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ORIGINAL ARTICLE

Targeted intestinal barrier repair *via* probiotic-derived engineered outer membrane vesicles: A 3A1M strategy with antioxidant, anti-inflammatory, anti-ferroptotic, and microbiome modulation effects



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Abstract Intestinal barrier disruption, driven by oxidative stress, ferroptosis, immune imbalance, and gut microbiota dysbiosis, plays a crucial role in inflammatory bowel disease (IBD) pathogenesis. Current treatments are often ineffective and cause side effects, emphasizing the need for novel therapies. Here, we have developed an engineered probiotic-derived outer membrane vesicle (OMV), GDO@CM, combining antioxidant gallic acid (GA) and anti-inflammatory H₂S for targeted intestinal barrier repair. Constructed from *Escherichia coli* Nissle 1917 (EcN)-derived OMVs, GA and diallyl trisulfide (DATS) are incorporated into the hydrophilic inner cavity and lipid bilayer, respectively, while mannose-decorated chitosan (CM) is electrostatically attached to the OMVs surface, enhancing stability and enabling targeted delivery to damaged colonic lesions. GDO@CM efficiently enters activated immune cells and epithelial cells, where GA scavenges reactive oxygen species and inhibits ferroptosis, while H₂S amplifies anti-inflammatory effects. OMVs further synergize with GA and DATS to suppress pathogenic bacteria. These combined actions facilitate effective barrier repair and alleviate IBD symptoms. Single-cell RNA

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sequencing reveals that GDO@CM reduces inflammation, increases the proportion of reparative M2 macrophages and intestinal stem cells, and promotes epithelial cell proliferation *via* the APP/CD74 axis. Our findings establish GDO@CM as a promising multi-target therapeutic for IBD, offering a novel strategy for intestinal barrier restoration.

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1. Introduction

Inflammatory bowel disease (IBD) encompasses a group of chronic, relapsing inflammatory disorders of the gastrointestinal tract, primarily including ulcerative colitis (UC) and Crohn's disease (CD). The hallmark of IBD is persistent inflammation of the intestinal mucosa, which clinically manifests as symptoms such as abdominal pain, diarrhea, weight loss, and fatigue¹. Chronic inflammation can lead to extraintestinal complications, affecting systems such as the joints, skin, and eyes, and significantly increasing the risk of colorectal cancer². These clinical manifestations profoundly impact patients' quality of life and social functioning. Worldwide, IBD affects an estimated 10 million individuals, and its incidence continues to rise. Despite the increasing prevalence, effective treatment options remain limited³. Current therapeutic strategies primarily involve pharmacological interventions, such as 5-aminosalicylic acid (5-ASA), corticosteroids (*i.e.*, dexamethasone), and immunosuppressive agents, including thiopurines and biologics^{4,5}. However, the efficacy of these treatments varies widely among patients, with many either failing to respond or experiencing relapse. Moreover, long-term use of these medications is often associated with significant adverse effects, such as increased susceptibility to opportunistic infections, hepatotoxicity, and immune suppression⁶. These challenges underscore the urgent need for the development of novel, safer, and more effective therapeutic strategies for IBD.

The precise etiology and pathogenesis of IBD remain incompletely understood. However, the role of reactive oxygen species (ROS) and the oxidative stress they induce in compromising the intestinal barrier is increasingly recognized as a key factor in the onset and progression of IBD^{7–9}. During chronic colonic inflammation, infiltrated macrophages, neutrophils, and dendritic cells become activated and produce excessive ROS¹⁰. These ROS directly damage lipids, proteins, and DNA in intestinal epithelial cells (IECs), leading to cellular injury, apoptosis, or necrosis^{11,12}. The accumulation of ROS also decreases the activity of glutathione peroxidase 4 (GPX4), which facilitates the Fenton reaction, generating more ROS and triggering ferroptosis, further compromising the intestinal epithelial barrier^{13,14}. Additionally, ROS disrupts tight junction proteins (TJPs), causing these junctions to loosen and increasing intestinal permeability¹⁵. The overproduction of ROS exacerbates intestinal inflammation by persistently activating key signaling pathways, including nuclear factor- κ B (NF- κ B) and p38 MAPK, which promote the release of pro-inflammatory chemokines and cytokines^{16–18}. These mediators amplify immune dysregulation and further impair intestinal homeostasis. Concurrently, ROS contribute to intestinal dysbiosis by promoting the growth of pathogenic bacteria while disrupting beneficial microbial populations^{19,20}. This increased intestinal permeability facilitates the translocation of harmful microorganisms and substances, further disturbing the microbial

ecosystem^{21–23}. Given the multifaceted roles of ROS in IBD pathogenesis, developing innovative therapeutic strategies that scavenge ROS, inhibit ferroptosis, restore immune homeostasis, and regulate the intestinal microbiota is essential. Such approaches hold significant promise for repairing the intestinal barrier and managing IBD progression.

Gallic acid (GA), a naturally occurring polyphenolic compound found abundantly in various plants, has garnered significant attention for its exceptional antioxidant and antimicrobial properties^{24,25}. The antioxidant mechanism of GA is complex, involving both physicochemical actions—such as directly scavenging free radicals through its phenolic hydroxyl groups and chelating metal ions—as well as the regulation of various signaling pathways and the enhancement of antioxidant enzyme activities, thereby effectively alleviating oxidative stress^{26,27}. These attributes position GA as a promising candidate for managing oxidative stress-related diseases. Additionally, GA has notable effects on gut microbiota, inhibiting the growth of pathogenic bacteria while promoting the proliferation of probiotics, further supporting its potential therapeutic application in IBD²⁸. However, the therapeutic needs of IBD extend beyond antioxidant and microbiota-modulating functions. As a complex, chronic inflammatory disorder, effective IBD management requires the comprehensive modulation of multiple pathological processes, including robust anti-inflammatory activity, restoration of immune homeostasis, and repair of the intestinal barrier^{29–32}. While GA can attenuate the sustained activation of pro-inflammatory signaling pathways through its ROS-scavenging properties, its intrinsic anti-inflammatory effects are insufficient to fully resolve the persistent inflammation characteristic of IBD³³. Furthermore, GA's limited stability in the gastrointestinal tract, susceptibility to degradation, and lack of specific targeting to inflamed tissues present significant challenges. These limitations result in uneven drug distribution, difficulty in maintaining therapeutic concentrations, and a potential risk of off-target effects on healthy tissues. Therefore, the development of innovative therapies capable of efficiently targeting IBD lesions and significantly enhancing the anti-inflammatory activity of GA is crucial for achieving precise and effective treatment of IBD.

Hydrogen sulfide (H₂S) gas therapy has emerged as a promising green therapeutic approach, attracting significant attention due to its profound effects across diverse physiological and pathological contexts³⁴. H₂S exhibits some antioxidant effects by activating the Nrf2/HO-1 signaling pathway and enhancing the activity of antioxidant enzymes, such as GPX4³⁵. Additionally, H₂S demonstrates robust anti-inflammatory properties by promoting the polarization of pro-inflammatory M1 macrophages into the anti-inflammatory M2 phenotype. It suppresses inflammatory signaling pathways, reduces the production of pro-inflammatory cytokines, and enhances the expression of anti-inflammatory cytokines through pathways such as PI3K–Akt^{36,37}. Moreover, H₂S

contributes to intestinal barrier repair by promoting the proliferation and migration of IECs³⁸. Diallyl trisulfide (DATS), an oil-soluble organosulfur compound derived from allicin, serves as a potent H₂S donor with broad-spectrum antimicrobial properties, releasing H₂S in response to glutathione (GSH)³⁹. Combining DATS as a targeted H₂S donor with GA represents a novel therapeutic strategy. This combination integrates the complementary antioxidant and anti-inflammatory effects of both agents, while enhancing the regulation of gut microbiota and promoting intestinal barrier repair. Such a regimen holds significant potential for treating IBD and related gastrointestinal disorders, addressing multiple pathological mechanisms and improving the efficacy of IBD therapies.

To facilitate the integration of GA with DATS-based H₂S gas therapy, OMVs derived from EcN have emerged as an ideal drug delivery platform⁴⁰. As a probiotic-derived natural product, EcN-OMVs exhibit low immunogenicity, excellent biocompatibility, and intrinsic capabilities to regulate gut microecology and promote intestinal health^{41–44}. More importantly, EcN-OMVs serve as an excellent drug delivery platform; their phospholipid bilayer structure and hydrophilic lumen enable the co-encapsulation of hydrophobic (*e.g.*, DATS) and hydrophilic drugs (*e.g.*, GA), thereby protecting the encapsulated drugs from environmental degradation⁴⁵. Crucially, EcN-OMVs possess innate targeting abilities for intestinal epithelial and immune cells, allowing precise delivery of therapeutic agents to the inflamed regions affected by IBD⁴⁶. This targeted approach ensures the localized and sustained release of DATS and GA at pathological sites, enhancing therapeutic efficacy⁴⁷. However, the delivery efficiency of EcN-OMVs can be compromised in the complex intestinal environment due to enzymatic activity, bile acids, and other destabilizing factors that may degrade OMVs and reduce their effectiveness. To address this challenge, research indicates that surface modifications of EcN-OMVs, such as the application of polymer coatings (*i.e.*, chitosan, polyethylene glycol, or glycosylation), can markedly improve their stability within the gastrointestinal tract^{48,49}. These surface modifications not only bolster the structural stability and functional integrity of OMVs but also enhance their utility as advanced, IBD-targeted drug delivery systems⁵⁰. This approach holds considerable promise for achieving precise, sustained, and effective treatment of IBD through the synergistic delivery of DATS and GA.

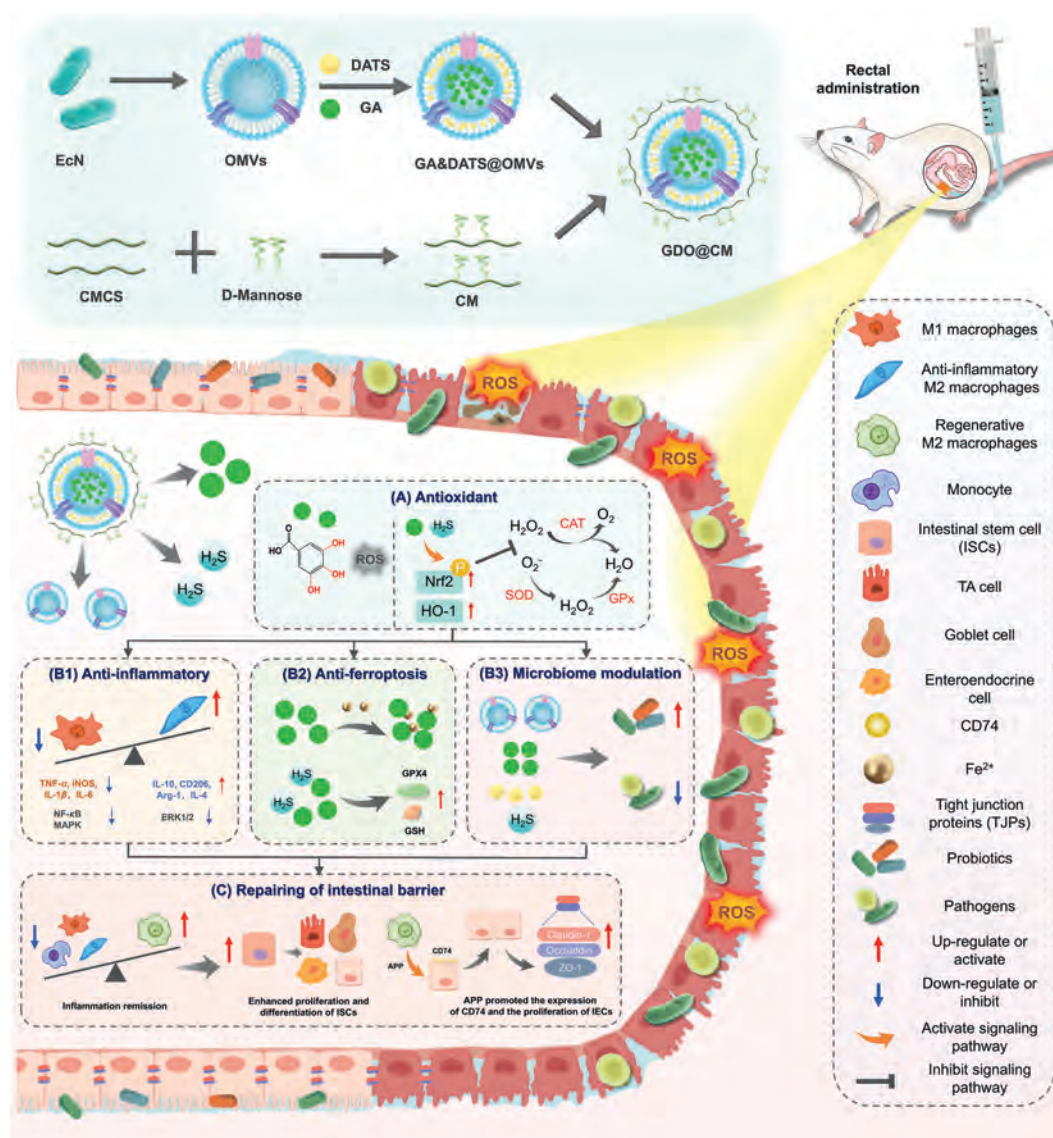
This study presents the development of engineered EcN-OMVs, termed GDO@CM, designed for targeted intestinal barrier repair in IBD. GDO@CM addresses multiple key factors, including ROS scavenging, ferroptosis inhibition, immune homeostasis regulation, and gut microbiota modulation. The preparation involves loading GA and DATS into EcN-OMVs *via* a manual liposome extruder, followed by adsorption of CM onto the OMVs surface (Scheme 1). This engineered formulation is specifically designed to target IBD lesion sites upon rectal administration, leveraging mannose receptors on macrophages to enhance delivery and internalization by both immune cells and IECs. Inside these cells, GA acts as a potent antioxidant and anti-ferroptotic agent, while DATS releases H₂S, providing anti-inflammatory benefits. In both *in vitro* and *in vivo* experiments, GDO@CM demonstrated impressive antioxidant activity, effectively inhibited inflammation, suppressed ferroptosis, reduced pathogenic bacterial growth, and promoted intestinal barrier repair. In a mouse colitis model, GDO@CM showed significant therapeutic potential for IBD treatment. 16S rRNA sequencing revealed that GDO@CM notably reshaped gut microbiota

composition, and scRNA sequencing of colonic tissues showed substantial shifts in immune cell populations, with a reduction in pro-inflammatory M1 macrophages and an increase in reparative M2 macrophages. Gene set enrichment analysis (GSEA) further confirmed the anti-inflammatory effects of GDO@CM, highlighting the suppression of several pro-inflammatory pathways. Significant changes were also observed in epithelial cell composition, with enhanced proportions of intestinal stem cells (ISCs) and mucus-secreting cells, indicating improved intestinal regeneration. Notably, the study found that APP may elevate CD74 expression on IECs through direct cell–cell interactions and secretory-mediated modulation, which in turn could promote IECs' proliferation and accelerate intestinal barrier repair. Collectively, these findings position GDO@CM as a promising therapeutic option for IBD, offering a multifaceted mechanism to restore intestinal barrier function.

2. Experimental section

2.1. Reagents and chemicals

EcN was obtained from the Bena Culture Collection (Beijing, China). Glass-bottom culture dishes were purchased from Guangzhou Jet Bio-Filtration Co., Ltd. (Guangzhou, China). The Exosome Isolation and Purification Kit (UR52121), Exosome Protein-Specific Lysis Buffer (UR33101), and Exosome Fluorescent Labeling Dye DiR (UR21017) were purchased from Umibio (Shanghai) Co., Ltd. (Shanghai, China). A manual liposome extruder (NZ-1) was purchased from NEXSTAR (Shanghai, China). DATS was sourced from Shanghai Dibo Chemicals Technology Co., Ltd. (Shanghai, China). GA was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Simulated intestinal fluid (SIF) was acquired from YuanYe Bio Co., Ltd. (Shanghai, China). Cell culture dishes and flasks were obtained from Bioland Biotech (Hangzhou) Co., Ltd. (Hangzhou, China). Oil Red O Stain, DAPI, Folin–Ciocalteu Reagent (1 mol/L), Calcein-AM/PI Cell Viability, and Actin-Tracker Red-555 were obtained from Beyotime Biotech Co., Ltd. (Shanghai, China). Carboxymethyl chitosan (CMCS, 240 kDa), 4-isothiocyanatophenyl α -D-mannopyranoside (Mannose), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 5-ASA were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Antibodies against HO-1, Nrf2, and p-Nrf2 were obtained from Proteintech Inc. (Wuhan, China). Antibodies against outer membrane protein A (OmpA) and outer membrane protein C (OmpC) were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Lipopolysaccharide (LPS) from *E. coli* O111:B4 was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). PKH67 was obtained from Mele Biotech Co., Ltd. (Hubei, China). WSP-5, Hoechst 33342, and the Cell Counting Kit-8 were purchased from Jiangsu KeyGen Biotech Co., Ltd. (Nanjing, China). The ROS DCFH-DA Probe was acquired from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The ROS Green H₂O₂ Probe was obtained from Shanghai Maokang Biotech Co., Ltd. (Shanghai, China). ABTS, CAT, SOD, GSH-Px, and Fe²⁺ Assay Kits were sourced from Suzhou Grace Biotech Co., Ltd. (Suzhou, China). Antibodies against iNOS and CD206 were obtained from Bioss Co., Ltd. (Beijing, China). RSL3 was sourced from Psaitong Biotech Co., Ltd. (Beijing, China). The GPX4 ELISA Kit was purchased from ZCIBIO Technology Co., Ltd. (Shanghai, China). The Cell Light™ Edu Apollo® 567 Imaging Kit was obtained from



Scheme 1 Schematic illustration of the construction of GDO@CM and its mechanisms of action in repairing the intestinal barrier. (A) Antioxidant: GA scavenges ROS via its phenolic hydroxyl groups, while H₂S synergizes with GA to activate the Nrf2/HO-1 signaling pathway, thereby upregulating the activity of CAT, SOD, and GSH-Px. (B1) Anti-inflammatory: GDO@CM modulates inflammatory responses by promoting macrophage polarization toward the M2 phenotype and inhibiting inflammatory cytokine expression through inhibition of the NF- κ B, MAPK, and ERK1/2 signaling pathways. (B2) Anti-ferroptosis: GA chelates Fe²⁺ through its phenolic hydroxyl groups, while H₂S synergizes with GA to prevent ferroptosis by upregulating GPX4 and enhancing GSH-Px activity. (B3) Microbiome modulation: OMVs, in combination with GA, DATS, and H₂S, restore gut microbiota balance by eliminating pathogenic bacteria and increasing microbial diversity and richness. (C) Intestinal barrier repair: GDO@CM reduces inflammation, increases the proportion of reparative M2 macrophages and intestinal stem cells, and promotes epithelial cell proliferation via the APP/CD74 axis, effectively restoring intestinal barrier integrity.

Guangzhou Ribobio Co., Ltd. (Guangzhou, China). The Live/Dead BacLight Viability Assay Kit was obtained from Invitrogen Co., Ltd. (Carlsbad, CA, USA). Dextran sulfate sodium (DSS) salt was obtained from Meilun Bio Co., Ltd. (Dalian, China). Sodium L-012 and a Protease Inhibitor Cocktail were obtained from APEX BIO Technology LLC (Houston, TX, USA). Mouse IL-1 β , TNF- α , IL-10, IL-4, and MPO ELISA Kits were purchased from Proteintech Inc. (Rosemont, IL, USA). FITC-Dextran was acquired from MedChemExpress Co., Ltd. (Shanghai, China). *S. aureus* (ATCC 25923), *C. rodentium* (ATCC 51116), and *E. coli* (ATCC 25922) were obtained from the ATCC. RAW264.7 murine macrophages and human

colon adenocarcinoma cells (Caco-2) were obtained from the ATCC.

2.2. Synthesis of GDO@CM

2.2.1. Isolation and identification of OMVs

EcN was cultured in Nutrient Broth (NB) at 37 °C with continuous shaking at 200 rpm. The extraction of OMVs was carried out according to the manufacturer's protocol provided by Umi-bio. Based on the previously established growth curve of EcN, the culture was grown to the late logarithmic phase (12 h, OD₆₀₀ = 0.9) at 37 °C. To remove cell debris, the culture was

centrifuged at $3000\times g$ for 10 min at 4 °C, and this process was repeated twice using a high-speed centrifuge (ThermoFisher Scientific, Waltham, MA, USA). The supernatant was transferred to fresh centrifuge tubes containing exosome concentration solution (ESC) and incubated for 18 h. EcN-derived OMVs were then pelleted by centrifugation at $10,000\times g$ for 1 h and resuspended in PBS. To further remove impurities, the resuspended OMVs were centrifuged again at $12,000\times g$ for 2 min. The crude OMVs preparation was subsequently purified using an Exosome Purification Filter (EPF column) by centrifugation at $3000\times g$ for 10 min at 4 °C. The purified OMVs were stored at -80 °C for further analysis. The protein content of the purified OMVs was determined using a BCA protein assay kit.

2.2.2. Synthesis of GA&DATS@OMVs (GDO)

Prior to the synthesis of GDO, the loading parameters of DATS and GA required systematic optimization. For DATS loading, DATS and OMVs were mixed at varying mass ratios (0.5/1, 1/1, 2/1, and 4/1) and extruded through a manual liposome extruder for 50 cycles to promote efficient loading. The mixture was incubated at 37 °C for 1 h, followed by centrifugation at $12,000\times g$ for 10 min (4 °C) to remove excess DATS. The DATS concentration in the supernatant was quantified using a UH5700 UV-Vis spectrophotometer (Hitachi High-Tech Corporation, Tokyo, Japan) and the loading content (LC) and loading efficiency (LE) were calculated. Results indicated an optimal DATS/OMVs mass ratio of 1/1. Subsequently, GA, DATS, and OMVs were co-loaded at different ratios (0.3/1/1, 0.6/1/1, and 0.9/1/1, w/w/w) using the same extrusion-centrifugation protocol. The resulting GA&DATS@OMVs complex was abbreviated as GDO. The loading contents of DATS and GA were determined by the UV-Vis spectrophotometry and the Folin-Ciocalteu method⁵¹, respectively.

2.2.3. Synthesis and characterization of CS-mannose (CM)

CMCS and mannose were mixed at a mass ratio of 1/1 in alkaline water (pH 9.0) and stirred at room temperature for 4 h to obtain CM. The grafting ratio of mannose was determined by nuclear magnetic resonance (¹H NMR) analysis.

2.2.4. Synthesis of GDO@CM

To optimize the synthesis of GDO@CM, CM and GDO were mixed at a series of weight ratios ranging from 1/1 to 16/1 and stirred at 4 °C for 1 h. The resulting mixture was then centrifuged at $12,000\times g$ for 15 min at 4 °C, and the supernatant was discarded. The precipitate was washed twice with PBS and centrifuged again at $12,000\times g$ for 10 min at 4 °C to obtain the final GDO@CM.

As control materials, GO@CM was prepared in parallel using a GA/OMVs weight ratio of 0.6/1 and a CM/GO weight ratio of 4/1, while DO@CM was prepared using a DATS/OMVs ratio of 1/1 (w/w) and a CM/DO ratio of 4/1 (w/w).

The particle size and zeta potential of OMVs, GDO@CM, GO@CM, and DO@CM were measured using a Zetasizer Nano ZS90 instrument (Malvern Panalytical Ltd., Malvern, Worcestershire, United Kingdom). Their morphology was observed by transmission electron microscopy (JEM-2100 Plus, JEOL Ltd., Tokyo, Japan). Western blot analysis was conducted to detect the presence of OMVs in these engineered OMVs.

2.3. Stability, *in vitro* antioxidant activity, and H₂S release capability of GDO@CM

To verify the improvement in the stability of OMVs through CM encapsulation, Oil Red O dye was encapsulated into OMVs using a manual liposome extruder to synthesize Oil Red O-loaded OMVs (ORO@OMVs). Similarly, Oil Red O-loaded OMVs@CM (ORO@OMVs@CM) was synthesized. Both ORO@OMVs and ORO@OMVs@CM were incubated in SIF (pH 6.5) for varying durations, and color changes were observed. Additionally, GDO@CM was incubated in SIF (pH 6.5) for different periods, and its particle size distribution and zeta potential were measured using DLS to assess its stability.

To determine the *in vitro* antioxidant capacity of GDO@CM, 3 mL of 0.1 μmol/L DPPH ethanol solution was mixed with 1 mL of 1 mg/mL OMVs, GO@CM, DO@CM, and GDO@CM. The mixtures were allowed to react in the dark for approximately 30 min, after which the absorbance was measured at 517 nm using a UV-Vis spectrophotometer. PBS served as the blank control, and a saturated GA solution was used as a positive control. The DPPH scavenging capacity was calculated accordingly. Moreover, the ABTS scavenging capacity of GDO@CM was assessed using a specialized assay kit. Reagents were added sequentially according to the manufacturer's instructions, and the scavenging ability was measured.

To evaluate the GSH-responsive H₂S release capacity of GDO@CM, 2 mL aliquots of GDO@CM (1 mg/mL) in SIF were placed in a sealable cylindrical glass container equipped with an H₂S detector. Simultaneously, 1 mL GSH at different concentrations (0, 1, 1.5, and 2 mmol/L) was added to the GDO@CM solution to stimulate H₂S release. The H₂S concentration (ppm) was recorded at various time points, and the amount of H₂S released was calculated using a previously established formula.

2.4. Cell viability of GDO@CM

RAW264.7 cells were cultured in complete medium (Wuhan Pricella Biotech Co., Ltd., CM-0190, Wuhan, China) at 37 °C in a 5% CO₂ incubator. Caco-2 cells were cultured under identical conditions in their specific medium (Wuhan Pricella Biotech Co., Ltd., CM-0050, Wuhan, China). Cytotoxicity was assessed using the CCK-8 assay. Non-activated macrophages (1×10^4 cells/well) and Caco-2 cells (1×10^4 cells/well) were initially seeded in 96-well plates and cultured for 24 h. The cells were then treated with PBS and GDO@CM (6.25, 12.5, 25, 50, 100, 200 μg/mL) for an additional 24 h. Cell viability was evaluated using the CCK-8 assay.

For live/dead staining, Caco-2 cells (1×10^4 cells/well) were seeded in 96-well plates and cultured for 24 h. The cells were then treated with PBS, OMVs (50 μg/mL), GO@CM (50 μg/mL), DO@CM (50 μg/mL), and GDO@CM (50 μg/mL) for 24 h. After treatment, the cells were stained with Calcein-AM and propidium iodide (PI). The stained cells were observed using an inverted fluorescence microscope (AxioObserver3, Carl Zeiss, Jena, Thuringia, Germany).

2.5. Cellular internalization and H₂S imaging

To evaluate the cellular uptake of GDO@CM, RAW264.7 cells (5×10^5 cells/well) were activated with 5 μg/mL LPS for 24 h. After activation, the cells were treated with serum-free DMEM containing 2 mg/mL PKH67-labeled GDO@CM for 4 h. After

incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min, and permeabilized with 0.5% (v/v) Triton X-100 in PBS (Beyotime Biotech) for 15 min. Subsequently, the cells were stained with Actin-Tracker Red-555 for 30 min and Hoechst for 5 min. Cellular uptake was observed using a confocal laser scanning microscope (CLSM, LSM 700, Carl Zeiss AG, Jena, Germany). The intracellular fluorescence intensity of PKH67 was quantified using ImageJ software.

For intracellular H₂S imaging, activated RAW264.7 cells were incubated with serum-free DMEM containing 2 mg/mL GDO@CM for 4 h. Afterward, the medium was replaced with 250 μ L DMEM containing 6 μ mol/L WSP-5. The cells were incubated for an additional 30 min. After incubation, the cells were washed with PBS, and intracellular fluorescence was visualized using an inverted fluorescence microscope. The fluorescence intensity of WSP-5 inside the cells was quantified using ImageJ software.

Control groups included cells treated with OMVs (50 μ g/mL), GO@CM (50 μ g/mL), DO@CM (50 μ g/mL), and GDO@OMVs (50 μ g/mL).

2.6. *In vitro* antioxidant

To evaluate the intracellular ROS and H₂O₂ scavenging capacity of GDO@CM, activated RAW264.7 cells were treated with DMEM containing 50 μ g/mL GDO@CM for 24 h. Following incubation, cells were exposed to 300 μ L of DMEM containing 5 μ mol/L DCFH-DA and ROS Green H₂O₂ probe for 30 min. After incubation, cells were washed with PBS to remove excess probes and observed under an inverted fluorescence microscope. The fluorescence intensities of intracellular ROS and H₂O₂ were quantified using ImageJ software.

To measure the enzymatic activities of CAT, SOD, and GSH-Px, activated RAW264.7 cells were first treated as described above, then the cells were lysed *via* ultrasonication on ice (300 W, 5 s on/25 s off cycles) for 30 min. The lysates were centrifuged, and the supernatant was collected for further biochemical analysis. Protein concentrations were quantified using a bicinchoninic acid (BCA) protein assay kit. To determine the enzymatic activities of CAT, SOD, and GSH-Px, aliquots of the collected supernatant were mixed with the corresponding reagents according to the manufacturer's instructions. Absorbance was measured at 510 nm for CAT, 450 nm for SOD, and 412 nm for GSH-Px using a microplate reader. Enzyme activities were calculated based on the provided formulas and expressed as follows: CAT activity in μ mol/min/mg protein (μ mol/min/mg), SOD activity in U/mg protein (U/mg), and GSH-Px activity in nmol/min/mg protein (nmol/min/mg).

To assess the effect of GDO@CM treatment on the expression of HO-1 and p-Nrf2, activated RAW264.7 cells were first treated as described above, and then the cells were collected for Western blot analysis. Cells were lysed on ice for 10 min in RIPA buffer containing a protease inhibitor. After centrifugation at 1500 $\times$ g for 10 min, the supernatant was collected for protein extraction. Protein concentration was determined using a BCA protein assay kit. Proteins were separated by SDS-PAGE, transferred onto membranes, and blocked. Membranes were then incubated with primary antibodies against HO-1, β -actin, Nrf2, and p-Nrf2. Protein bands were visualized using an enhanced chemiluminescence (ECL) kit and imaged using a gel imaging system. Band intensities were quantified using ImageJ

software, with β -actin and Nrf2 serving as the internal control for normalization.

The control groups included normal (non-activated) RAW264.7 cells, as well as activated RAW264.7 cells treated with PBS, OMVs (50 μ g/mL), GO@CM (50 μ g/mL), and DO@CM (50 μ g/mL).

2.7. *In vitro* anti-inflammatory

To evaluate the anti-inflammatory effects of GDO@CM, activated RAW264.7 cells were treated as described above. Morphological changes, including cell elongation and the number of synapses, were assessed using an inverted fluorescence microscope and quantitatively analyzed. Additionally, immunofluorescence staining was performed using antibodies against iNOS and CD206, followed by imaging with a CLSM. Quantification of pro-inflammatory and anti-inflammatory cytokine expression (TNF- α , IL-1 β , IL-6, Arg-1, IL-10, and IL-4) was performed *via* RT-qPCR, following previously reported protocols. The specific primers used for RT-qPCR are provided in the Supporting Information Table S1.

The control groups included normal (non-activated) RAW264.7 cells, as well as activated RAW264.7 cells treated with PBS, OMVs (50 μ g/mL), GO@CM (50 μ g/mL), and DO@CM (50 μ g/mL).

2.8. *In vitro* intestinal barrier repair

To investigate the effect of GDO@CM on inhibiting ferroptosis in Caco-2 cells, Caco-2 cells (1×10^4 cells/well) were seeded into 96-well plates and cultured for 24 h. Ferroptosis was induced by treating the cells with 45 μ mol/L RSL3, followed by the addition of GDO@CM (50 μ g/mL) for an additional 24-h incubation. After treatment, cells were washed twice with PBS, and cell viability was assessed using the CCK-8 assay. For live/dead staining, cells were stained with Calcein-AM and PI, and fluorescence images were captured using an inverted fluorescence microscope.

To assess the effect of GDO@CM on GPX4 protein expression, Fe²⁺ levels, and GSH-Px enzyme activity in Caco-2 Cells, Caco-2 cells (5×10^5 cells/well) were seeded into T25 flasks and cultured for 24 h. Ferroptosis was induced using the previously described method, followed by treatment with GDO@CM (50 μ g/mL) for an additional 24 h. The culture medium was collected, and GPX4 levels were quantified using an ELISA kit. For GSH-Px enzyme activity and Fe²⁺ concentration measurement, cells underwent the same treatment and were subsequently lysed by ultrasonic disruption in an ice bath (300 W, 5 s/25 s cycle) for 30 min. The lysates were centrifuged, and the supernatant was collected for protein quantification using a BCA protein assay kit. According to the manufacturer's instructions, cell supernatants from different treatment groups were mixed with the assay reagents, and absorbance was measured at 412 nm using a microplate reader. GSH-Px enzyme activity was calculated based on the provided formula and expressed as nanomoles per min per milligram of protein (nmol/min/mg). Following the instructions of the Fe²⁺ assay kit, the reagent was mixed with cell supernatants from different treatment groups, and the absorbance was measured at 550 nm. The Fe²⁺ concentration was then calculated using the previously established Fe²⁺ standard curve. Control groups included PBS, OMVs (50 μ g/mL), GO@CM (50 μ g/mL), and DO@CM (50 μ g/mL)-treated Caco-2 cells undergoing RSL3-induced ferroptosis.

To investigate the effects of GDO@CM on cell proliferation, cell migration, and tight junction protein expression, we established an H₂O₂-induced oxidative damage model. For the cell proliferation assay, Caco-2 cells (1×10^5 cells/well) were seeded into 96-well plates and cultured for 24 h. Subsequently, the cells were exposed to 200 μ L of culture medium containing 1 mmol/L H₂O₂ and 50 μ g/mL GDO@CM for 24 h. After treatment, the cells were incubated for an additional 6 h in 200 μ L of the resulting medium containing 10 mmol/L EdU. Following incubation, cells were fixed, permeabilized, stained with Hoechst dye, and finally visualized the EdU fluorescence using an inverted fluorescence microscope.

For the cell migration assay, Caco-2 cells (1×10^5 cells/well) were seeded into 12-well plates with a 3-well Ibidi culture insert and incubated at 37 °C for 24 h. After removing the culture insert to create a 0.5 mm scratch, the cells were treated as previously described. Following treatment, the cells were stained with crystal violet and observed under an inverted microscope. ImageJ software was used to quantitatively analyze the fluorescence intensity and measure the width of the scratch in the images.

For the quantification of tight junction protein expression, Caco-2 cells (1×10^5 cells/well) were seeded into 6-well plates and cultured at 37 °C for 24 h. Afterward, the cells were treated as previously described. Following the incubation, total RNA was extracted, and the mRNA expression levels of ZO-1, Occludin, and Claudin-1 were analyzed using RT-qPCR. The primer sequences used in this study are provided in Table S1.

Control groups included PBS, OMVs (50 μ g/mL), GO@CM (50 μ g/mL), DO@CM (50 μ g/mL), and GDO@OMVs (50 μ g/mL)-treated Caco-2 cells subjected to H₂O₂-induced oxidative damage.

2.9. *In vitro* antimicrobial effect

S. aureus, *E. coli*, *C. rodentium*, and EcN were cultured overnight at 37 °C in Luria-Bertani (LB) broth. After incubation, bacterial cells were harvested by centrifugation at 3000 rpm for 10 min. *S. aureus*, *E. coli*, and *C. rodentium* suspension (10^6 CFU/mL) was then mixed with 100 μ L of GDO@CM (2 mg/mL) and incubated at 37 °C for 4 h. Following incubation, the bacterial suspensions were serially diluted to concentrations ranging from 10^6 to 10^2 CFU/mL and evenly plated onto LB agar plates. The plates were incubated at 37 °C for 24 h, and colonies were counted to assess bacterial survival rates, serving as a quantitative measure of the antimicrobial activity of GDO@CM. For the live/dead staining assay, after centrifugation, the bacterial cells were stained with a combination of SYTO-9 (1 mmol/L) and PI (1.5 mmol/L) for 30 min. EcN (10^8 CFU/mL) was co-cultured with GDO@CM, and the optical density (OD₆₀₀) was measured at 3 h intervals to assess bacterial growth kinetics. The stained bacterial samples were examined under an inverted fluorescence microscope. PBS was used as a control group.

2.10. *In vivo* therapeutic effect on DSS-induced colitis in mice

All animal experiments in this study were approved by the Animal Care and Use Committee of Wenzhou Medical University Animal Ethics Committee (Approval No. wydw 2025-0021). Thirty female BALB/c mice (20–25 g) were purchased from Beijing SPF Biotech Co., Ltd. (Beijing, China) and acclimatized for 7 days prior to the experiment.

Acute colitis was induced by administering 3% (w/v) DSS in drinking water for one week^{52–54}. Twenty-four colitis mice exhibiting significant weight loss and fecal occult blood positivity were then randomly divided into four groups ($n = 6$), with continued DSS administration for an additional 10 days to sustain the inflammatory condition. On Days 1, 3, 5, 7, and 9, each mouse with colitis received a single rectal administration of 100 μ L 5-ASA (20 mg/mL), OMVs (1 mg/mL), or GDO@CM (1 mg/mL). The Normal control group ($n = 6$) received 100 μ L PBS throughout the entire treatment period.

To evaluate the retention of GDO@CM at the damaged colonic tissues, colitis mice were first fasted for 24 h, followed by rectal administration of DiR-GDO@CM (1 mg/mL) at a volume of 100 μ L. The *in vivo* distribution of DiR-GDO@CM was imaged using an *in vivo* imaging system (Guangzhou Biolight Biotech. Co., Ltd., AniView 100pro, Guangzhou, China) at 0, 1, 3, 6, and 9 h post-administration. For *in vivo* tracking of DiR-labeled GDO@CM, we utilized an emission window of 745–820 nm (λ_{em}) to leverage the characteristic near-infrared fluorescence profile of the DiR dye. This spectral range is optimal for deep-tissue imaging, enabling accurate quantification of nanoparticle biodistribution and retention kinetics. Upon sacrifice, the intestines were collected, and the fluorescence intensity was quantified.

Body weight, stool consistency, and rectal bleeding were monitored daily throughout the experimental period. DAI was calculated based on previously described methods, which include assessments of body weight loss, stool consistency, and the presence of rectal bleeding. The survival rate for each group was recorded daily and calculated based on the number of surviving mice.

On Day 11, mice were orally administered FITC-Dextran (5 mg/100 g) to assess the ability of GDO@CM to restore the colonic epithelial barrier. Four hours later, orbital blood samples were collected, and the fluorescence intensity of FITC-Dextran in the serum was measured using a multimode microplate reader. Plasma concentrations of FITC-Dextran were determined using a pre-established standard curve.

On Day 11, mice received an intraperitoneal injection of 100 μ L L-012 probe solution (5 mg/100 g body weight). *In vivo* ROS imaging was then performed using an emission wavelength range of 535–600 nm (λ_{em}), corresponding to L-012's peak fluorescence emission spectrum, to quantify intestinal luminescence and evaluate GDO@CM's ROS scavenging capacity in inflamed tissues.

At the end of treatment, orbital blood samples were collected from mice for routine blood tests. Besides, all mice were euthanized, and their colon tissues and spleens were collected for measurement of colon length and spleen size, respectively. For histological analysis, colon segments and major organs were rinsed, fixed, embedded, sectioned, and stained with H&E. Histological scores were then assessed using previously reported methods.

To evaluate the anti-inflammatory effects of GDO@CM, colon segments were homogenized, centrifuged, and the supernatants were analyzed for MPO, IL-6, IL-1 β , IL-10, TNF- α , and GPX4 levels using ELISA kits. The MDA levels and enzyme activity levels of CAT, SOD, and GSH-Px were measured according to the manufacturer's instructions⁵⁵. Immunofluorescence staining was performed on colon sections using antibodies against GPX4, F4/80, iNOS, CD206, ZO-1, Occludin, and Claudin-1, and the stained

samples were observed under an inverted fluorescence microscope.

2.11. *ScRNA-seq*

A total of 14542 cells were included in the analysis, with 5758 cells from the DSS group and 8784 cells from the GDO@CM group. Quality control filters retained cells with gene counts ranging from 200 to 7000, RNA counts exceeding 1000, and mitochondrial gene expression comprising less than 5% of total reads. The merged dataset was normalized, and the top 2000 highly variable genes were selected for downstream analysis. Libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego) at 150 bp paired-end read lengths by Shanghai Personal Biotech (Shanghai, China).

For dimensionality reduction and clustering, Uniform Manifold Approximation and Projection (UMAP) was employed following principal component analysis (PCA). A shared nearest neighbor (SNN) graph was constructed using the FindNeighbors and FindClusters functions to identify distinct cellular subpopulations. Initial cluster identities were determined based on canonical marker genes, further refined through Gene Ontology (GO) enrichment analysis and pseudotemporal trajectory inference to ensure robust annotation.

To characterize transcriptional differences between the DSS and GDO@CM groups, we conducted a comparative analysis to identify differentially expressed genes (DEGs). Functional perturbations were systematically assessed through multi-modal enrichment strategies, including GO term annotation, KEGG pathway analysis, and GSEA, to elucidate pathway-level activation dynamics. Cell communication networks were constructed using CellChat (version 1.6.1), identifying significantly overexpressed genes and computing cell–cell communication probabilities based on ligand–receptor, polymer, and cofactor interactions. Finally, the expression patterns of CD74 in both the DSS and GDO@CM groups were computationally extracted and visualized to further investigate its regulatory role in cell signaling.

2.12. *Microbiome analysis*

For 16S rRNA gene sequencing, fecal samples were collected from mice after euthanasia and divided into four groups: a healthy control group (Normal), a DSS-induced colitis group without treatment (DSS), an OMVs treatment group (OMVs), and the GDO@CM group (GDO@CM). The samples were immediately frozen on dry ice and stored at -80°C . Total genomic DNA was extracted from these samples using the E.Z.N.A. Soil DNA Kit. DNA quality and concentration were assessed using agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer. The V3–V4 region of the 16S rRNA gene was amplified with primers 338F and 806R using an ABI GeneAmp 9700 PCR thermal cycler. Microbial community composition analysis was performed according to the standard protocol provided by Majorbio Bio-Pharm Technology (Shanghai, China), utilizing the Illumina MiSeq sequencing platform. Operational taxonomic units (OTUs) were clustered at a 97% similarity threshold using UPARSE software (version 7.1). Taxonomic classification of representative sequences from the OTUs was performed using the RDP Classifier (version 2.2) against the Silva database (132/16S bacterial version) with a confidence threshold set to 0.7. Comprehensive data analysis was conducted on the Majorbio Biocloud platform to

explore the microbial community structure and diversity across the sample groups.

2.13. *Statistical analysis*

The results are presented as mean $\pm$ standard deviation (SD), based on at least three independent experiments. Statistical analysis was conducted using unpaired *t*-tests and one-way analysis of variance (ANOVA) as applicable. Data analysis was conducted using Prism software (version 9; GraphPad, La Jolla, CA, USA). *P* values were considered statistically significant at $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

3. Results and discussion

3.1. *Preparation and characterization of GDO@CM*

The preparation of GDO@CM involves several key steps. First, EcN-OMVs are isolated using an Exosome Isolation and Purification Kit. Next, GA and DATS are loaded into the hydrophilic inner cavity and the lipid bilayer of EcN-OMVs, respectively, using a manual liposome extruder to generate GA&DATS@OMVs (referred to as GDO). Finally, CM is electrostatically adsorbed onto the surface of GDO, forming GDO@CM. As shown in Fig. 1A, EcN-OMVs are cup-shaped, spherical vesicles with an average particle size of 146.1 nm, a polydispersity index (PDI) of 0.49, and a zeta potential of -15.67 mV. Reportedly, the loading content (LC) of hydrophilic small molecules in the hydrophilic inner cavity of OMVs typically ranges from 20% to 30%, while the theoretical LC for hydrophobic drugs in the lipid bilayer is between 5% and 20%. To optimize GDO synthesis, we systematically evaluated the effects of different DATS/OMVs mass ratios (0.5/1, 1/1, 2/1, and 4/1, *w/w*) and GA/DATS/OMVs mass ratios (0.3/1/1, 0.6/1/1, and 0.9/1/1, *w/w*) on the LC and loading efficiency (LE) of DATS and GA. The actual LC and LE values of GA and DATS were quantified using the Folin–Ciocalteu method and UV–Vis absorption spectroscopy, respectively. Supporting Information Figs. S1 and S2 present the standard curve and UV–Vis spectra for DATS and DATS@OMVs, respectively, while Supporting Information Fig. S3 shows the standard curve for GA. Our results demonstrated that the optimal DATS LC (20.06%) and LE (25.11%) were achieved at a DATS/OMVs ratio of 1/1 (*w/w*), beyond which no significant LC enhancement was observed (Supporting Information Table S2). For GA, the ratio of GA/DATS/OMVs = 0.6/1/1 (*w/w/w*), yielding optimal performance with 23.46% LC and 53.57% LE (Supporting Information Tables S3 and Fig. S4). Higher ratios did not significantly improve these parameters. Notably, DATS LE remained stable at approximately 25.11% across all tested GA ratios, indicating that GA loading did not compromise DATS incorporation. Based on these findings, a mass ratio of GA/DATS/OMVs = 0.6/1/1 (*w/w/w*) was identified as optimal for GDO preparation.

After loading GA and DATS, the particle size and zeta potential of OMVs did not show significant changes (Fig. 1B), but the PDI significantly decreased to 0.14, indicating that the particle size distribution of OMVs became more uniform following treatment with the manual liposome extruder. Supporting Information Fig. S5 shows the proton peak at 6.9–7.2 ppm in the ^1H NMR spectrum of CM, corresponding to protons on the mannose. Further calculations revealed that the grafting rate of mannose onto chitosan (CS) was 21%. To optimize the adsorption of CM

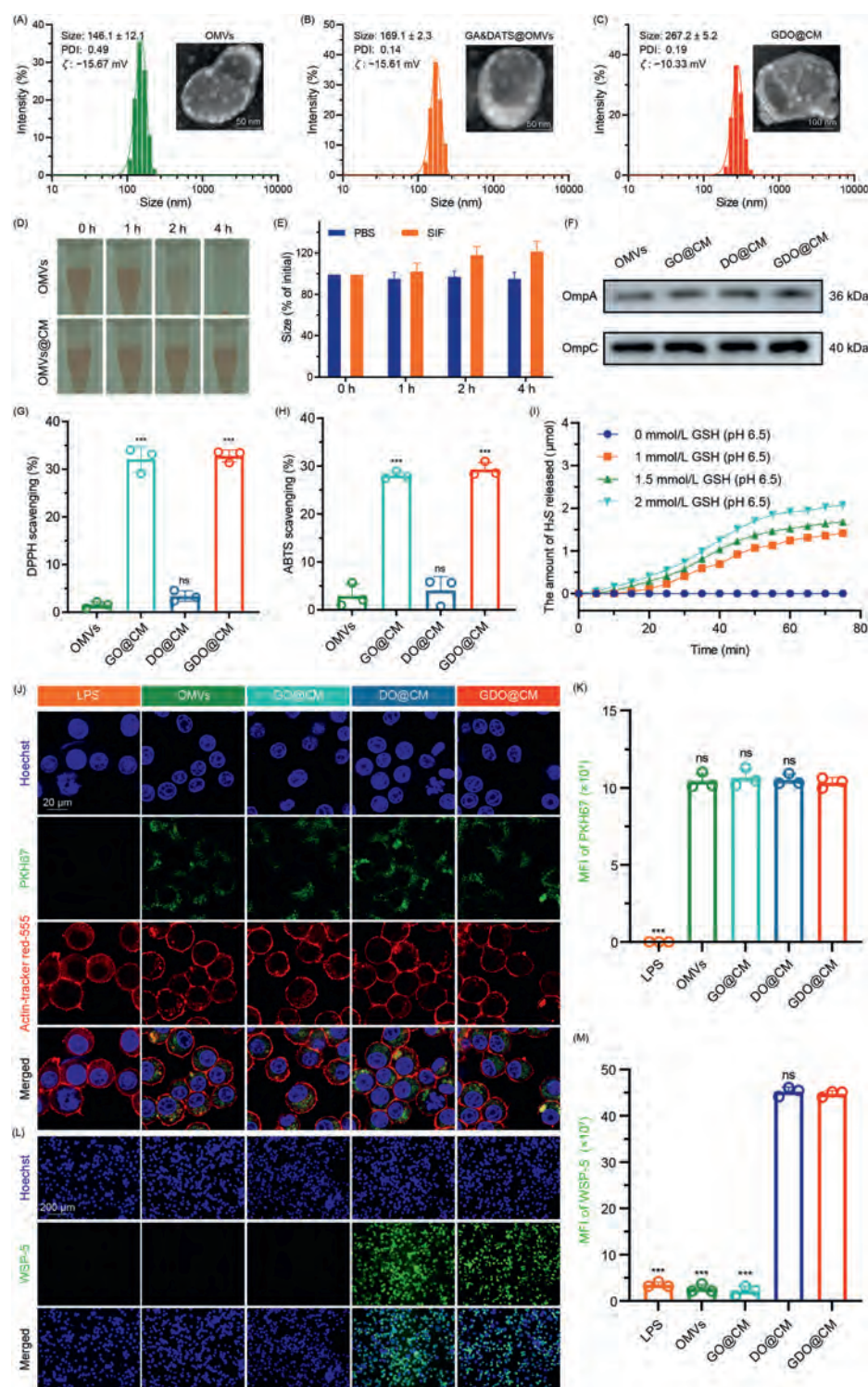


Figure 1 Preparation and physicochemical characterization of GDO@CM. (A–C) Particle size distributions and TEM images of OMVs, GDO, and GDO@CM. (D) Stability of OMVs and OMVs@CM in simulated intestinal fluid (SIF). (E) Size variation of GDO@CM in SIF. (F) Representative Western blots analysis showing OMVs markers (OmpA and OmpC) in OMVs, GO@CM, DO@CM and GDO@CM. (G, H) DPPH and ABTS radical scavenging abilities of OMVs, GO@CM, DO@CM, and GDO@CM. (I) H₂S release profiles of GDO@CM in SIF with varying concentrations of GSH. (J, K) Cellular internalization of GDO@CM by LPS-induced activated RAW 264.7 cells, with OMVs labeled with PKH67 for green fluorescence (scale bar = 20 μ m). (L, M) Intracellular imaging of H₂S release and fluorescence intensity of WSP-5 probes (scale bar = 200 μ m). Statistical analyses were performed by comparing the OMVs group (G, H) or the GDO@CM group (K, M) with the other groups. Data are presented as mean $\pm$ SD ($n = 3$). *** $P < 0.001$ vs. indicated, ns, not significant.

onto GDO, we tested five different mass ratios of CM/GDO (1/1, 2/1, 4/1, 8/1, and 16/1, w/w). For GDO@CM prepared at ratios of 1/1 (w/w) and 2/1 (w/w) (Supporting Information Table S4), the particle size and zeta potential did not significantly differ from those of GDO, indicating only minimal CM adsorption onto the GDO surface. When the mass ratio increased to 4/1, the particle size of GDO@CM significantly increased to 267.2 nm and formed a rough, uneven membrane surface—contrasting with the smooth morphology of bare GDO, consistent with previous reports⁴⁷. Simultaneously, the surface zeta potential increased significantly to -10.33 mV (Fig. 1C). The increase in particle size was primarily attributed to the introduction of CM, which enhanced the hydrophilicity of GDO. Further increases in the CM/GDO mass ratio did not result in significant changes in particle size and zeta potential. Therefore, we concluded that the optimal ratio for preparing GDO@CM was 4/1 (w/w).

The encapsulation of CM significantly improved the stability of OMVs and their drug-loaded nanoparticles. As shown in Fig. 1D, OMVs loaded with Oil Red O dye rapidly degraded in SIF, resulting in significant dye release, which settled at the bottom of the centrifuge tube. In contrast, OMVs@CM loaded with Oil Red O dye exhibited almost no dye release after 4 h of incubation with SIF. Further investigation revealed that GDO@CM, when incubated for 4 h either in PBS or SIF, showed no significant changes in particle size or zeta potential (Fig. 1E and Supporting Information Fig. S6A). The exceptional stability of GDO@CM is further evidenced by its ability to maintain a consistent particle size even after being stored at 4°C for one week and at -80°C for seven weeks (Fig. S6B and S6C). These results indicate that CM encapsulation greatly enhanced the stability of GDO, particularly in SIF, which is crucial for improving the therapeutic efficacy of GDO. It is important to note that subsequent studies primarily focused on evaluating the biological performance of GDO@CM, without assessing that of GDO. Additionally, to verify the antioxidant and anti-inflammatory properties conferred by GA and DATS to EcN-OMVs, we prepared GO@CM and DO@CM using the same method. The particle size, zeta potential, and drug loading capacities are presented in Supporting Information Fig. S7A and S7B, and Table S5. The results in Fig. 1F confirmed the presence of OMVs in GO@CM, DO@CM, and GDO@CM, as evidenced by the presence of two typical exosome-enriched markers—OmpA and OmpC—on the surface of these engineered OMVs.

The *in vitro* antioxidant activity of these engineered OMVs was assessed using DPPH and ABTS radical scavenging assays. The results in Fig. 1G and H shows that GO@CM and GDO@CM exhibited excellent antioxidant capacity, with DPPH and ABTS scavenging rates of 31.21% and 33.65%, and 27.9% and 29.4%, respectively. After confirming the antioxidant activity of GDO@CM, we used a specialized H_2S detector to monitor its real-time H_2S release in SIF under different concentrations of GSH (0, 1, 1.5, and 2 mmol/L) (Fig. 1I). Under 0 mmol/L GSH conditions, GDO@CM released almost no H_2S within 1 h. However, under 1, 1.5, and 2 mmol/L GSH conditions, the cumulative release of H_2S after 1 h was 1.25, 1.52, and 1.94 μmol , respectively. These results indicate that the DATS in GDO@CM exhibits GSH-responsive H_2S release, confirming its ability to effectively release H_2S at the IBD site.

In addition to its remarkable antioxidant activity and GSH-responsive H_2S release, GDO@CM demonstrates excellent biocompatibility. As shown in Supporting Information Fig. S8A–S8C, the viability of Caco-2 and RAW264.7 cells remained above 90% when treated with GDO@CM at

concentrations below 50 $\mu\text{g/mL}$. Live/dead staining further confirmed that nearly no Caco-2 cells were PI-labeled in the GDO@CM-treated group at 50 $\mu\text{g/mL}$. Furthermore, even at concentrations as high as 1 mg/mL, the hemolysis rate in the GDO@CM-treated group remained below 1%, well below the 5% threshold typically regarded as hemolytic (Supporting Information Fig. S9A and S9B). These results indicate that GDO@CM exhibits excellent biocompatibility.

The intracellular uptake of GDO@CM and its ability to release H_2S in LPS-activated macrophages were evaluated using CLSM and an inverted fluorescence microscope. All OMVs were labeled with PKH67. Notably, strong green fluorescence from PKH67 was detected in all groups, indicating that OMVs exhibit high cellular uptake capacity and that CM encapsulation does not impair this important feature (Fig. 1J and K). Additionally, as shown in Fig. 1L and M, minimal H_2S generation was observed in activated macrophages treated with PBS and GO@CM. In contrast, significant H_2S production was observed in macrophages treated with DO@CM and GDO@CM, suggesting that the DATS encapsulated in OMVs can efficiently and rapidly release H_2S within activated macrophages, and that encapsulation with CM does not affect H_2S release.

3.2. *In vitro* antioxidant and anti-inflammatory effects of GDO@CM

High levels of ROS continuously secreted by activated immune cells at the site of IBD lesions are key contributors to oxidative damage and the exacerbation of inflammation⁵⁶. In our *in vitro* experiments, we have already demonstrated the exceptional ability of GDO@CM to scavenge ROS. To further validate this crucial property, we used DCFH-DA and ROS Green™ H_2O_2 probes to assess the ROS and H_2O_2 scavenging capabilities of GDO@CM in activated macrophages. As shown in Fig. 2A and B, strong green fluorescence signals generated by the DCF and H_2O_2 probes were observed in activated macrophages, indicating significantly elevated levels of ROS and H_2O_2 . In contrast, activated macrophages treated with GO@CM and DO@CM showed a marked decrease in fluorescence intensity, suggesting that GA can effectively scavenge ROS and H_2O_2 within the cells, and that the H_2S released from DATS also has some ROS scavenging ability. Notably, under the dual action of GA and H_2S , GDO@CM almost completely eliminated ROS and H_2O_2 in activated macrophages, with fluorescence intensities approaching that of normal macrophages (Fig. 2C and D). Further analysis revealed that GA's remarkable antioxidant activity primarily stems from its significant upregulation of key antioxidant enzymes, including CAT, SOD, and GSH-Px. This was verified by the significantly higher expression levels of these enzymes in activated macrophages treated with GO@CM compared to the other treatment groups. As shown in Fig. 2E–G, the expression levels of CAT, SOD, and GSH-Px in the GO@CM-treated group increased by 10.92-, 4.48-, and 8.72-fold, respectively, compared to the LPS-treated group. Treatment with OMVs showed almost no antioxidant activity, only slightly increasing CAT enzyme activity. While H_2S can also upregulate SOD and GSH-Px expression, its effect is notably weaker than that of GA, indicating that the antioxidant effect of GDO@CM is mainly attributed to GA. Notably, GDO@CM treatment also significantly activated the expression of HO-1 and phosphorylated Nrf2 (p-Nrf2) in activated macrophages. As shown in Fig. 2H and I, and Supporting Information Fig. S10, the expression levels of HO-1 and p-Nrf2 in the GDO@CM-treated group increased by 2.91- and 2.62-fold, respectively, compared

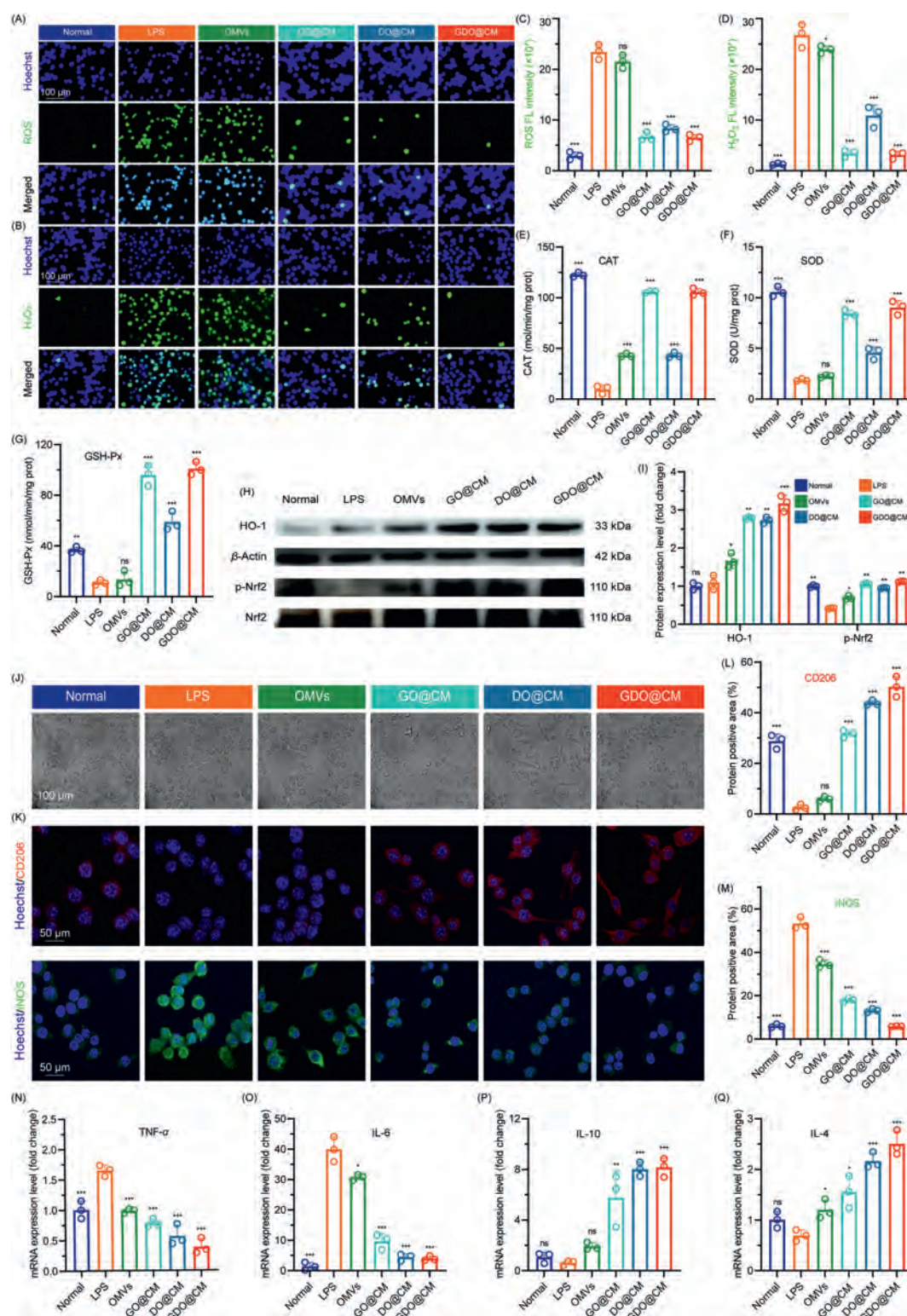


Figure 2 *In vitro* antioxidant and anti-inflammatory activities of GDO@CM. (A, B) ROS and H₂O₂ scavenging abilities in GDO@CM-treated activated macrophages (scale bar = 100 μ m). (C, D) Corresponding fluorescence intensity of DCFH-DA and ROS Green H₂O₂ probes in GDO@CM-treated activated macrophages (scale bar = 100 μ m). (E–G) Enzymatic activity levels of SOD, CAT, and GSH-Px in GDO@CM-treated activated macrophages. (H, I) Expression levels of HO-1 and p-Nrf2 in the GDO@CM group. (J) Morphological changes of activated macrophages after GDO@CM treatment (scale bar = 100 μ m). (K–M) Immunofluorescence staining of iNOS-positive and CD 206-positive cells in the GDO@CM group, along with intracellular fluorescence intensity of iNOS and CD206 (scale bar = 50 μ m). (N–Q) mRNA expression levels of TNF- α , IL-6, IL-10, and IL-4 in the GDO@CM group. Statistical analyses were performed by comparing the LPS group with the other groups. Data are presented as mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. indicated, ns, not significant.

to the LPS-treated group. Nrf2, as a transcription factor, can sense intracellular oxidative stress signals and initiate the transcription of antioxidant genes, thereby regulating the cellular antioxidant response⁵⁷. HO-1 is one of the key antioxidant enzymes regulated by Nrf2 activation. The Nrf2/HO-1 signaling pathway plays a complex role in IBD, reducing oxidative damage through antioxidant actions and alleviating immune responses by inhibiting inflammation, thus effectively mitigating IBD symptoms⁵⁸. In summary, GDO@CM can significantly activate the Nrf2/HO-1 signaling pathway in activated macrophages, thereby effectively regulating the expression of various antioxidant enzymes and exerting significant antioxidant effects. Notably, GDO@CM demonstrates superior radical-scavenging performance compared to some existing materials containing comparable concentrations of either GA or DATS alone^{59,60}, an effect we attribute to the synergistic interaction between GA and H₂S.

After validating the exceptional antioxidant activity of GDO@CM, we further evaluated its anti-inflammatory effects. As shown in Fig. 2J, LPS-induced activated macrophages exhibited a dendritic morphology with numerous pseudopodia, which are characteristic of the M1 macrophage phenotype. In the GDO@CM treatment group, significant morphological changes were observed, characterized by a substantial increase in cell elongation and a marked reduction in cell protrusions. As shown in Supporting Information Fig. S11A and S11B, the maximum elongation rate of the GDO@CM-treated cells reached 4.94, with the average number of cell protrusions being only 1.67. In comparison, the LPS group showed a maximum elongation rate of just 1.33, with the average number of cell protrusions being as high as 7. To further confirm the effects of GDO@CM on macrophage phenotype, we performed immunofluorescence labeling on the treated cells. As shown in Fig. 2K–M, compared to the LPS group, the fluorescence intensity of CD206-labeled M2 macrophages was significantly enhanced in the GDO@CM-treated group, while the fluorescence intensity of iNOS-labeled M1 macrophages was notably reduced. These results further indicate that GDO@CM treatment effectively promotes macrophage polarization toward the M2 phenotype. Further RT-qPCR analysis showed that GDO@CM treatment significantly downregulated the expression levels of pro-inflammatory cytokines TNF- α , IL-6, and IL-1 β , reducing their levels by 4.33-, 10.75-, and 5.40-fold, respectively, compared to the LPS group (Fig. 2N and O, and Supporting Information Fig. S12A). In addition, GDO@CM treatment significantly upregulated the expression levels of M2 macrophage markers IL-4, IL-10, and Arg-1, with increases of 3.60-, 4.27-, and 12.96-fold, respectively, compared to the LPS group (Fig. 2P and Q, and Fig. S12B). In summary, GDO@CM demonstrated remarkable anti-inflammatory effects by significantly reducing the proportion of M1 macrophages, inhibiting the expression of pro-inflammatory cytokines, promoting macrophage polarization toward the M2 phenotype, and enhancing the expression of anti-inflammatory cytokines. Notably, the anti-inflammatory effects of GDO@CM are primarily attributed to the action of H₂S. This is supported by the fact that the effects observed in the DO@CM treatment group—whether in regulating cell phenotype polarization, suppressing pro-inflammatory cytokine expression, or promoting anti-inflammatory cytokine expression—were notably superior to those in the GO@CM group, and comparable to the results in the GDO@CM-treated group. In conclusion, GDO@CM exhibits excellent antioxidant and anti-inflammatory activities, with its antioxidant effects primarily attributed to GA and its anti-inflammatory effects mainly driven by the H₂S released from DATS.

3.3. *In vitro anti-ferroptosis and reparative effects of GDO@CM on the intestinal barrier*

ROS is a key driver of ferroptosis and can also exacerbate cellular oxidative damage by activating inflammatory responses. Besides, inflammatory cytokines can also promote the occurrence of ferroptosis by regulating iron metabolism⁶¹. Therefore, after confirming the significant antioxidant and anti-inflammatory capabilities of GDO@CM, we explored whether it could effectively repair the intestinal mucosal barrier by inhibiting ferroptosis. We used Caco-2 cells as a model, as these cells have differentiation abilities and can form a monolayer structure similar to small IECs, exhibiting various functions related to the intestinal epithelium⁶². Thus, they are widely used to model human colonic and rectal epithelial barriers. In this experiment, RSL3 was used as an inducer of ferroptosis to induce ferroptosis in Caco-2 cells, and to assess whether GDO@CM could inhibit ferroptosis. As shown in Supporting Information Fig. S13A, the cell viability of Caco-2 cells significantly decreased with the increase in RSL3 concentration. After treatment with 45 μ mol/L RSL3 for 18 h, the cell survival rate sharply dropped to 46.75%. However, after treatment with 50 μ g/mL GO@CM or DO@CM for a further 12 h, the cell viability of Caco-2 cells was significantly improved, increasing to 82.71% and 72.72%, respectively (Fig. 3A), indicating that the antioxidant effect of GA and the anti-inflammatory effect of H₂S play significant roles in inhibiting RSL3-induced ferroptosis. Importantly, the synergistic effect of both GA and H₂S significantly improved the cell viability in the GDO@CM treatment group to 90.72%. The live/dead staining results further confirmed that GDO@CM significantly inhibited ferroptosis. As shown in Fig. 3B and Fig. S13B and a large number of PI-positive dead cells were observed in the RSL3 and OMVs groups, while only a small number of PI-positive dead cells were observed in the DO@CM, GO@CM, and especially the GDO@CM groups.

By assessing ferroptosis-related markers, we identified that the primary mechanism underlying the significant inhibition of ferroptosis by GDO@CM is the upregulation of intracellular GSH-Px activity and GPX4 protein expression levels (Fig. 3C and D). As key antioxidant enzymes, GSH-Px and GPX4 efficiently reduce lipid peroxides on the cell membrane, thereby preventing lipid peroxidation and inhibiting ferroptosis. Following RSL3 treatment, the Fe²⁺ concentration in Caco-2 cells significantly increased, potentially due to the inhibition of GPX4 activity, which impairs iron storage protein function, releases stored iron, and raises free Fe²⁺ levels. Additionally, GPX4 inhibition may promote the reduction of Fe³⁺ to Fe²⁺ by altering the cellular redox state, further elevating Fe²⁺ concentrations. Importantly, both GO@CM and GDO@CM treatments significantly reduced intracellular Fe²⁺ concentrations by upregulating GPX4 activity (Fig. 3E). The reduction in Fe²⁺ concentration inhibits lipid peroxidation by decreasing the highly reactive hydroxyl radicals ($\cdot$ OH) generated in the Fenton reaction, thereby alleviating oxidative stress and preventing ferroptosis. In conclusion, the anti-ferroptosis effects of GO@CM and GDO@CM not only prevent lipid peroxidation by upregulating key antioxidant enzyme activity, but also further protect cells from ferroptosis by reducing Fe²⁺ concentrations and limiting free radical generation.

In addition to effectively inhibiting ferroptosis in Caco-2 cells, GDO@CM also significantly promotes cell proliferation, migration, and the expression of various TJPs, all of which are critical for repairing the intestinal barrier. As shown in Fig. 3F, in the H₂O₂-induced oxidative damage model, the proliferative capacity

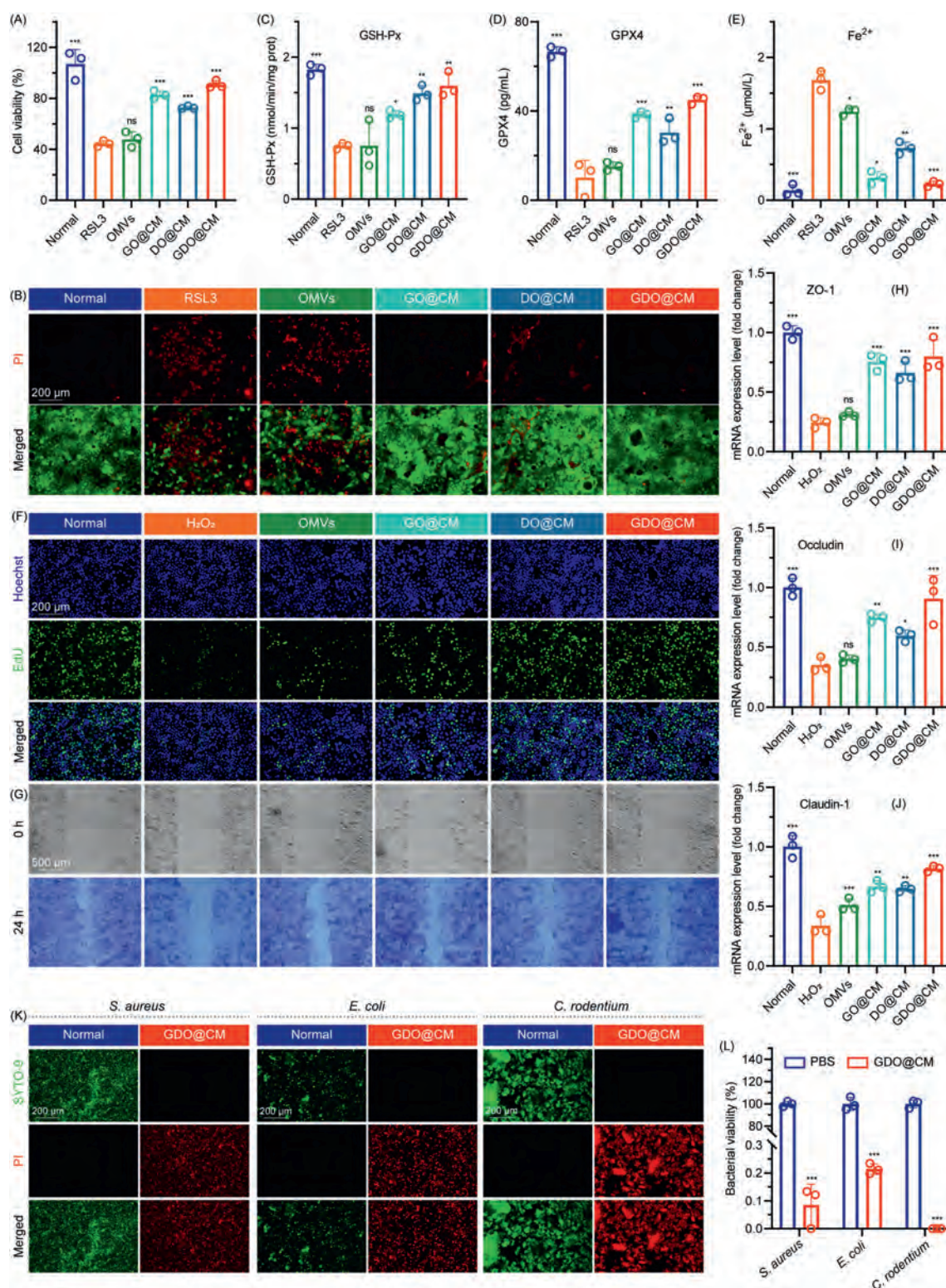


Figure 3 *In vitro* intestinal barrier repair activities of GDO@CM. (A, B) Cell viability and live/dead staining showing the protective effect of GDO@CM on Caco-2 cells against RSL3-induced ferroptosis (scale bar = 200 μm). (C–E) GSH-Px enzymatic activity, GPX4 expression levels, and Fe²⁺ levels in Caco-2 cells following treatment. (F) EdU staining (scale bar = 200 μm) and (G) scratch wound healing (scale bar = 500 μm) assays in Caco-2 cells treated with GDO@CM. (H–J) Intracellular expression levels of ZO-1, Occludin, and Claudin-1 in Caco-2 cells after treatment with GDO@CM. (K) Live/dead staining of *S. aureus*, *E. coli*, and *C. rodentium* following various treatments (scale bar = 200 μm). (L) Corresponding survival rates of *S. aureus*, *E. coli*, and *C. rodentium*. Statistical analyses were performed by comparing the RSL3, H₂O₂, or PBS group with the other groups. Data are presented as mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. indicated, ns, not significant.

of Caco-2 cells was significantly inhibited, as indicated by a marked decrease in EdU-positive cell count. However, in the GDO@CM, GO@CM, and DO@CM treatment groups, cell proliferation was effectively restored, and the number of EdU-positive cells was close to the normal group. Fluorescence intensity analysis further confirmed that the EdU fluorescence intensity in the GDO@CM treatment group was significantly higher than in the other groups (Supporting Information Fig. S14A). In the cell scratch healing assay, the scratch healing rate in the GDO@CM group reached 89.34% after 24 h of incubation, close to the normal group (91.12%), and significantly higher than that of the H₂O₂ group (51.58%) (Fig. 3G and Fig. S14B). These results suggest that GDO@CM has significant potential in promoting Caco-2 cell migration. More importantly, GDO@CM treatment also significantly upregulated the expression levels of ZO-1, Occludin, and Claudin-1 in the H₂O₂-induced oxidative damage Caco-2 cells. Compared to the H₂O₂ group, GDO@CM treatment upregulated the mRNA expression levels of these genes by 3.28-, 2.59-, and 2.40-fold, respectively (Fig. 3H–J). The upregulation of these key TJPs plays an important role in repairing the membrane structure of IECs and strengthening the tight junctions of the epithelial barrier. In summary, GDO@CM not only effectively inhibits ferroptosis-induced intestinal epithelial barrier damage but also promotes Caco-2 cell proliferation, accelerates scratch healing, and significantly increases the expression of TJPs. These results provide important evidence for the potential application of GDO@CM in repairing ROS-induced intestinal barrier damage.

An increasing number of studies have highlighted the critical role of gut microbiota dysbiosis in intestinal barrier disruption⁶³. Abnormal microbiota, particularly pathogenic bacteria and their endotoxins, can invade the intestinal mucosa, increase mucosal permeability, and exacerbate IBD symptoms. Given that GA interferes with bacterial cell wall synthesis, DATS disrupts bacterial cell membranes and inhibits bacterial membrane transport systems, and CS exhibits certain antimicrobial activity, we hypothesize that GDO@CM may help restore intestinal barrier integrity by eliminating pathogenic bacteria that heavily colonize the IBD site and remodeling gut microbiota homeostasis. To test this hypothesis, we assessed the antimicrobial effects of GDO@CM against three common IBD-related pathogens: *S. aureus*, *E. coli*, and *C. rodentium* *in vitro*. As shown in Fig. 3K, after co-incubation with GDO@CM for 8 h, nearly all pathogens exhibited strong red fluorescence from PI staining, indicating bacterial death. Colony counting results (Supporting Information Fig. S15 and Fig. 3L) further demonstrated that, within 8 h, GDO@CM achieved bactericidal rates of 99.99%, 99.96%, and 99.93% for *S. aureus*, *E. coli*, and *C. rodentium*, respectively. Further investigations revealed that GDO@CM exerts moderate inhibitory effects on EcN growth kinetics (Supporting Information Fig. S16), primarily attributed to the inherent broad-spectrum antimicrobial properties of its DATS and GA. In IBD-affected regions where pathogenic bacteria significantly outnumber beneficial bacteria, GDO@CM's efficient eradication of pathogens not only facilitates inflammation resolution but also creates favorable conditions for probiotic proliferation, thereby systematically restoring gut microbiota homeostasis.

3.4. Therapeutic effect of GDO@CM in the DSS-induced IBD mouse model

After confirming the excellent effects of GDO@CM *in vitro*, including antioxidant, anti-inflammatory properties, ferroptosis

inhibition in IECs, and intestinal mucosal barrier repair, we further established a DSS-induced colitis mouse model to evaluate its *in vivo* therapeutic effects. To establish the colitis model, mice were administered 3% DSS water for approximately 7 days to induce acute colitis, with the model evaluated by monitoring blood in feces and changes in body weight (Supporting Information Fig. S17). Subsequently, all colitis mice were randomly divided into four groups ($n = 6$): the PBS treatment group (DSS group), OMVs treatment group (OMVs group, 1 mg/mL), GDO@CM treatment group (GDO@CM group, 1 mg/mL), and 5-ASA treatment group (5-ASA group, 20 mg/mL, as a positive control)^{64,65}. Healthy mice served as the normal control group (Normal group). Given that 5-ASA is a commonly used drug for clinical IBD treatment, albeit with a relatively slow onset and noticeable side effects, it was used for comparison. During the 11-day treatment period, all colitis mice continued to drink 3% DSS water to maintain the inflammatory condition. The OMVs, GDO@CM, and 5-ASA groups were treated rectally with the corresponding dose (100 μ L) on Days 1, 3, 5, 7, and 9 (Fig. 4A). To assess whether GDO@CM could effectively treat IBD, we first evaluated its targeting and retention capabilities at the IBD site, which is crucial for long-lasting therapeutic effects. We used an *in vivo* imaging system to assess the targeting ability of GDO@CM. The results showed that 9 h after rectal administration, DiR-labeled GDO@CM (DiR-GDO@CM) was largely retained at the colonic site in colitis mice (Fig. 4B and Supporting Information Fig. S18), suggesting that GDO@CM exhibits significant targeting and retention. This phenomenon may be attributed to the strong electrostatic interaction between the negatively charged GDO@CM and the positively charged glycoproteins on the damaged intestinal mucosa, as well as the inherent targeting effect of mannose toward activated immune cells. Additionally, 6 h after rectal administration, only a small amount of DiR-GDO@CM remained in the colonic site of healthy mice, further confirming the targeted retention of GDO@CM at the damaged colonic mucosa.

To evaluate the therapeutic effects of GDO@CM, we performed a comprehensive analysis of its performance in the IBD mouse model using multiple indicators, including survival rate, body weight changes, disease activity index (DAI), colon length, spleen size, myeloperoxidase (MPO) expression levels, and histological scores. During the treatment period, no deaths occurred in the Normal group. In contrast, the DSS group began to experience mortality on Day 6, with only 2 mice surviving by Day 11. The OMVs group also showed mortality starting on Day 7, with only 3 mice surviving by Day 11. In comparison, the GDO@CM group exhibited significantly improved survival, with only 1 mouse dying by Day 11, showing a survival rate similar to that of the 5-ASA group (Fig. 4C). These data indicate that GDO@CM significantly enhances the survival rate of IBD mice, providing preliminary evidence for its potential in IBD treatment. Weight loss is a key pathological feature in colitis mice and is generally correlated with the severity of the disease and the intensity of the inflammatory response. All colitis mice exhibited weight loss. The DSS group showed a 30% weight loss by Day 11, indicating a severe condition. The 5-ASA and OMVs groups had lesser weight loss, at 15% and 23%, respectively. Notably, the GDO@CM group showed the least weight loss, with a gradual recovery beginning on Day 9, resulting in only a 13% reduction by the end of the study (Fig. 4D). These results suggest that GDO@CM effectively alleviates weight loss induced by colitis. The DAI score, which is a comprehensive indicator of colitis activity and severity, is calculated based on weight changes, stool characteristics, and

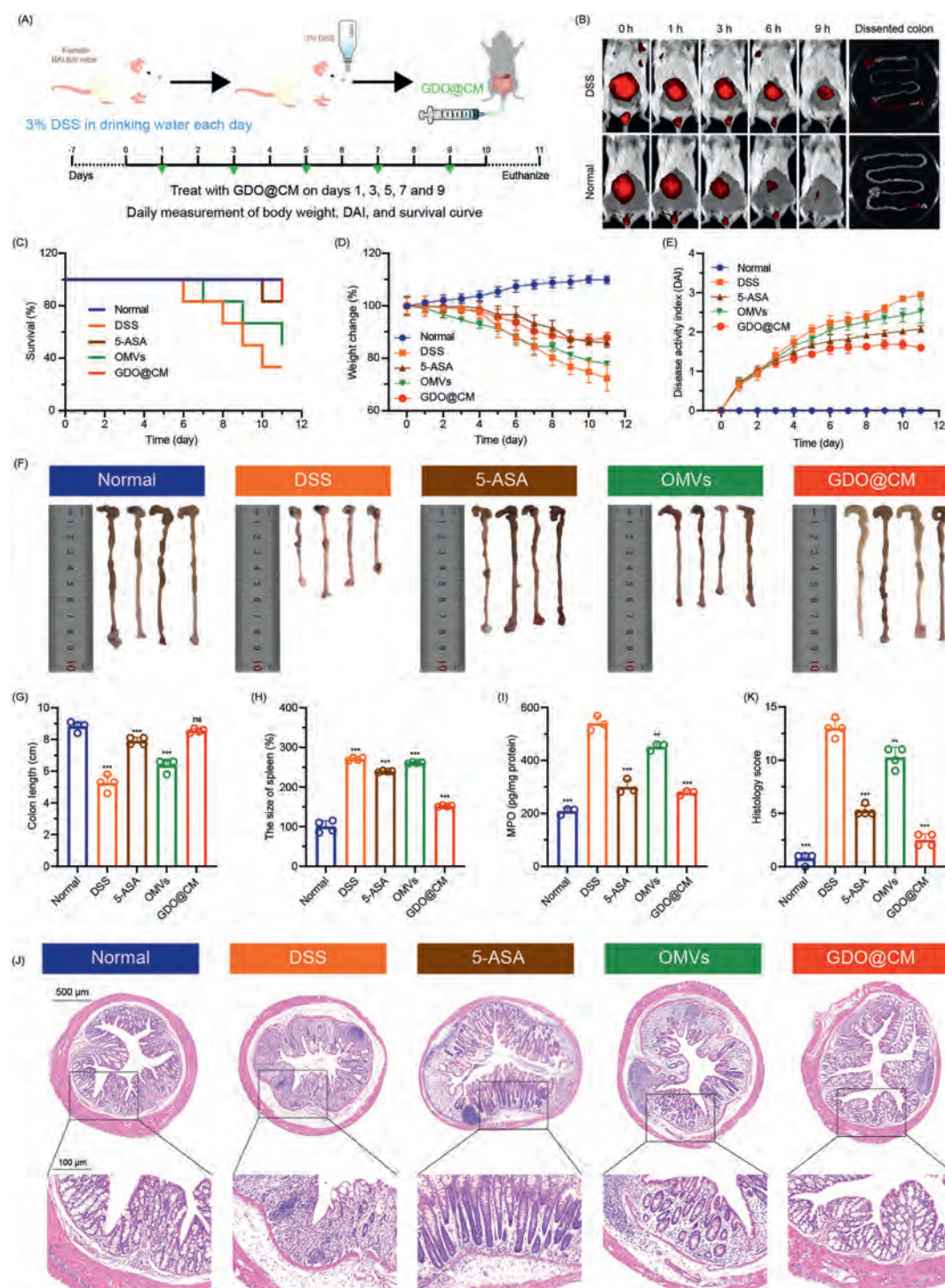


Figure 4 Therapeutic efficacy of GDO@CM in mice with DSS-induced colitis. (A) Experimental scheme. (B) Biodistribution of DiR-GDO@CM in the colon at different time points following rectal administration. (C) Survival rate, (D) body weight, and (E) DAI index changes throughout the treatment period. (F, G) Colonic length, (H) spleen size, and (I) tissue MPO activity in mice at the end of treatment. (J) H&E staining images of colon tissues (scale bar = 500 and 100 μm) and (K) histological score on Day 11. Statistical analyses were performed by comparing the Normal or DSS group with the other groups. Data are presented as mean ± SD ($n = 3$ for I; $n = 4$ for G, H, and K). ** $P < 0.01$, *** $P < 0.001$ vs. indicated. ns, not significant.

rectal bleeding⁶⁶. Throughout the treatment period, the GDO@CM group consistently had a lower DAI score compared to the other treatment groups. Particularly on Day 11, the GDO@CM group had a score of 1.6, significantly lower than the DSS group

(3.0), 5-ASA group (2.1), and OMVs group (2.7) (Fig. 4E). This result further supports the conclusion that GDO@CM significantly alleviates colitis symptoms and effectively reduces the activity of intestinal inflammation.

Ex vivo analysis of colon tissue further confirmed the significant therapeutic effects of GDO@CM. As shown in Fig. 4F and G, compared to the healthy mouse colon length, the DSS, 5-ASA, and OMVs groups showed reductions in colon length by 37.4%, 10.5%, and 27.8%, respectively. In contrast, the colon length in the GDO@CM group was only reduced by 3.3%, demonstrating its exceptional ability to repair intestinal barrier integrity. Spleen size, another critical indicator of colitis severity, correlates with systemic inflammation⁶⁷. An enlarged spleen typically reflects severe inflammation, while a reduction in spleen size indicates inflammation alleviation. *Ex vivo* analysis of spleen tissue revealed that, compared to the spleen volume of healthy mice, the DSS, 5-ASA, and OMVs groups exhibited a 2.7-, 2.4-, and 2.6-fold increase in spleen volume, respectively. In contrast, the GDO@CM group showed only a 1.5-fold increase in spleen volume (Fig. 4H and Supporting Information Fig. S19A). This suggests that GDO@CM treatment significantly alleviates systemic inflammation and reduces spleen enlargement. Further analysis of MPO expression levels supported the reduction of inflammation. MPO, a marker of neutrophil infiltration, is commonly used to assess inflammation⁶⁸. Compared to the DSS group, the GDO@CM group exhibited a reduction of approximately 1.94-fold in MPO expression in colon tissue, nearly reaching the MPO levels seen in healthy mice (Fig. 4I). This indicates that GDO@CM effectively reduces neutrophil infiltration and mitigates local inflammation. H&E staining (Fig. 4J) revealed that the GDO@CM treatment group maintained better colon epithelial integrity, with a significant reduction in inflammatory cell infiltration. The colon injury score was 2.5 (Fig. 4K), much lower than the DSS group (13.3), 5-ASA group (5.25), and OMVs group (10.25). Colon tissues from the DSS, 5-ASA, and OMVs groups displayed severe crypt destruction and widespread immune cell infiltration, further validating the significant effects of GDO@CM in alleviating intestinal damage and suppressing inflammation. In conclusion, GDO@CM exhibits significant therapeutic effects in DSS-induced colitis, including: (1) enhancing survival rates, (2) alleviating weight loss, (3) reducing inflammatory activity, (4) protecting colon tissue, and (5) suppressing spleen enlargement. It is noteworthy that the overall therapeutic effect of GDO@CM is slightly superior to that of 5-ASA, demonstrating its significant potential for clinical application.

In addition to its excellent therapeutic effects, GDO@CM also demonstrated superior biocompatibility throughout the treatment period, as evidenced by the following observations: (1) During the entire treatment period, the occult blood rate in the GDO@CM group remained at the lowest level (Fig. S19B), indicating minimal intestinal irritation and no significant hemorrhagic response; (2) At the end of the treatment, the red blood cell count, white blood cell count, and hemoglobin levels in the plasma of the GDO@CM group were nearly identical to those of healthy mice (Supporting Information Fig. S20), with no significant hematological abnormalities; (3) ALT and AST levels demonstrated no significant differences between GDO@CM-treated and Normal groups, with ALT levels being 37.3 ± 4.91 U/L (GDO@CM) versus 26.14 ± 3.73 U/L (Normal) and AST levels being 105.84 ± 12.71 U/L (GDO@CM) versus 85.54 ± 7.31 U/L (Normal) (Supporting Information Fig. S21); (4) H&E examination of major organs (heart, liver, spleen, lung, and kidney) from GDO@CM-treated IBD mice (Supporting Information Fig. S22) revealed no evidence of tissue necrosis or inflammatory cell infiltration, well-preserved tissue architecture, and histological features indistinguishable from normal controls. In conclusion, GDO@CM, as a therapeutic nanomedicine

for IBD, not only exhibits significant therapeutic advantages but also outstanding biocompatibility. More importantly, it outperforms 5-ASA in most evaluation metrics, highlighting its tremendous potential as a clinical treatment strategy.

To elucidate the potential mechanisms underlying GDO@CM's effects in IBD treatment, we further investigated its impact on antioxidation, anti-inflammation, intestinal barrier repair, and microbiota modulation at the IBD site. First, we assessed the ROS scavenging activity of GDO@CM using the L-012 chemiluminescence probe. As shown in Fig. 5A and Supporting Information Fig. S23, after 11 days of treatment, mice in the DSS group still exhibited strong L-012 fluorescence signals in the IBD region, indicating a substantial accumulation of ROS in the inflamed areas. In contrast, GDO@CM treatment resulted in a 13.50-fold reduction in L-012 fluorescence intensity, suggesting that ROS were effectively scavenged from the IBD site. These results demonstrate that GDO@CM possesses strong *in vivo* ROS scavenging ability, which alleviates oxidative stress at the IBD site. MDA measurements in colonic tissues further confirmed GDO@CM's potent antioxidant activity *in vivo*, as evidenced by colonic MDA levels in the GDO@CM group (1.51 ± 0.15 nmol/mg protein) that were significantly lower than those in the DSS group (4.09 ± 0.25 nmol/mg protein) and approached physiological baseline levels observed in normal mice (0.91 ± 0.12 nmol/mg protein) (Supporting Information Fig. S24). Further analysis of key antioxidant enzyme activities in colon tissue revealed that GDO@CM significantly increased the expression of CAT, SOD, and GSH-Px by 2.61-, 10.1-, and 12.07-fold, respectively, compared to the DSS group (Fig. 5B–D). Additionally, immunofluorescence staining and ELISA results (Fig. 5E and F) showed that GDO@CM notably increased the expression of GPX4 in colon tissue, with expression levels rising by 2.54-fold compared to the DSS group. These findings suggest that GDO@CM exerts exceptional antioxidant activity in colitis mice by enhancing the activity of multiple key antioxidant enzymes, thus alleviating oxidative stress and inhibiting ferroptosis in IECs.

In addition to its efficient antioxidant effects and ferroptosis inhibition, GDO@CM also demonstrated significant effects in alleviating inflammation at the IBD site. As shown in Fig. 5G–J, after treatment, the DSS group still exhibited high levels of pro-inflammatory cytokines in colon tissue (TNF- α : 804.7 pg/mL, IL-6: 830.9 pg/mL), while anti-inflammatory cytokine levels (IL-4: 302.7 pg/mL, IL-10: 430.5 pg/mL) remained relatively low. In contrast, the GDO@CM treatment group showed a marked reduction in pro-inflammatory cytokine levels (TNF- α : 376.9 pg/mL, IL-6: 275.3 pg/mL) and a significant increase in anti-inflammatory cytokine levels (IL-4: 899.5 pg/mL, IL-10: 886.1 pg/mL). Immunofluorescence analysis further revealed that GDO@CM treatment promoted the differentiation of M2 macrophages (CD206-positive) while inhibiting the differentiation of M1 macrophages (iNOS-positive) in colon tissue (Fig. 5K and Supporting Information Fig. S25). These results suggest that GDO@CM effectively polarizes macrophages from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype, thereby alleviating excessive inflammation and promoting tissue repair. Due to GDO@CM's excellent antioxidant, ferroptosis-inhibiting, and anti-inflammatory properties, GDO@CM treatment also significantly increased the expression of several TJPs (such as ZO-1, Occludin, and Claudin-1) in colon tissue. As shown in Fig. 5L and M, at the end of treatment, the DSS group exhibited almost undetectable levels of ZO-1,

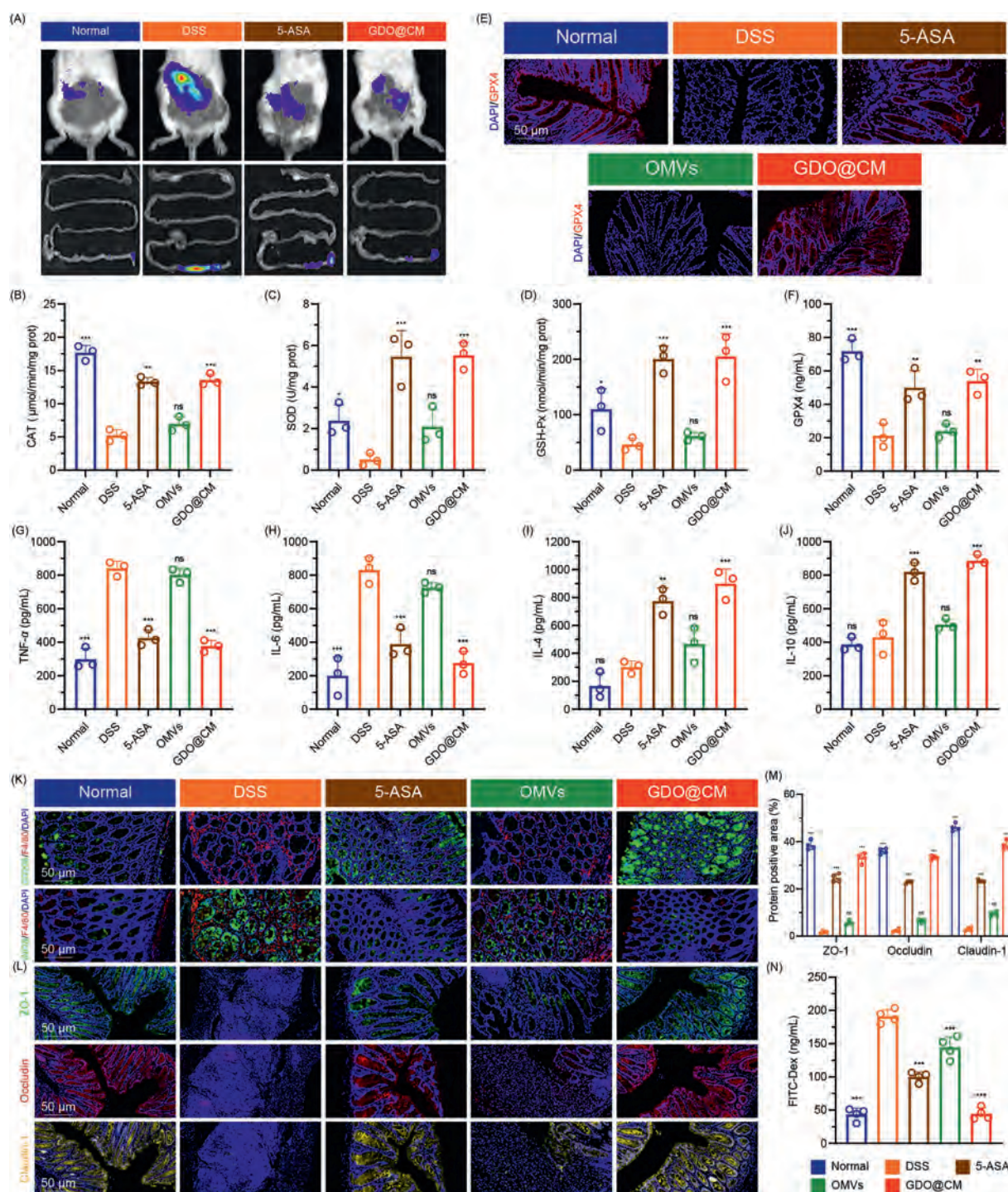


Figure 5 *In vivo* antioxidant, ferroptosis inhibition, anti-inflammatory, and intestinal barrier repair effects of GDO@CM. (A) ROS elimination in colon tissue. (B–D) Enzymatic activity of CAT, SOD, and GSH-Px in colon tissue. (E, F) GPX4 expression in colon tissue (scale bar = 50 μm). (G–J) Expression levels of TNF-α, IL-6, IL-4, and IL-10 in colon tissue. (K) Immunofluorescence staining of iNOS-positive and CD206-positive cells in colon tissue (scale bar = 50 μm). (L, M) Expression levels of ZO-1, Claudin-1, and Occludin in colon tissue (scale bar = 50 μm). (N) Concentration of FITC-Dex in the supernatant from blood collected from colitis mice. Statistical analyses were performed by comparing the DSS group with the other groups. Data are presented as mean ± SD ($n = 3$ for B–D and F–J; $n = 4$ for M and N). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. indicated, ns, not significant.

Occludin, and Claudin-1 expression in colon tissue. In contrast, the GDO@CM-treated group showed a significant increase in the expression of these TJPs, with expression levels 18.76-, 14.81-, and 12.34-fold higher than those in the DSS group. Moreover, the

expression of these proteins was notably higher than that in the 5-ASA group, even approaching the levels observed in the healthy control group. To further assess the effect of GDO@CM on intestinal barrier repair, we used FITC-labeled dextran (FITC-Dex,

Mw = 4 kDa) as a fluorescent probe to evaluate intestinal permeability⁶⁹. FITC-Dex does not penetrate an intact intestinal barrier, but when the barrier is compromised, it enters the bloodstream. As expected, the DSS group showed the highest fluorescence intensity of FITC-Dex in serum, indicating severe disruption of the intestinal barrier and increased intestinal permeability (Fig. 5N). In contrast, the fluorescence intensity in the GDO@CM group was significantly reduced, nearly identical to the healthy control group, suggesting that GDO@CM effectively restores intestinal barrier function and significantly reduces intestinal permeability. In summary, GDO@CM exhibits robust antioxidant, anti-inflammatory, ferroptosis-inhibiting, and intestinal barrier-repairing effects *in vivo*. These findings further support the potential of GDO@CM as an effective treatment option for IBD and provide new theoretical insights and research directions for clinical treatment of IBD.

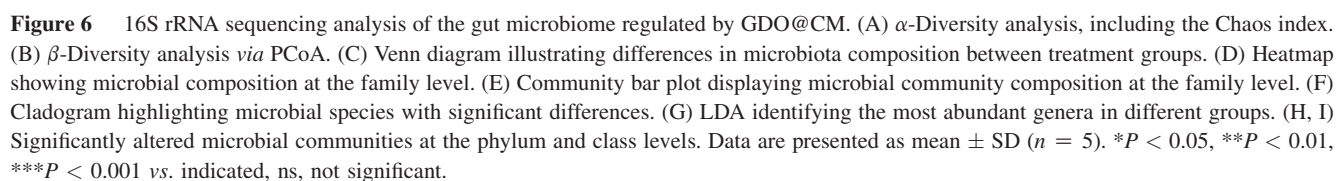
3.5. *In vivo modulation of gut microbiota composition and function*

The gut microbiota and intestinal mucosal barrier are closely interconnected and work synergistically to maintain intestinal health, which in turn has significant effects on systemic physiological functions^{70,71}. A reduction in the abundance of beneficial microbes or the proliferation of harmful bacteria can compromise the integrity of the intestinal barrier, leading to increased intestinal permeability. This allows toxins, pathogens, and undigested food particles to enter the bloodstream, triggering systemic inflammatory responses⁷². Conversely, impaired intestinal barrier function, as observed in conditions such as IBD or drug-induced damage, can disrupt the microbiota, exacerbate inflammation, and aggravate pathological changes⁷³. After confirming that GDO@CM possesses antioxidant, anti-inflammatory, ferroptosis-inhibiting, and intestinal epithelial barrier-repairing capabilities, we further explored its regulatory effects on the gut microbiota through 16S ribosomal RNA (rRNA) gene sequencing analysis. Based on the Chaos, Shannon, and Simpson (Fig. 6A and Supporting Information Fig. S26A and S26B) indices, we observed a significant reduction in these indices in the DSS group, indicating a marked decrease in gut microbiota diversity in the DSS-induced IBD model. In contrast, the indices in the GDO@CM group were significantly elevated, even surpassing those in the Normal group, suggesting that GDO@CM notably restored the diversity of the gut microbiota, demonstrating its superior therapeutic potential. Although the OMVs group showed higher indices compared to the DSS group, the large error bars indicated substantial individual variability or instability in the effects of OMVs on the gut microbiota, suggesting that further optimization of the treatment protocol is needed to improve reproducibility and stability. Principal coordinate analysis (PCoA) based on β -diversity analysis was conducted to further assess and compare the microbial community composition among the control, DSS, OMVs, and GDO@CM groups (Fig. 6B). Overall, the microbial composition of the GDO@CM group closely resembled that of the Normal group, indicating its significant role in regulating the gut microbiota and effectively restoring microbiota structure. In contrast, the DSS and OMVs groups exhibited microbial compositions that deviated significantly from the normal state, indicating marked dysbiosis in the DSS group, while OMVs had a relatively limited effect on gut microbiota modulation. Further Venn diagram analysis (Fig. 6C) confirmed a significant increase in species richness in the GDO@CM treatment group, with the OMVs group

also showing some improvement compared to the DSS group. Heatmaps and bar charts (Fig. 6D and E) provided a detailed visualization of the microbial composition, particularly at the family level, for the Normal, DSS, OMVs, and GDO@CM groups. Additionally, linear discriminant analysis effect size (LEfSe) and linear discriminant analysis (LDA) scores were used to identify differentially abundant taxa, highlighting the prominent taxonomic groups and their variations from phylum to genus (Fig. 6F and G). Notably, compared to the DSS group, GDO@CM significantly altered the microbial composition at the phylum and class levels (Fig. 6H and I). Specifically, harmful microorganisms such as *Enterobacteriaceae_A*, *Proteobacteria*, and *Actinomycetia* were significantly reduced, while beneficial bacteria such as *Firmicutes_D*, *Bacteroidota*, and *Actinobacteriota* were markedly increased. These beneficial microbes contribute to gut health by inhibiting the proliferation of harmful bacteria and modulating intestinal immune functions, thereby promoting immune homeostasis. In conclusion, GDO@CM effectively enhances the richness and diversity of the gut microbiota, reduces the abundance of pathogenic bacteria, and increases the abundance. The observed effects are primarily attributed to the direct antimicrobial actions and probiotic colonization-promoting effects of proteins, LPS, and enzymes present in EcN-OMVs^{74,75}. Furthermore, GA exerts its antimicrobial properties through the scavenging of ROS and the chelation of metal ions, which alters the microenvironment favorable to bacterial survival and proliferation^{76,77}. On the other hand, DATS impedes the signaling pathways of pathogenic bacteria, thereby inhibiting their colonization. Moreover, DATS, through the action of endogenous GSH, releases H₂S, which interferes with bacterial metabolism and physiological functions⁷⁸. The combined actions of the aforementioned components regulate the balance of the gut microbiota. Although previous studies have reported that EcN-OMVs possess the ability to modulate the gut microbiota and promote intestinal health, our study did not observe similar effects in this study. We hypothesize that this discrepancy may be attributed to the insufficient capacity of EcN-OMVs in addressing key pathological aspects of IBD, such as antioxidant activity, inhibition of ferroptosis in IECs, immune modulation, and intestinal barrier repair. The superior capacity to modulate the gut microbiota is insufficient to effectively treat IBD and alleviate clinical symptoms.

3.6. *ScRNA-seq to elucidate the underlying mechanisms of GDO@CM to restore the intestinal barrier integrity*

To comprehensively elucidate the underlying mechanisms of GDO@CM treatment in restoring intestinal barrier integrity, scRNA-seq was performed on colon tissue harvested on day 11 post-treatment using a droplet-based scRNA-seq platform (10 × Genomics) (Fig. 7A). The sequencing data were processed and analyzed using Seurat. After excluding cells with low RNA content or high mitochondrial gene expression, a total of 14,542 cells were retained, with 22,788 genes quantified for each cell. We then identified the top 2000 highly variable genes and performed dimensionality reduction using Uniform Manifold Approximation and Projection (UMAP), which revealed 13 distinct cell subpopulations. These included: cDCs cells (Cd86), T cells (Cd3e, Cd3d, Cd2), B cells (Cd79a, Cd79b), Fibroblasts (Col1a1, Dcn, Lum), Epithelial cells (Epcam), Neutrophils (Csf3r, Cxcr2, Camp, Lcn2), Monocytes/Macrophages (Mrc1, C1qa, Adgre1, Cd14, Ccr2, Ly6c2), Plasma cells (Jchain), Pericytes (Rgs5, Adcc9, Kcnj8), Endothelial cells (Cldn5, Cdh5), Pancreatic



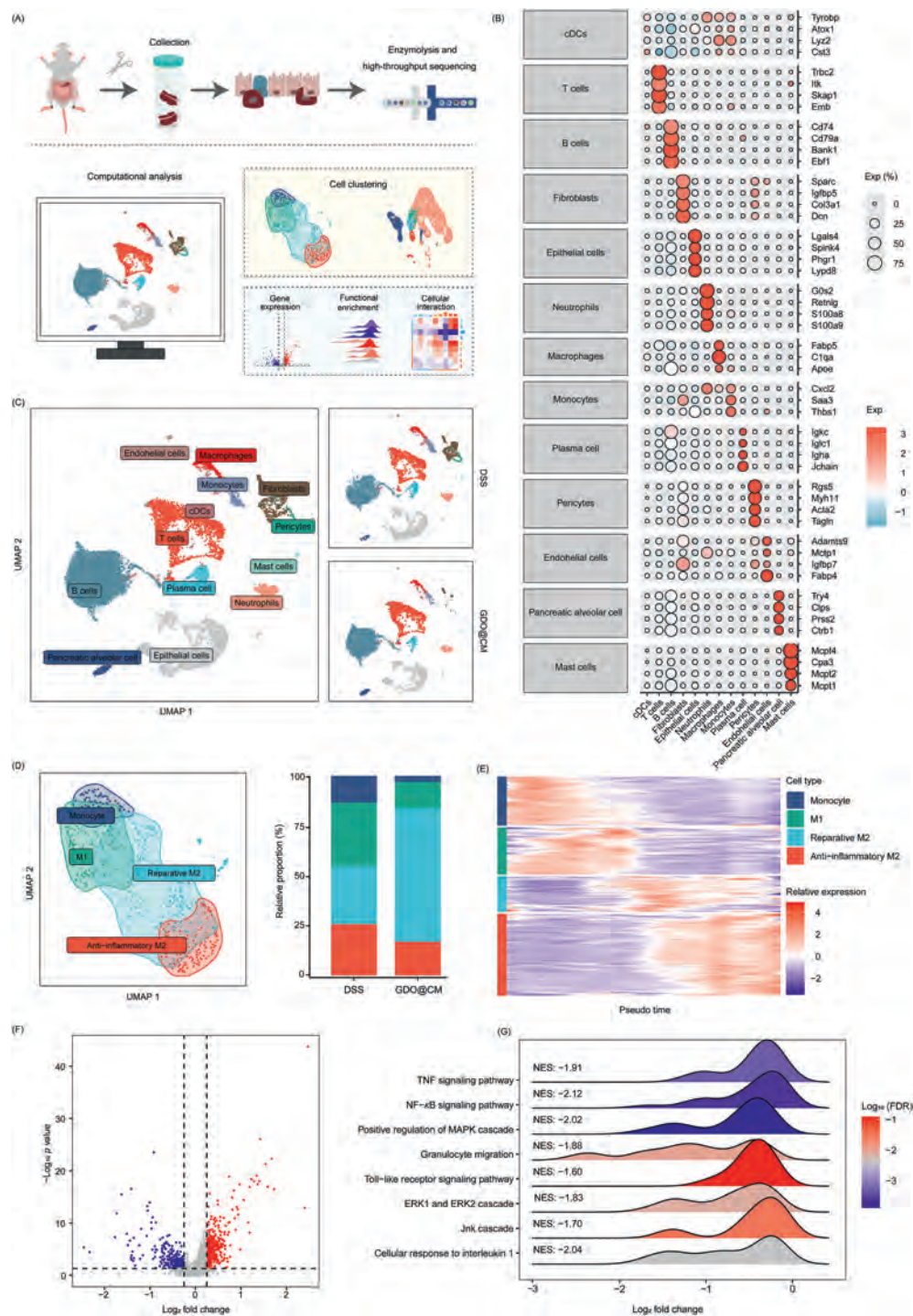


Figure 7 ScRNA sequencing analysis of GDO@CM-treated mice colonic tissues. (A) Graphical overview of the scRNA-seq experimental design. (B) Dot plots displaying 13 cellular clusters with 50 signature gene expressions. The size of each dot represents the proportion of cells expressing specific marker genes, while the color gradient indicates the average expression levels of the markers (converted to Z-scores). (C) UMAP plots showing 13 major cell types identified in the colon of GDO@CM-treated mice, with each dot representing an individual cell. (D) Plot depicting four subpopulations of monocyte-macrophages, their proportions in different treatment groups, and the expression levels of marker genes across cells at various differentiation stages. (E) Heatmap displaying the expression levels of marker genes for four macrophage sub-clusters across different pseudo time points. (F) Volcano plot showing DEGs in macrophages between treatment groups. (G) GSEA reveals downregulation of multiple signaling pathways after GDO@CM treatment.

alveolar cells (Pla2g1b, Pnliprp2, Cpa2), Mast cells (Cpa3) (Fig. 7B and C)⁷⁹.

Macrophages are key mediators of inflammation in IBD and important therapeutic targets. Compared to the DSS group, the GDO@CM treatment group showed a significant reduction in the number of macrophages and monocytes, suggesting that GDO@CM may exert its anti-inflammatory effects by inhibiting the infiltration or activation of these cells. Additionally, fibroblasts, which play a central role in intestinal fibrosis—an often-observed complication of IBD—also exhibited significantly inhibited proliferation, indicating that GDO@CM may possess antifibrotic properties (Supporting Information Fig. S27). To further investigate the effects of GDO@CM on macrophage phenotype, we isolated the monocytes and macrophage subpopulations and performed additional UMAP dimensionality reduction. We identified four distinct macrophage subpopulations: monocytes (Ly6c2), M1 macrophages (Tnf), reparative M2 macrophages (M2a macrophages, Il4ra), and anti-inflammatory M2 macrophages (M2b macrophages, Mrc1) (Fig. 7D and Supporting Information Figs. S28 and S29). To validate the accuracy of the subpopulation assignments, pseudotime analysis was performed on macrophages, and the expression of marker genes for each cell type was visualized (Fig. 7E). The results revealed that the marker genes for monocytes were predominantly expressed at the early stages of differentiation, corresponding to the blood monocyte state. M1 macrophage markers showed high expression during the injury phase, where pro-inflammatory macrophages produce TNF- α , IL-1 β , IL-6, and other cytokines to recruit immune cells and eliminate pathogens and damaged cells. Subsequently, M2 macrophages emerged, reflecting the transition of infiltrating macrophages to the anti-inflammatory (M2) phenotype, playing a crucial role in inflammation resolution and tissue repair⁸⁰. After GDO@CM treatment, the proportions of monocytes, M1 macrophages, and anti-inflammatory M2 macrophages were downregulated, while the proportion of reparative M2 macrophages significantly increased. These findings suggest that GDO@CM effectively controlled colonic inflammation in IBD and promoted the differentiation of macrophages into a repair phenotype, thereby facilitating the recovery of the intestinal barrier from inflammatory damage.

To assess the effect of GDO@CM treatment on macrophage function, differentially expressed genes (DEGs) analysis was conducted using the FindAllMarkers function to compare gene expression profiles between the GDO@CM and DSS treatment groups. A total of 463 upregulated genes and 256 downregulated genes (with a Log₂ fold change threshold of 0.25 and a *P*-value threshold of 0.05) were identified, indicating significant alterations in the macrophage transcriptome following GDO@CM treatment (Fig. 7F). To further elucidate the biological significance of these transcriptomic changes, GSEA was performed using GO and KEGG (Kyoto Encyclopedia of Genes and Genomes) gene sets (Supporting Information Figs. S30 and S31). The results revealed that GDO@CM treatment significantly inhibited several key signaling pathways, including Toll-like receptor, TNF, NF- κ B, MAPK, granulocyte migration, ERK1/2, JNK cascade, and interleukin 1 signaling pathways (Fig. 7G). These pathways not only play a critical role in the inflammatory response but also contribute to oxidative stress by promoting ROS generation. By modulating oxidative stress levels, these pathways exacerbate cellular damage, further propagating inflammation. The significant inhibition of these pathways suggests that GDO@CM may exert its potent anti-inflammatory effects by reducing ROS generation

and suppressing pro-inflammatory signaling cascades in macrophages. Taken together, GDO@CM treatment effectively alleviates the inflammatory response in IBD by promoting the polarization of macrophages from the pro-inflammatory M1 phenotype to the reparative M2 phenotype, while inhibiting the activation of multiple signaling pathways involved in oxidative stress and pro-inflammatory responses. Ultimately, the remission of inflammation may facilitate the repair of the intestinal barrier.

Given that epithelial cells are a major component of the colon, playing crucial roles in barrier protection and tissue repair—especially in pathological conditions such as IBD, where epithelial damage and repair are key factors in maintaining intestinal health, we further isolated epithelial cells and performed dimensionality reduction and clustering using similar methods. Five epithelial cell subtypes were identified: enterocytes (Vil1, Krt20), goblet cells (Atoh1, Muc2, Tff3), ISCs (Lgr5, Ascl2), transit-amplifying cells (Mki67, Top2a), and enteroendocrine cells (Chga, Chgb, Pyy, Gcg) (Fig. 8A and Supporting Information Fig. S32). Following GDO@CM treatment, the proportions of goblet cells, ISCs, and transit-amplifying (TA) cells increased (Fig. 8A). Goblet cells secrete mucus, forming a protective mucosal layer essential for maintaining the intestinal barrier. ISCs within crypts differentiate into TA cells, which subsequently give rise to various IECs, including absorptive enterocytes and goblet cells. This suggests active epithelial cell regeneration, contributing to intestinal barrier repair. These findings indicate that GDO@CM treatments promote epithelial regeneration and mucus secretion, thus facilitating the restoration of the intestinal barrier.

To further investigate the functional changes in epithelial cells following GDO@CM treatment, we performed GSEA using GO and KEGG gene sets (Supporting Information Figs. S33 and S34) to analyze DGEs between the GDO@CM and DSS treatment groups. GDO@CM treatment significantly downregulated the expression of genes associated with ferroptosis and apoptosis, while activating key signaling pathways, including MAPK, ERK1/2, and cadherin-mediated cell adhesion (Fig. 8B). The downregulation of ferroptosis and apoptosis-related genes protects the intestinal epithelium from oxidative stress-induced damage, promoting cell survival and aiding the repair of the intestinal barrier. The MAPK pathway, a crucial signaling cascade in eukaryotic cells, regulates processes such as cell proliferation, migration, and immune responses—all essential for intestinal barrier repair. Specifically, ERK1/2 activation promotes the proliferation and migration of IECs, accelerating epithelial repair. Moreover, cadherins, calcium-dependent adhesion molecules, form strong intercellular connections that are vital for maintaining intestinal barrier integrity. Activation of cadherins reduces barrier permeability and facilitates the repair of damaged cell junctions, supporting barrier recovery. These findings underscore the protective role of GDO@CM in IECs, highlighting its ability to inhibit cell death, promote cell survival, and enhance cell–cell adhesion. These effects contribute to the restoration and maintenance of the intestinal barrier, ultimately supporting overall gut health.

In the context of IBD, macrophages play a pivotal role by secreting high levels of pro-inflammatory cytokines such as interleukin-12 (IL-12) and IL-23, which contribute to inflammation and tissue damage. Concurrently, macrophages secrete growth factors, including insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF)- α , which regulate epithelial cell proliferation and repair processes. This dual role highlights the importance of macrophages not only in mediating

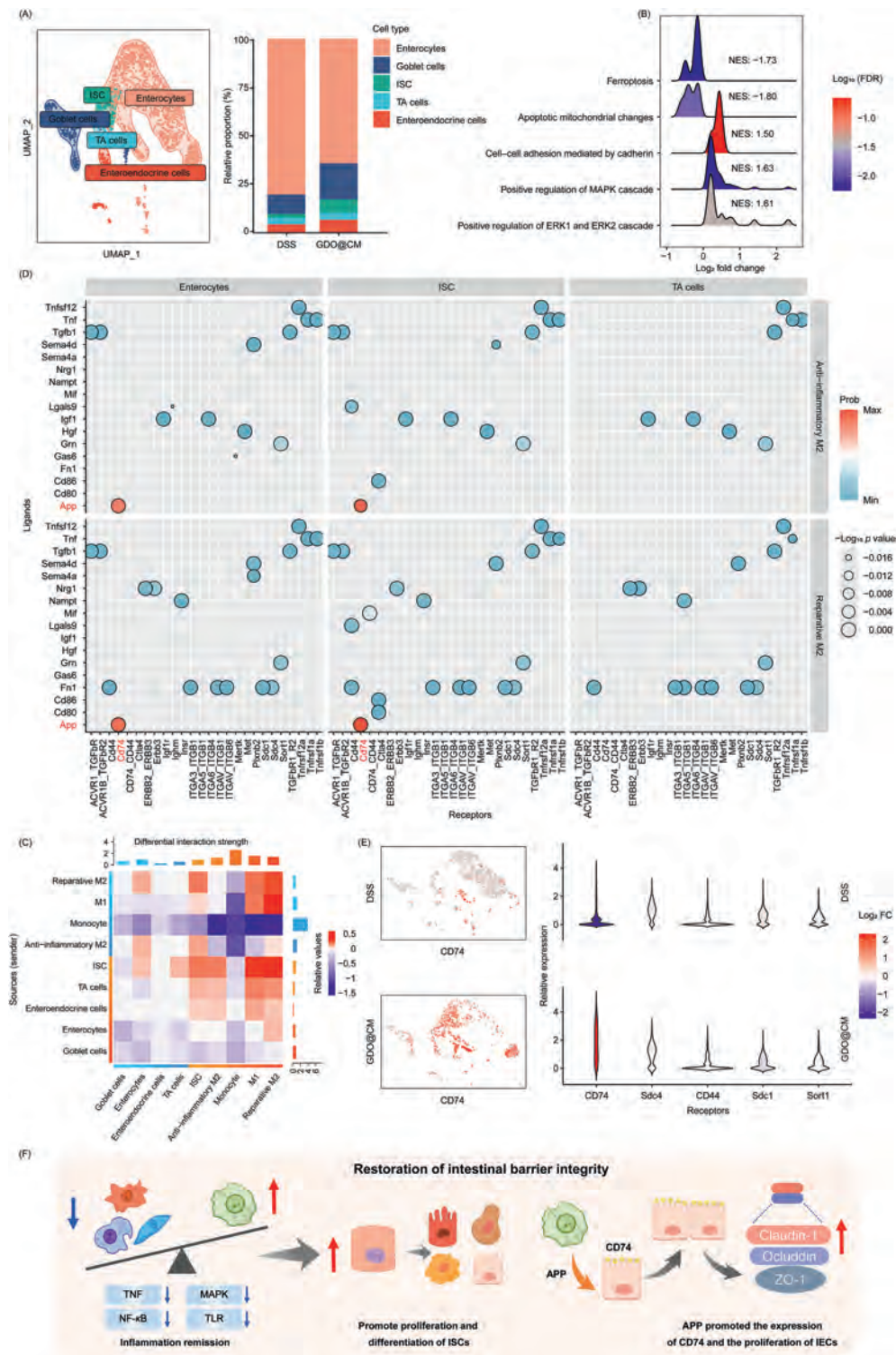


Figure 8 GDO@CM remodels the epithelial-macrophage crosstalk to promote intestinal barrier repair. (A) Plot displaying five subpopulations of epithelial cells and their proportions in different treatment groups. (B) GSEA indicating functional changes in epithelial cells following GDO@CM treatment. (C) Differences in interaction strength between the GDO@CM and DSS treatment groups (red indicates increased interactions, blue indicates decreased interactions). (D) Predicted interactions between macrophages and epithelial cells, where the size of the circles represents the P value, and the color intensity indicates the likelihood of interaction. Red indicates a higher likelihood, while blue indicates a lower likelihood. (E) Expression levels of CD74 in the DSS and GDO@CM treatment groups, alongside receptors potentially involved in macrophage-epithelial cell interaction in both groups. (F) Schematic diagram illustrating the efficient intestinal epithelial barrier healing mechanism of GDO@CM.

inflammation but also in supporting tissue repair. In our previous analysis, we observed a significant upregulation of reparative M2 macrophages following GDO@CM treatment. To further investigate whether macrophages are responsible for mediating these reparative effects, we utilized the CellChat tool to analyze the interactions between M2 macrophages and epithelial cells. By extracting cells from both the DSS and GDO@CM groups and calculating communication probabilities, we compared the intensity of cell–cell communication before and after GDO@CM treatment (Supporting Information Figs. S35 and S36). The results demonstrated a marked increase in signal transmission within repair-type M2 macrophages and anti-inflammatory M2 macrophages. Simultaneously, epithelial cells, including ISCs, TA cells, and enterocytes, exhibited enhanced signal reception (Fig. 8C).

Notably, the analysis of the top 100 signaling pathways involved in macrophage-to-epithelial communication revealed a significant interaction between CD74, a key receptor expressed on the surface of IECs, and amyloid precursor protein (APP) secreted by M2 macrophages (Fig. 8D). Further validation through a violin plot confirmed the upregulation of CD74 expression in the GDO@CM-treated group, suggesting enhanced activation of downstream CD74 signaling pathways (Fig. 8E). APP is a transmembrane protein widely expressed in various cell types, with its highest expression observed in neurons⁸¹. In addition to nervous, APP is expressed in other cell types, including monocytes and endothelial cells^{82,83}. APP exists not only as a membrane-bound protein but also as soluble fragments that can be secreted into the extracellular space^{84–86}. Beyond its well-established role in the nervous system, APP has been implicated in immune regulation, cell growth, and tissue repair^{86,87}. In M2 macrophages, membrane-bound APP may directly interact with receptors on the surface of IECs or promote CD74 expression indirectly by inducing the secretion of endogenous signaling molecules, such as cytokines and chemokines. Moreover, soluble APP fragments may circulate or be locally released, binding to CD74 receptors on IECs and further enhancing their expression. CD74, a surface receptor predominantly found on immune cells like dendritic cells and macrophages, plays a critical role in immune responses^{88,89}. Although CD74 is primarily involved in immune cell functions, it also participates in the repair of intestinal damage. During this repair process, CD74 interacts with other signaling pathways to regulate the proliferation, migration, and restoration of IECs^{90,91}. The upregulation of CD74 expression is likely to enhance the interaction between IECs and surrounding cells, thus accelerating the repair of the intestinal barrier. Based on these findings, we hypothesize that APP may significantly elevate CD74 expression on the surface of IECs through multiple mechanisms, including direct cell–cell contact and secretory-mediated modulation. This enhanced CD74 expression could, in turn, promote the proliferation of IECs and expedite the repair of the intestinal barrier.

In summary, we propose that the primary mechanisms by which GDO@CM efficiently restores intestinal barrier integrity involve several key steps: (1) Alleviation of chronic inflammation: GDO@CM alleviates the inflammatory response in IBD by promoting the polarization of macrophages from the pro-inflammatory M1 phenotype to the reparative M2 phenotype. At the same time, it inhibits the activation of multiple signaling pathways involved in oxidative stress and pro-inflammatory responses, further reducing intestinal inflammation. (2) Promotion of ISCs proliferation and differentiation: GDO@CM significantly increases the proportion and number of ISCs and goblet cells, while downregulating the expression of genes related to

ferroptosis and apoptosis. In parallel, signaling pathways associated with cell proliferation and adhesion are significantly activated, facilitating the repair of the intestinal barrier. (3) Strong interaction of the APP/CD74 signaling axis: The robust interaction between APP and CD74 significantly promotes the proliferation of IECs, accelerating the restoration of the intestinal barrier (Fig. 8F).

4. Conclusions

In this study, we present the development of GDO@CM, an engineered probiotic-derived OMV formulation designed to efficiently repair the intestinal barrier and manage IBD by targeting key pathophysiological factors such as oxidative stress, ferroptosis, immune homeostasis disruption, and gut microbiota dysbiosis. GDO@CM is constructed by utilizing EcN-derived OMVs as a carrier, incorporating GA and DATS into the hydrophilic inner cavity and lipid bilayer of the OMVs, respectively. CM is electrostatically attached to the OMV surface to enhance stability in colon fluids and to facilitate targeted delivery to damaged colonic sites. Upon rectal administration, GDO@CM preferentially accumulates at the injured colonic mucosa, where it is efficiently internalized by activated immune cells. GA efficiently scavenges ROS and inhibits ferroptosis, while DATS, in response to GSH, releases H₂S, thereby enhancing antioxidant effects and exerting a robust anti-inflammatory effect. Furthermore, the combination of GA, DATS, and CM synergistically inhibits the colonization of pathogenic bacteria at IBD sites. *In vitro* and *in vivo* experiments demonstrated that GDO@CM exhibits exceptional antioxidant activity, effectively inhibits ferroptosis, and restores immune homeostasis and gut microbiota balance. These combined actions significantly promote intestinal barrier repair and alleviate IBD-related symptoms. 16S rRNA sequencing revealed that GDO@CM remodels the gut microbiota by inhibiting harmful bacterial populations and enhancing the abundance of beneficial bacteria. ScRNA-seq further demonstrated that GDO@CM significantly reduces inflammation, increases the proportion of reparative M2 macrophages and ISCs, and promotes epithelial cell proliferation and barrier repair through the activation of the APP/CD74 axis by M2 macrophages. In conclusion, GDO@CM offers a promising, multifaceted therapeutic strategy for IBD, leveraging its potent ROS scavenging, anti-inflammatory, and intestinal barrier restoration properties. This engineered OMVs formulation holds significant potential as an effective treatment for clinical IBD, providing a novel and targeted approach to managing this chronic condition.

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Author contributions

Li Yu: Investigation, methodology, visualization, writing-original draft. He Zhang, Chengge Shi, Qiang Zhou, Jiayu Li, Bin Lu: Methodology, visualization, and formal analysis. Hongyang Lu, Ting Jin, Yinci Zhu, Tianci Zuo, Mengzhu Xu, Mingli Su, and Yanmei Zhang: Methodology, resources, and validation. Quazi T.

H. Shubhra, Xiaowen Hu, and Hui Deng: Supervision. Xiaojun Cai: Conceptualization, resources, supervision, funding acquisition, visualization, writing-review, and editing. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2026.01.031>.

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