in IBD expand its therapeutic relevance beyond CDI, suggesting broader applications in treating other inflammatory conditions.

Monoclonal antibodies

Monoclonal antibodies are engineered molecules designed to mimic the immune system's capacity to combat pathogens, such as viruses and cancer cells. These antibodies precisely target surface proteins on pathogens, either marking them for immune destruction or blocking their ability to infect host cells. In CDI, monoclonal antibodies are being explored as therapeutic agents due to their potential to neutralize specific toxins or surface proteins. By binding to these targets, they can inhibit toxin binding to host cells, neutralize

Table 1. Summary of studies investigating the effective of antibody in CDI

Monoclonal antibodies	Study	Major findings	Study limitation described by author	Reference
PA-50 and B mAb PA-41	Starting on day 1, or mAbs administered intraperitoneally every other day (QOD) × 4, starting on day −1 for hamsters' model with CDI.	In a hamster model for CDI, 95% of animals treated with a combination of humanized PA-50 and PA-41 showed long-term survival relative to 0% survival of animals treated with standard antibiotics or comparator mAbs.	Need to be further applied in clinical trial	Olson et al,[87] (2012)
IgG1	Humanized IgG1 monoclonal antibodies (MAbs) which neutralize TcdA and TcdB	Reduction of the severity, duration of diarrhea, death rates, rate of recurrence in hamster model	Need to be further applied in clinical trial	Davies et al,[88] (2013)
Bezlotoxumab	Double-blind, randomized, placebo-controlled, phase 3 trials, MODIFY I and MODIFY II, involving 2655 adults; MAb Bezlotoxumab neutralize TcdB	Bezlotoxumab was associated with a substantially lower rate of recurrent infection than placebo and had a safety profile similar to that of placebo	Standard-of-care antibiotic was not standardized; no assessment of the effect of neutralization of toxin B or toxin A on the severity and duration of the baseline episode; underestimating the proportion of participants with a severe baseline episode of CDI; safety assessments were limited; other therapies for prevention of recurrent CDI were not allowed	Wilcox et al,[89] (2017)
Actoxumab	Double-blind, randomized, placebo-controlled, phase 3 trials, MODIFY I and MODIFY II, involving 2655 adults; MAb Actoxumab neutralize TcdA	Actoxumab did not improve efficacy	In light of the advanced age and coexisting conditions of the participants who died, a causal association between actoxumab treatment and death could not be established or ruled out	Wilcox et al,[89] (2017)
Polyclonal antibodies				
ABA	Tetravalent and bispecific heavy-chain-only single domain (V_HH) antibody to both TcdA and TcdB. Fulminant CDI in mice infected with an epidemic 027 strain was injected a single of the antibody; ABA bound to both toxins	ABA binded to both toxins simultaneously and displayed a significantly enhanced neutralizing activity both in vitro and in vivo	ABA was able to broadly neutralize toxins from clinical *C. difficile* isolates that expressed both TcdA and TcdB but failed to neutralize the toxin from $TcdA^-TcdB^+$ *C. difficile* strains	Yang et al,[90] (2014)
TxA4, TxB4	Sheep be immunized using the antigens TxA4 and TxB4	The production of TxA4 and TxB4 showed high binding titers in an immunoassay and protected Vero cells against the two natural toxins in-vitro	Need to be further applied in clinical trial	Lyras et al,[56] (2016)
Endogenous antibodies eAb-A and eAb-B	Randomized, double-blind, placebo-controlled, phase 3 trials; Serum samples were collected predose (day 1) and at weeks 4 and 12 postdose for measurement of eAb-A and eAb-B	Higher eAb titers against toxin B, but not toxin A, were associated with protection against recurrence CDI	The assays employed in this analysis measured polyclonal antibodies to *C. difficile* toxins A and B, and did not discriminate between neutralizing antibodies and nonfunctional antibodies	CP. Kelly et al,[91] (2019)

pathogenic effects, and potentially enhance immune responses against *C. difficile* (Figure 3). While some monoclonal antibodies have shown promise in clinical trials, further research is needed to optimize their efficacy and safety. Antibody-based therapies targeting toxins such as TcdA and TcdB offer promising alternatives due to their minimal risk of inducing gastrointestinal dysbiosis and antibiotic resistance. Current research focuses on optimizing antibody-based therapies for toxin neutralization in CDI and developing treatments for recurrent infections.

Passive immune therapy. Evidence suggests that immunoglobulins IgA, IgG, and IgM might provide protection against CDI. This is supported by findings showing an association between low levels of fecal IgA and decreased colonic IgA-producing cells with prolonged CDI symptoms and higher recurrence rates.[80,81] High titers of IgG antibodies against both toxins offer protection against primary and recurrent CDI. Elevated levels of serum IgG antibodies against *C. difficile* toxin A and B are observed in individuals with asymptomatic *C. difficile* colonization compared to those who develop diarrhea due to CDI. In patients with CDI, detectable levels of IgM and IgG antibodies against *C. difficile* toxin A are associated with protection against CDI recurrences.[82] The effectiveness of these antibodies in preventing or recurring CDI is currently under scrutiny. Detailed descriptions of several antibodies that have undergone or are undergoing clinical evaluation in humans are provided below (Table 1).

Anti-TcdA. Several pharmaceutical companies have developed monoclonal antibodies targeting TcdA to combat *C. difficile*-induced colitis. Medarex, Inc. (now part of Bristol-Myers Squibb), in collaboration with the University of Massachusetts Medical School, developed two monoclonal antibodies: actoxumab, targeting *C. difficile* toxin A, and bezlotoxumab, targeting toxin B.[83-86]

Actoxumab (MK-3415/GS-CDA1/CDA1) is a fully human monoclonal antibody developed to neutralize the cytotoxic effects of *C. difficile* toxin A by targeting the CROP domain of TcdA, thereby blocking its interaction with host cells and preventing the intoxication cascade.[92] Preclinical studies, including

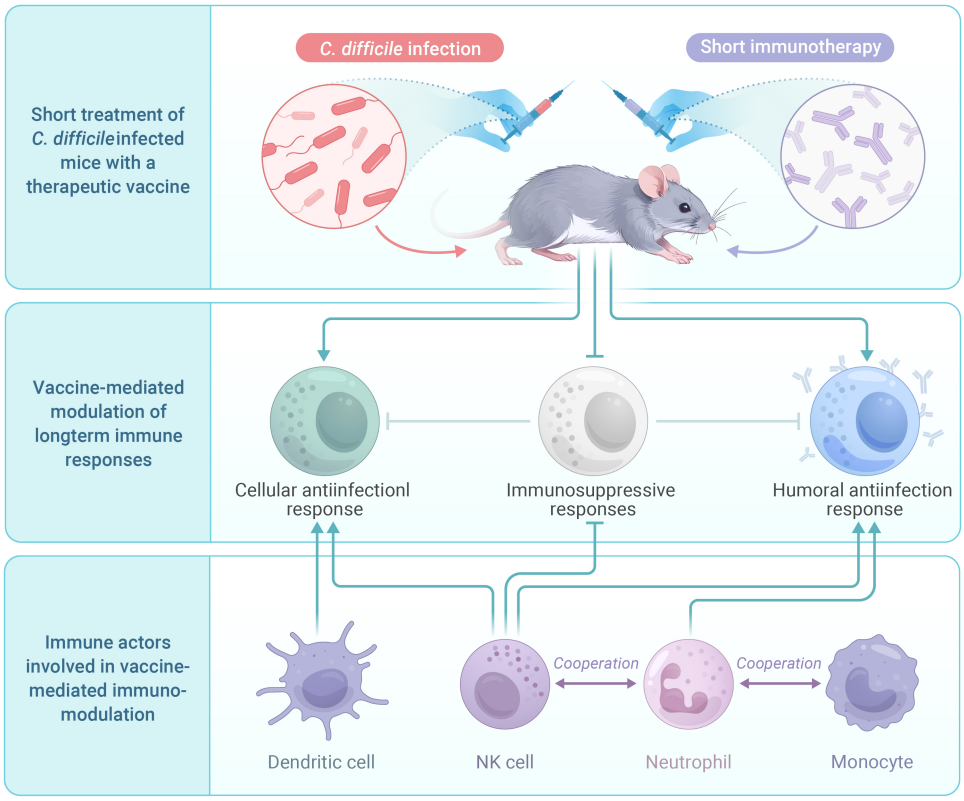

Figure 4. Mechanisms Involved in the Induction of Vaccinal Effects A short treatment with a therapeutic vaccine in mice infected with *C. difficile* induces a long-term protective immune response through several mechanisms: 1. Establishment of a Humoral Anti-Infection Response: The therapeutic vaccine stimulates the production of antibodies that specifically target the *C. difficile* pathogen, preventing its further spread. 2. Inhibition of Immunosuppressive Responses: The vaccine prevents the activation of regulatory T cells, which can suppress the immune system's ability to combat infection. 3. Induction of a Cellular Anti-Infection Response: The vaccine activates T cells, B cells, and other immune cells to recognize and attack CDI cells. Research in this mouse model of CDI has elucidated the mechanisms underlying protective immunity: Neutrophil Helper Functions: Neutrophils, traditionally known for their role in inflammation and bacterial defense, acquire helper functions that enhance antibody production by B cells. Cooperation with Monocytes and NK Cells: Neutrophils work collaboratively with monocytes and natural killer (NK) cells to strengthen the protective immune response against *C. difficile*. NK cells not only contribute to the induction of humoral and cellular responses but also help prevent immunosuppressive responses by controlling the spread of infection. Activation of Dendritic Cells by Immune Complexes: Dendritic cells, which present antigens to T cells, are activated by immune complexes formed between the *C. difficile* vaccine and target antigens, leading to an enhanced cellular anti-infection response.

those conducted in hamster and gnotobiotic piglet models, demonstrated significant protection against CDI-associated morbidity and mortality, with the gnotobiotic piglet model being particularly valued for closely mimicking human disease.[93-95] However, in a phase 3 double-blind, randomized, placebo-controlled clinical trial involving 2,655 adults with primary or recurrent CDI, actoxumab failed to show statistically significant improvements over the control group receiving standard-of-care antibiotics. This discrepancy between preclinical success and clinical outcomes highlights the challenges of translating animal model findings to human therapeutic efficacy.[96] The results underscore the complexity of CDI treatment and the limitations of current animal models in predicting clinical effectiveness. Further research is needed to clarify the causes of this divergence and explore strategies to enhance the clinical efficacy of actoxumab or similar antibody-based therapies.

Anti-TcdB. Bezlotoxumab, a fully humanized IgG monoclonal antibody targeting *C. difficile* toxin B, has shown significant potential in inhibiting toxin activity and protecting colonic cells. X-ray crystallography reveals that Bezlotoxumab's Fab fragments bind specifically to two epitopes within the N-terminal region of the TcdB CROP domain, neutralizing toxin B by blocking its carbohydrate-binding pockets and preventing attachment to colonic mucosa. Additional mechanisms, such as inducing conformational changes or impairing cell adhesion, may further enhance its neutralizing effects.

Global trials, including MODIFY II, demonstrated the efficacy and safety of Bezlotoxumab. The results showed that Bezlotoxumab, when combined with standard antibacterial treatment, was more effective in preventing CDI recurrence compared to antibiotics alone. Clinical cure rates were higher with Bezlotoxumab monotherapy than with the combination of Actoxumab and Bezlotoxumab or placebo. Additionally, sustained response rates—defined as clinical cure without recurrence within 12 weeks were significantly higher with Bezlotoxumab.[88] In October 2016, Bezlotoxumab became the first approved agent for reducing CDI recurrence, with Merck securing global development and commercialization rights for both Bezlotoxumab and Actoxumab.[97]

Vaccines for CDI

Researchers are actively pursuing vaccines against CDI to stimulate immune responses that neutralize bacterial toxins or prevent infection (Figure 4). While significant progress has been made in preclinical studies and early-phase clinical trials, developing an effective, widely available vaccine remains challenging. However, this remains a critical area of focus due to the potential for long-term protection against recurrent CDI.

Toxin-based vaccines (Toxoid Vaccines). Efforts to develop vaccines targeting *C. difficile* toxins have led to several toxoid-based candidates. Pfizer conducted a phase III clinical trial evaluating a genetically modified, full-length TcdA and TcdB toxoid vaccine in adults aged 50 and older at risk for CDI.[98] This vaccine demonstrated high immunogenicity, inducing neutralizing antibodies against both toxins. In a phase II trial (July 2015 to March 2017), 855 adults aged 65-85 were randomized to receive either the vaccine (100 or 200 μg) or placebo, with dosing schedules at 0, 1, and 6 months or 1, 8, and 30 days. The vaccine showed a favorable safety profile, good tolerability, and strong immunogenicity in the targeted population.[99]

Sanofi also initiated a phase III trial (July 2013 to November 2017) to assess the efficacy, immunogenicity, and safety of its toxoid vaccine candidate in adults over 50 at risk for CDI. However, this trial was terminated by an independent data monitoring committee due to a low likelihood of meeting its efficacy objectives.[100,101] Sanofi Pasteur also developed a bivalent vaccine consisting of formalin-inactivated toxoids A and B, which demonstrated robust immune responses and excellent tolerability in clinical trials.[102]

Purified bacterial protein vaccines. Research into microbiome-based therapeutics has also led to the development of SER-109, a formulation containing live purified proteins from *Firmicutes* bacterial spores, designed to prevent recurrent CDI. The ECOSPOR III trial demonstrated that SER-109 reduced the recurrence rate of CDI to 12%, compared to 40% in the placebo group, with both treatments showing similar safety profiles.[103] Additionally, researchers have explored the use of *Salmonella* enterica serovar *Typhimurium* flagellin (FliC) subunit D1 as an immunoadjuvant in a recombinant fusion vaccine targeting the receptor-binding domains (RBDs) of TcdA and TcdB. Mice immunized intraperitoneally with this vaccine exhibited high serum levels of anti-TcdA and anti-TcdB IgG antibodies, highlighting the potential of FliC as an immunoadjuvant in CDI vaccines.[104]

Bacterial vaccines. Bacterial vaccine development has progressed from purified protein-based strategies to engineered bacteria that induce toxin-specific immunity or enhance resistance to colonization. Wang et al. genetically modified a non-toxigenic *C. difficile* strain (CCUG37785) to express immunogenic fragments of TcdA and TcdB, creating a promising oral mucosal vaccine candidate for preventing both primary and recurrent CDI.

Another approach involves the use of *Clostridium butyricum* MIYAIRI588 (CBM588), a butyrate-producing strain, as a live biotherapeutic.[105] Butyrate enhances epithelial barrier function, induces antimicrobial peptides, and reduces inflammation.[106,107] In mouse models, CBM588 has been shown to alleviate CDI severity by recruiting neutrophils, Th1, and Th17 cells, reducing inflammation and mortality through butyrate-mediated immune responses.[108] Increasing focus has been placed on live bacterial vaccines for enhancing host immunity against CDI. For instance, a live attenuated *Salmonella Typhimurium* strain (YS1646) has been developed as a vaccine vector to deliver *C. difficile* toxin antigens. This vaccine induced robust serum IgG responses and provided complete protection in a murine model.[109] These findings underscore the potential of bacterial vaccines in CDI prevention.

DNA vaccine. DNA vaccines are emerging as promising strategies for immunization against *C. difficile*, as they encode antigenic epitopes into plasmid vectors to induce antigen-specific antibody production and cellular immune responses. In 2009, a synthetic gene encoding the RBD of *C. difficile* toxin A was optimized for human cell expression. Mouse studies demonstrated that this DNA construct elicited strong antibody responses and provided protection against lethal toxin A challenge.[110] Subsequent efforts developed a DNA vaccine encoding the RBDs of both toxin A and toxin B, which generated robust serum antibody responses with neutralizing activity both in vitro and in vivo.[111,112] Pfizer's PF-06425090, incorporating genetically modified toxins A and B, has progressed through several Phase I and II clinical trials, with ongoing Phase II and III trials. Preliminary Phase III data indicate a significant increase in anti-toxin A titers, suggesting potential efficacy in preventing CDI.[113] These findings highlight the promise of DNA and replicating vaccines in combating CDI. However, further research and clinical trials are required to comprehensively evaluate their efficacy and safety for human use.

Comparative Analysis of Immunotherapeutic Modalities for CDI

Immunotherapy is a promising alternative to conventional antibiotics for treating CDI, particularly due to its potential to reduce recurrence while preserving the gut microbiota. Monoclonal antibodies, such as bezlotoxumab, target *C. difficile* toxins A and B, neutralizing their effects and effectively lowering CDI recurrence rates when used alongside standard antibiotics. FMT, which aims to restore gut microbial diversity, has shown cure rates exceeding 90% for recurrent CDI.[114,115] By rebalancing the microbiota, FMT enhances resistance to *C. difficile* colonization. However, FMT carries potential risks, including the transmission of infectious agents or unintended microbiota alterations, necessitating thorough donor screening. Vaccines targeting *C. difficile* toxins or surface proteins are in development, with early trials showing promising immunogenicity. Larger studies are required to assess their effectiveness in preventing CDI or its recurrence. Vaccines are generally well-tolerated, with typical side effects similar to those of other vaccines.

Current evidence supports the effectiveness of immunotherapies like bezlotoxumab in reducing short- to mid-term CDI recurrence, especially when combined with antibiotics. FMT has demonstrated strong efficacy in preventing recurrence and improving long-term gut health through microbial diversity restoration.[116] However, the long-term impacts of vaccines and newer therapies, such as engineered microbiota and combination treatments, remain under investigation.

In conclusion, the choice of immunotherapeutic approach should consider infection severity, recurrence risk, comorbidities, and resource availability. Future strategies are expanding beyond monoclonal antibodies and vaccines, exploring combination therapies such as FMT with immunomodulatory agents, probiotics, and prebiotics to enhance microbial resilience. The development of engineered microbiota or targeted bacterial strains also holds promise for personalized treatment. Ongoing research is essential to evaluate the safety and efficacy of these innovative approaches.

THE ROLE OF INTESTINAL STEM CELLS (ISCs) IN CDI

ISCs are key players in the dynamic process of intestinal renewal and repair. The epithelial layer of the intestines is subjected to constant wear and tear due to digestive processes, and ISCs are essential for replenishing this layer. They reside at the base of the intestinal crypts, where they continuously produce new cells that rise up to replace older cells that are shed from the surface of the gut lining.[117] These stem cells possess the unique ability to either self-renew, maintaining the stem cell population, or differentiate into various types of IECs.[118] This differentiation is essential for replenishing cells lost through natural turnover or damage. In CDI, ISCs are critical due to their regenerative capacity, which restores the damaged epithelial lining, essential for recovery. By re-establishing epithelial integrity, ISCs help restore barrier function, reducing pathogen spread and promoting mucosal defense. Additionally, ISCs represent promising therapeutic targets to accelerate healing and strengthen intestinal resilience against CDI, suggesting new strategies for improved clinical outcomes.

Mucosal Repair

In CDI, toxins from *C. difficile* extensively damage the intestinal mucosa, causing inflammation, ulceration, and barrier disruption. ISCs, located at the base of intestinal crypts, are crucial for regenerating the epithelium by proliferating and differentiating into various cell types, including enterocytes, Paneth cells, goblet cells, and enteroendocrine cells.[119] This regenerative process restores barrier integrity, countering epithelial cell loss and tight junction disruption common in CDI.[120] Successful engraftment in experimental colitis models suggests that ISCs transplantation may be a promising therapeutic strategy to restore mucosal barrier function in patients with severe disease.[121]

Immune Modulation

ISCs can influence the production of antimicrobial peptides and cytokines, which are crucial for coordinating immune responses against pathogens such as *C. difficile*. Research has shown that IL-22 serves as a protective factor for ISCs during inflammatory intestinal damage.[122] Additionally, IL-33 activates ILC2, which help protect against colonic damage and reduce mortality.[123]

By enhancing local immune responses, ISCs contribute to infection control and limit its spread, potentially lowering the risk of recurrent CDI. This dual role in tissue repair and immune modulation underscores the importance of ISCs in maintaining gut health and preventing disease relapse.

Microbial Homeostasis

Gut microbiota profoundly influences ISC activity and intestinal morphology, contributing to homeostasis, regeneration, and immune defense. The microbiota, comprising diverse commensal microorganisms, strategically occupy functional niches throughout the intestine, including the ISC niche. These microbes inhabit the ISC niche, helping to maintain a balanced microenvironment essential for ISC function, and they produce a wide range of metabolic byproducts. Microbial metabolites engage in both direct and indirect interactions with ISCs, promoting their proliferation, differentiation, and overall activity. Gut microbes release signaling molecules such as SCFAs - including acetate, butyrate and propionate - which directly influence ISC behavior.[124] Microbial metabolites also facilitate interactions with other cell types, such as enterocytes, Paneth cells, and immune cells, shaping the local immune response and maintaining epithelial integrity.[125]

Microbial signals can also indirectly influence ISC activity by activating immune pathways, such as TLR signaling, which induces cytokine and growth factor production to support ISC proliferation and intestinal repair.[126] This complex interaction network is crucial for modulating epithelial function, promoting mucosal repair, and sustaining gut health.

Restoring a healthy microbiota is particularly important in preventing recurrent CDI. Understanding the mechanisms that regulate ISC-microbiota interactions may reveal new therapeutic strategies to manage CDI and prevent recurrence, underscoring ISCs' role in maintaining and repairing the intestinal epithelium and supporting immune modulation and microbial homeostasis.

CONCLUSION

In conclusion, understanding CDI's pathogenesis and host-pathogen interactions is crucial for developing effective preventive and therapeutic strategies. A comprehensive strategy that incorporates FMT, vaccine development, antibody therapies, and other innovative interventions is essential for overcoming the challenges of CDI management. Continued interdisciplinary

research and technological advances are paving the way for novel solutions to mitigate the global burden of CDI, ultimately improving patient outcomes and healthcare systems worldwide.

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AUTHOR CONTRIBUTIONS

Lamei Wang, Xinhua Chen and Qianyun Lin conceived the idea and edited the manuscript. Lamei Wang, Yangchun Cao, Javier A. Villafuerte Gálvez and Christina Lee collected the data and wrote the manuscript. Lamei Wang and Yangchun Cao drew the figures. Junhu Yao, Javier A. Villafuerte Gálvez and Ciaran P. Kelly validated and supervised the manuscript. All authors have read the final manuscript and approved it for publication.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

Not applicable.

DATA AND CODE AVAILABILITY

Not applicable.

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