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Natural products and intestinal mucosal immunity

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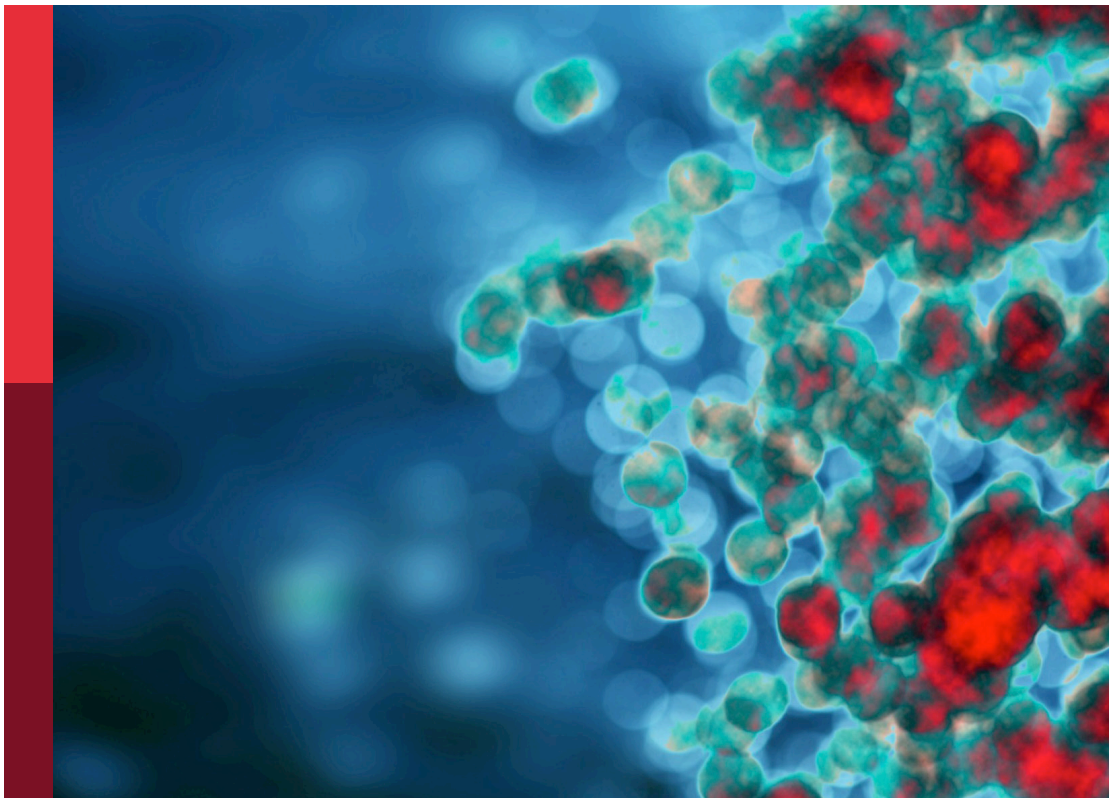

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Natural products and intestinal mucosal immunity

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Table of contents

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Editorial: Natural products and intestinal mucosal immunity

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Editorial on the Research Topic
Natural products and intestinal mucosal immunity

The gastrointestinal tract is the largest immunological interface of the body. Mucosal immunity maintains a delicate equilibrium in this surveilled environment by balancing tolerance to commensal bacteria with defense against pathogens (1). Disruption of this equilibrium can trigger the pathogenesis of inflammatory bowel disease (IBD), metabolic disease, and extra-intestinal inflammatory disorders (2). Natural agents, including phytochemicals, herbal extracts, and dietary constituents, are emerging as potent modifiers of intestinal immunity (3). Their pleiotropic mechanisms, favorable safety profiles, and ability to synergize with the host physiology confer specific advantages compared to conventional immunomodulatory approaches. This Research Topic compiles pioneering research that elucidates how natural molecules modulate intestinal immunity at the molecular, cellular, and multi-omic levels. The ten articles included here explore diverse mechanisms from mitochondrial homeostasis and cell death mechanisms to microbiome crosstalk and clinical translation, collectively advancing natural products from empirical remedies to rationally based therapeutics.

At the forefront of intestinal defense, disruption of the epithelial barrier initiates inflammatory cascades. Cai et al. demonstrated that hyperglycemia exacerbates barrier damage by activating neutrophil extracellular traps (NETs). These are extracellular chromatin structures that disrupt tight junctions. The flavonoid baicalin can prevent NET formation by inhibiting histone citrullination, a key modulator of NETosis; this preserves intestinal integrity in diabetes models. Zhou et al. revealed that aberrant mitochondrial dynamics play a critical role in ulcerative colitis (UC) pathogenesis. The accumulation of dysfunctional mitochondria was found to generate reactive oxygen species (ROS), which trigger the NLRP3 inflammasome and perpetuate inflammation. Phytochemicals such as curcumin and resveratrol enhance mitophagy via the PINK1/Parkin and AMPK pathways. This selective mitochondrial elimination reduces epithelial apoptosis and restores redox balance; thus, mitochondrial quality control was established as a target for treating IBD with natural compounds.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is a key target of natural compounds that modulate mucosal immunity. According to Long et al., berberine suppresses STAT1/STAT3 phosphorylation, and *Tetrastigma hemsleyanum* polysaccharide induces SOCS1 expression, serving as a natural JAK inhibitor. These agents promote Th17/Treg cell balance and improve colitis without the hematologic toxicity associated with synthetic JAK inhibitors. Pan et al. observed that the flavonoid kurarinone inhibits Th17 differentiation and increases IL-10-producing regulatory T cells by upregulating Blimp-1. Cell death pathways also revealed their multi-mechanistic roles, and Zhao and Lin found PANoptosis, an integrated cell death program involving pyroptosis, apoptosis, and necroptosis, to be a primary force in IBD. The authors demonstrated how berberine blocks caspase-8-mediated epithelial apoptosis, suggesting that natural products may intercept PANoptosis at multiple levels to ensure mucosal viability.

The microbiome, serving as both a target and a mediator of natural products, is essential in regulating intestinal immunity. According to Zhu et al., commensal fungi utilize pattern recognition receptors such as Dectin-1 and TLRs to modulate host gut immunity. Pan et al. demonstrated that kurarinone augments beneficial gut genera such as *Lactobacillus* and *Ruminiclostridium*, contributing to the modulation of intestinal mucosal inflammation. Guan et al. investigated the microbial-metabolite-immune axis of gastroesophageal reflux disease (GERD). Lower GI microbiome dysbiosis may indirectly induce GERD by changing gut dynamics, and this could be mitigated by ingredients in Chinese herbal medicine. These compounds restore microbial richness and enhance mucin secretion and thus illustrate how prebiotic-like natural compounds enhance mucosal protection by modulating the gut microbiome.

Natural compounds demonstrate clinical relevance beyond IBD to iatrogenic diseases such as immune checkpoint inhibitor-induced colitis (irColitis). Dong et al. described how traditional Chinese medicine formulas such as Gegen Qinlian decoction alleviate irColitis by inhibiting JAK/STAT and NF-κB signaling pathways. Similarly, Wang et al. demonstrated that cimifugin acts on the gut-organ axis to modulate UC-associated lung injury by inhibiting JAK1/STAT1-dependent macrophage M1 polarization, underscoring the systemic impacts of gut-targeted immunomodulation with botanical compounds.

Future research priorities for this field encompass several key areas. First is the development of precision-targeted nanoparticle formulations to achieve maximum bioavailability and tissue-selective targeting. Second is engineering protective microbiomes through the synergistic combination of phytochemical prebiotics, probiotics, and other applicable approaches. Third is identifying additional natural products with the ability to modulate intestinal mucosal immunity and establishing a multidimensional methodological approach to elucidate the mechanisms of action, thereby laying a foundation for further clinical applications. Fourth is conducting rigorous clinical benchmarking via randomized controlled trials comparing natural products with biologics in UC, irColitis, and related conditions, to further validate the clinical effectiveness of natural products.

This Research Topic illuminates the cutting-edge advances in this field. Natural products are evolving from complementary therapies to emerge as potent immunomodulatory agents with selective molecular targets. With applications ranging from the inhibition of NETosis and the regulation of PANoptosis to the activation of mitochondria and the alteration of the microbiome, natural products modulate the intestinal immune system with remarkable breadth. The included studies integrate ethnopharmacology with molecular medicine, offering promising approaches to addressing conditions that are associated with gut mucosal immunity.

Author contributions

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Global hotspots and trends in gut mycological research: a visual analytics and bibliometric approach

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Background: Recent findings highlight the significant impact of intestinal fungi on the complex makeup of the gut microbiota and human health, challenging past oversights. However, a lack of thorough systematic and quantitative analyses remains. This study aims to address this gap by thoroughly examining the current research on gut fungi. Through analyzing developments and unique features in this area, our goal is to foster a deeper understanding and identify future research pathways.

Methods: We performed an extensive bibliometric analysis on documents from 2000 to 2023, sourced from the Web of Science Core Collection (WoSCC). Utilizing advanced visualization tools such as VOSviewer, CiteSpace, and Bibliometrix R, we meticulously examined and illustrated the data in scientific landscapes and networks.

Results: A total of 1434 papers were analyzed, revealing a substantial increase in publication volume over the past two decades, particularly in 2020. Contributions came from 67 countries, 2178 institutions, and 8,479 authors. China led in publication output with 468 articles, followed by the University of California with 84 articles, and ZHANG F as the most prolific author with 17 articles. Emerging research areas such as "Fungal-Bacteria Interactions," "Gut Fungus and Gut-Brain Axis," and "Gut Fungus and Immunity" are expected to attract growing interest in the future.

Conclusion: This extensive bibliometric analysis offers a current overview of scholarly efforts concerning intestinal fungi, highlighting the predominant

landscape in this field. These insights can assist scholars in identifying appropriate publication avenues, forming collaborative relationships, and enhancing understanding of key themes and emerging areas, thereby stimulating future research endeavors.

KEYWORDS

gut, microbiota, gut microbiome, fungi, gut fungal ecology

1 Introduction

The gastrointestinal tract harbors a diverse array of microorganisms, collectively referred to as the gut microbiota, which includes bacteria, viruses, fungi, and archaea. The intricate gut microbiota plays a pivotal role in human health and disease, primarily exerting its influence in areas such as host digestion, the normal metabolism of various substances, the synthesis of essential vitamins, the recognition and resistance to pathogens, the maintenance of functional stability of the intestinal barrier, and the shaping and regulation of the immune system (1–3).

In previous studies, the primary focus of research on the gut microbiome has predominantly been on bacterial components. This is likely due to the relatively low abundance of fungi within the gut microbiome, which typically ranges from 0.1% to 1% (4). However, in recent years, scientists have progressively unveiled the non-negligible role of fungi, highlighting their significance in the complex ecosystem of the gut (5).Certain pathogenic fungal species have been implicated in various disorders, including cancer, inflammatory bowel disease, immune dysregulation, and atherosclerosis (6). Concurrently, commensal fungi may leverage pattern recognition receptors such as Dectin-1 and TLRs to exert significant roles in crucial physiological pathways, including the gut-brain axis, metabolic equilibrium, and immune modulation. For example, in 2019, the groundbreaking findings by Bacher and colleagues have elucidated that, within a cohort of 30 fungal community members, Candida albicans stands out as the most efficacious agonist for the induction of human CD4+ memory T helper 17 (Th17) cells (7). In parallel, the field of gut mycobiome research still harbors significant gaps that warrant exploration. These include the definitional issues surrounding the gut mycobiome, the taxonomic ascertainment of intestinal fungal species, and their contributions to human health and the pathogenesis of diseases (8).

In response to the vast amount of literature data in the field of gut mycobiome, we have considered bibliometric analysis, an emerging research methodology. Bibliometric analysis, a quantitative method for evaluating the impact and trends of academic publications, involves statistical examination of citations, author collaborations, and journal metrics (9). Traditional reviews, while offering a comprehensive overview of a particular field, often lack specific data support and visualization, requiring readers to possess a high level of expertise. Compared to them, bibliometric analysis offers a rigorous approach to understanding vast amounts of unstructured data, which can be employed to decipher and map the accumulated scientific knowledge and evolutionary nuances of established fields. Within the realm of gut mycobiome research, it facilitates scholars in acquiring a holistic overview at once, pinpointing knowledge gaps, formulating innovative research directions, and strategically positioning their anticipated contributions to the field (10).

Historically, bibliometric analyses within this domain have predominantly encompassed the gut microbiome, without specifically highlighting the role of fungi. The present study endeavors to elucidate the significant function of fungi within the gut microbiome by leveraging data from the Web of Science, employing visualization to delineate the distribution of publications, authors, institutions, and keywords from 2000 to 2023. By identifying trends and research foci, this analysis aims to systematically articulate the study of the gut mycobiome in physiological and pathological mechanisms and its association with clinical diseases.

2 Methods

2.1 data collection

Data on Gut microbiota and fungi were retrieved from the Science Citation Index Expanded (SCI-EXPANDED) and the Social Sciences Citation Index (SSCI) between January 1, 2000, and December 31, 2023. These datasets were extracted from the Web of Science Core Collection (WoSCC) database on February 20, 2024, using advanced search terms. The construction of advanced search terms involves the strategic combination of Boolean operators, synonyms, wildcards, and filters to precisely and efficiently retrieve relevant literature or data within a specific domain.

1. ((((((TS=(gut microbiota)) OR TS=(intestinal microbiota)) OR TS=(fecal microbiota)) OR TS=(gastrointestinal microbiota)) OR TS=(gut microbiome)) OR TS=(intestinal microbiome)) OR TS=(fecal microbiome)

2. ((TS=(fungus)) OR TS=(fungoid)) OR TS=(fungi)
3. #2 AND #1

All data elements, encompassing titles, keywords, authorship, geographical and institutional origins, publishing journals, publication dates, H-indices, and citation metrics, were meticulously extracted from the publications identified by the two authors (ZW and CJ). The search formula we applied yielded an initial corpus of 6,267 documents pertinent to our research theme. After imposing restrictions on document types, language preference for English, and temporal constraints, the collection was refined to 2,109 documents.

The subsequent phase involved a meticulous manual curation by the same authors to exclude articles that did not align with the thematic focus of our study. Discrepancies in the assessment were amicably addressed by an esteemed corresponding author (LY), following a systematic procedure. This procedure commenced with a critical analysis of the titles and abstracts to ascertain the thematic relevance of the articles. Those deemed suitable based on this initial evaluation were subjected to a thorough manual review to confirm their alignment with our research objectives.

In instances where the thematic relevance could not be ascertained from the title alone, the full texts were procured from the Southeast University Library for an in-depth examination to verify their pertinence to our study. Post the stringent application of our inclusion and exclusion criteria, a curated subset of 1,434 articles was selected for an in-depth analysis, thereby ensuring the scholarly rigor and thematic integrity of our literature review. The detailed procedures for subject enrollment and exclusion criteria are delineated in Figure 1.

2.2 Data analysis

The initial dataset was sourced from the WoSCC database and analyzed using VOSviewer (version 1.6.20), CiteSpace (version 6.1.6), and the Bibliometrix R package. These tools were selected for their specific contributions to our bibliometric analysis. VOSviewer was utilized for its network visualization capabilities, which facilitated the exploration of citation and co-citation networks. CiteSpace was employed to perform advanced co-citation analysis and to uncover trends in publication dynamics. The Bibliometrix R package provided a robust framework for data extraction and refinement within the R environment, allowing for detailed bibliographic analysis. Utilizing these three tools allows for the straightforward creation of visual representations of bibliometric data, a method widely employed in mainstream bibliometric analysis.

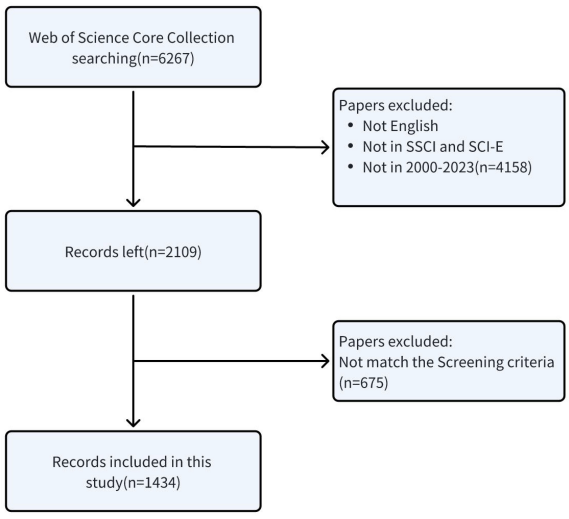

FIGURE 1
Flow diagram of literature search and screening.

3 Results

3.1 Annual development trend of publications and citation

In Figure 2A, we present the annual publication volume and cumulative number of published documents spanning the past 23 years, while Figure 2B delineates the annual average citations within this domain. Noteworthy is the substantial increase witnessed in both annual and cumulative publications over the years. Annual citations reached their zenith in 2006, 2008, 2014, and 2017, signifying notable strides in research during these periods. Critically speaking, this could be attributed to a significant increase in the number of new journals, as well as a tendency among researchers to pursue trending fields in academia. Particularly striking is the citation peak in 2014, which may be attributed to the first clinical application of Next-Generation Sequencing-based metagenomic detection (mNGS) that successfully saved the life of a 14-year-old boy (11, 12). These trends underscore the burgeoning significance of mycology as a global focal point within the sphere of human health.

3.2 Analysis of productive journals

A comprehensive analysis reveals a total of 1434 articles related to gut fungi, dispersed across 506 journals. Notably, Table 1 showcases the top ten most prolific journals in this domain, offering a glimpse into the scholarly landscape. Topping the list is Frontiers in Microbiology, boasting 104 articles, which translates to 7.25% of the total publications. Following closely behind is PLOS ONE, with 51 articles, representing 3.56% of the corpus. However, it is intriguing to note that MICROBIOME, despite ranking fourth in terms of the number of articles, stands out with an impressive impact factor (IF) of 15.5 and an average number of citations per article at 72, underscoring its substantial influence within the realm of intestinal mycology. Regarding these top-tier journals, they have a high volume of publications, and many scholars in the field will opt for these journals when submitting their work. The editorial policies of these journals can also indirectly influence the direction of research. This data not only highlights the breadth of research

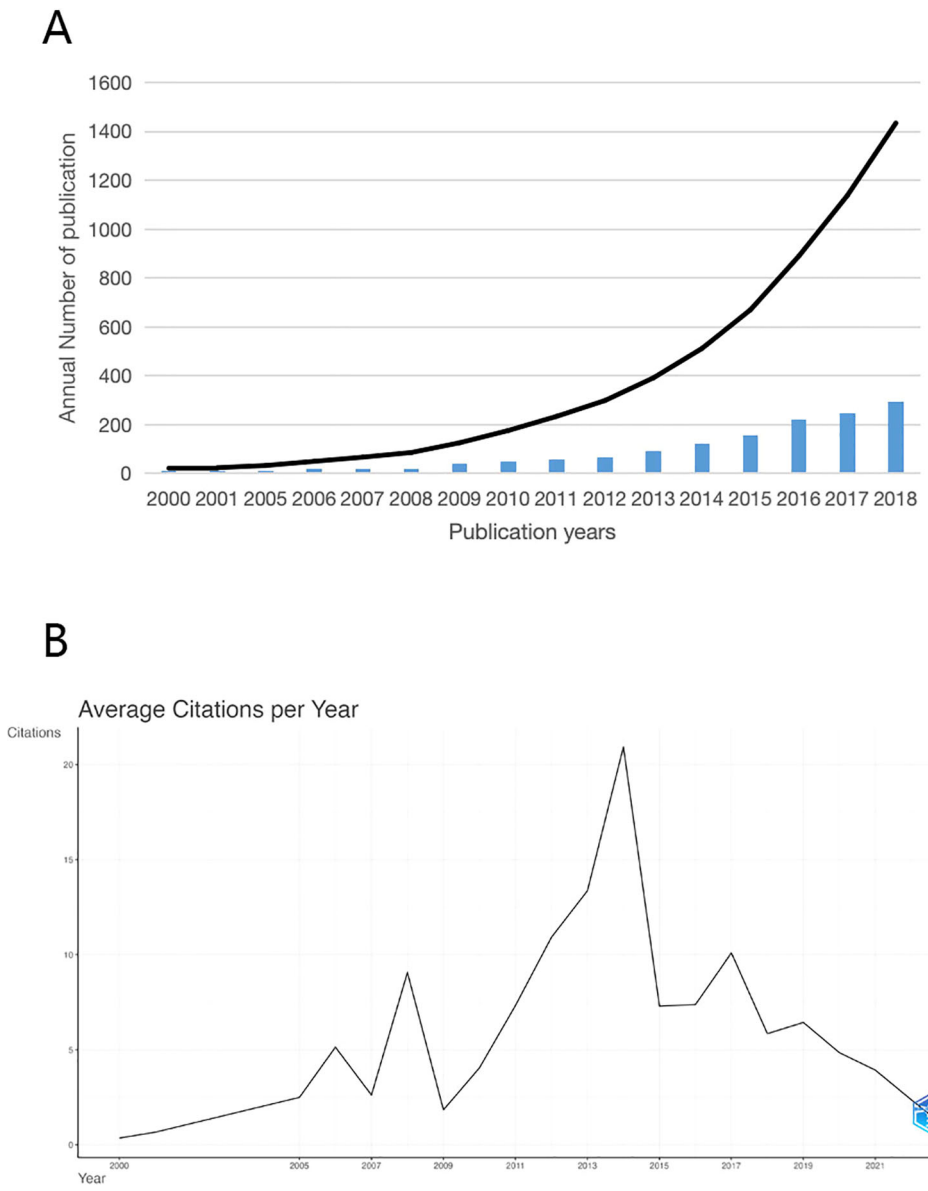

FIGURE 2
(A) Trends in the growth of publications in gut fungi **(B)** The number of average citations per year in gut fungi.

interest in gut fungi but also emphasizes the importance of key journals in shaping the discourse and impact of this field.

3.3 Global meta-analysis: countries, institutions, and authors

In the expansive field of gut microbiota research, exploration into fungal elements has attracted interest from 67 countries and regions worldwide. Table 2 delineates the publication output, highlighting that China tops the list with a total of 463 scholarly contributions, followed by the USA with a count of 299 articles. These two nations surpass the 100-article mark, indicating significant investment in fungal gut research. While China leads in volume, the USA achieves nearly five times the average citations per article, reflecting higher research impact. The leading position of China and the United States in terms of publications is primarily attributed to ample research funding and robust scientific infrastructure. Collaboration networks, illustrated in Figure 3, reveal the USA as the central hub for gut fungi research, closely collaborating with China, Germany, the United Kingdom, and Canada. The formation of these collaboration networks facilitates the ease with which scholars from around the globe can exchange their research directions. Consequently, whenever a new hot topic is identified, all scholars can quickly detect it and delve into further exploration. Recent years have seen a surge in international academic exchanges, demonstrating China's substantial potential in this field.

Table 3 meticulously outlines the affiliations of authors involved in intestinal fungi research. The University of

TABLE 1 The top ten journals based on total citations in gut fungal microbiota.

Sources	Articles	Citations	Citations per-publication	Journal IF
FRONTIERS IN MICROBIOLOGY	104	3001	29	5.2
PLOS ONE	51	2163	42	3.7
SCIENTIFIC REPORTS	41	1971	48	4.6
MICROBIOME	27	1939	72	15.5
MICROBIAL ECOLOGY	25	1711	68	3.6
JOURNAL OF FUNGI	23	1652	72	4.7
MICROBIOLOGY SPECTRUM	23	1428	62	3.7
MICROORGANISMS	20	1050	53	4.5
ANIMALS	18	1015	56	3
APPLIED AND ENVIRONMENTAL MICROBIOLOGY	17	973	57	4.4

California System leads the chart in both article count and citations, reflecting its substantial research output and scholarly influence. Following closely is the esteemed Chinese Academy of Sciences, with 71 articles and 310 citations. The leading positions of these two institutions are likely due to their large scale, with numerous branches or faculties and corresponding teams of experts engaged in this field. Notably, Chulalongkorn University in Thailand emerges as a strong contender, securing the third position with 57 articles and 309 citations, surpassing several Western counterparts.

Figure 4 intricately illustrates the collaboration network among esteemed universities and institutions involved in intestinal fungi research, delineating 25 distinct clusters, each represented by a unique color. At the core of this collaborative ecosystem lies the Chinese Academy of Sciences, serving as the linchpin and facilitating connections with six Chinese universities and institutions. Globally, leading institutions in agriculture and medicine conduct extensive research on intestinal fungal communities, showcasing interconnectedness and emphasizing the substantial importance of intestinal fungi in both agricultural and medical spheres.

The dataset in question encompasses a formidable assembly of 8,479 authors, each a luminary within the realm of intestinal fungal research. In Table 4, we present a curated selection spotlighting the top 10 authors based on their H-index. It is noteworthy that Iliev Id holds the top position in both h-index and total citation rates, and

TABLE 2 The top 10 productive countries/regions involved in gut fungi.

Country	Articles	SCP	MCP	Freq	MCP_Ratio	TC	Average Article Citations
CHINA	463	373	90	0.323	0.194	6918	14.80
USA	299	217	82	0.209	0.274	22341	74.50
GERMANY	50	25	25	0.035	0.5	1884	36.90
FRANCE	49	30	19	0.034	0.388	3408	69.60
ITALY	43	29	14	0.03	0.326	2811	65.40
BRAZIL	37	24	13	0.026	0.351	627	16.90
CANADA	37	23	14	0.026	0.378	855	23.10
INDIA	36	33	3	0.025	0.083	707	19.10
POLAND	33	26	7	0.023	0.212	365	10.70
UNITED KINGDOM	28	9	19	0.02	0.679	1085	38.80

MCP represents the number of co-authored papers with authors from other countries, SCP represents the number of co-authored papers with authors of the same nationality, MCP_Ratio reflects the rate of international cooperation, and TC usually refers to the total number of citations of a document. They are calculated based on the structure and patterns in the citation network and are used to quantify how much an article contributes to the citation network.

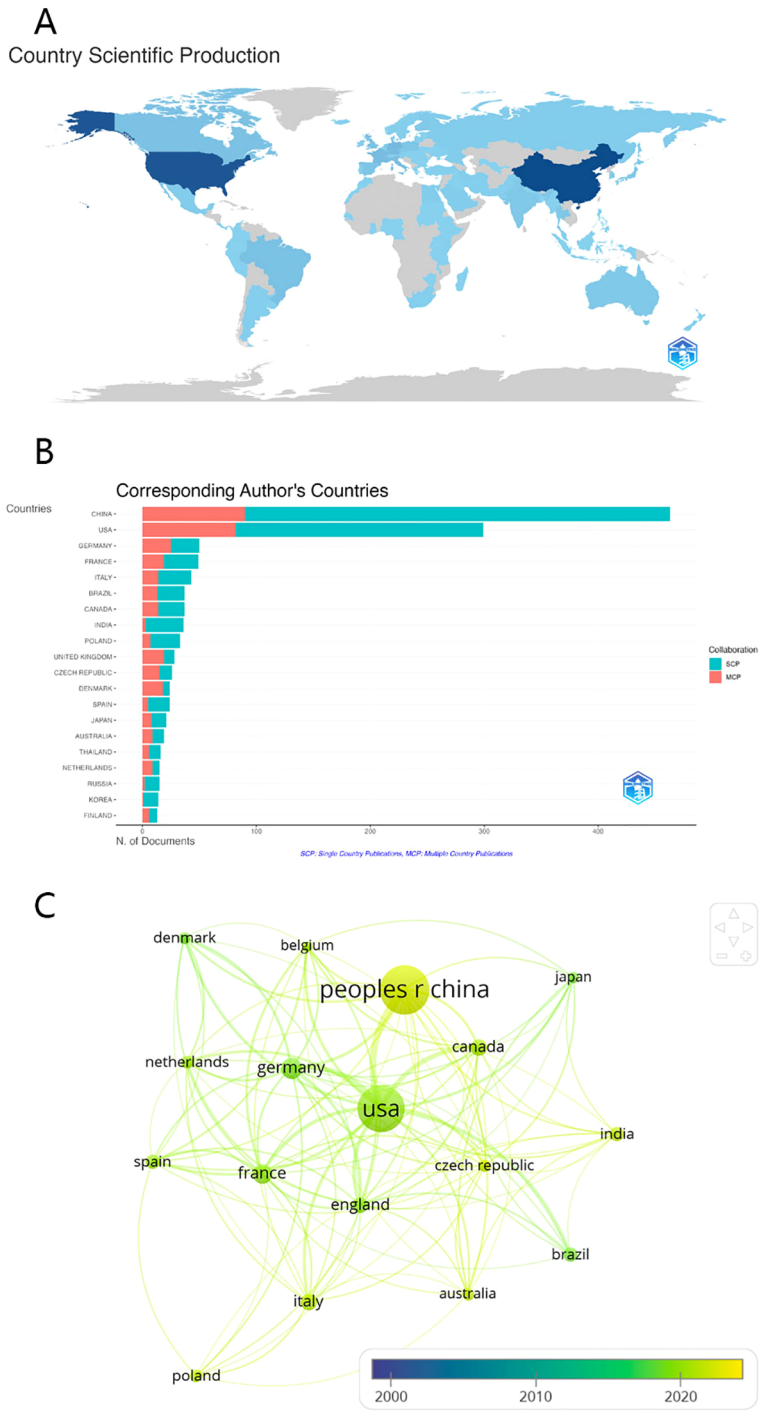

FIGURE 3
(A) Distribution of publications from different countries/regions **(B)** The top 20 countries with the most corresponding authors **(C)** International collaboration network of the top 20(3 excluded because of no links) countries over time.

commenced his investigation into fungal commensalism versus pathogenesis in 2012, thus establishing himself as a pivotal figure in the field. His seminal paper, published in *Nature Reviews Immunology* in 2014, titled "The Mycobiota: Interactions Between Commensal Fungi and the Host Immune System," summarizes the mechanisms of interactions between symbiotic fungi, pathogenic fungi, and the immune system (13). Simultaneously, Zhang F claims preeminence in the tally of Fractionalized articles, G-index, and NP, underscoring the profound impact of his contributions to the intestinal fungal community.

To illustrate the temporal evolution of scholarly productivity, our team devised a bubble chart delineating the trajectory of the top 10 most prolific authors in terms of article publication over time, as depicted in Figure 5A. Wang J and Iliev Id emerge as pioneers,

TABLE 3 The top 10 institutions of articles and citations involved in gut fungi.

Affiliation	Articles	Country
UNIVERSITY OF CALIFORNIA SYSTEM	84	USA
CHINESE ACADEMY OF SCIENCES	71	China
CHULALONGKORN UNIVERSITY	57	Thailand
CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)	54	France
WEILL CORNELL MEDICINE	52	USA
CORNELL UNIVERSITY	50	USA
HARVARD UNIVERSITY	50	USA
INRAE	50	France
INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)	46	France
UNIVERSITY OF WISCONSIN MADISON	43	USA

initiating their scholarly journey during the early stages of research around 2011-2012, when discussions on the subject were limited. However, by 2014, a growing cohort of scholars had shifted their focus to gut microbiome fungi, leading to a significant increase in articles pertaining to this emerging field.

In Figure 5B, we provide a visual depiction of collaborative dynamics among scholars. At its core is Professor Zhang Y from Shanxi University, a distinguished expert acclaimed for his knowledge in Cordyceps sinensis (14). Intriguingly, Western scholars appear as isolated nodes with sparse connections, indicative of a dearth in collaborative research initiatives, while Chinese scholars are interconnected by a dense network of lines, symbolizing their close-knit collaborative bonds. This may be attributed to the fact that the research funding for scholars in China is predominantly sourced from the National Natural Science Foundation of China and governmental subsidies, whereas the financial backing for Western scholars is more varied, including support from government entities, private research organizations, philanthropic contributions, and sponsorships from technology corporations. As for the quality of collaborations among top authors, an excellent illustration is provided by an article on the role of fungi and immunity in inflammatory bowel disease, published in Nature in 2022 by Ellen J. Scherl and Iliyan D. Iliev (15).

3.4 Conditions of references and co-cited references

Table 5 provides a comprehensive overview of the top 10 research articles, ranked by total citations from 2000 to 2023 (16–25). The leading article, authored by David L. and published in Nature, elucidates the gut microbiome's swift adaptability to dietary changes, potentially accommodating various human dietary preferences. The 16S rRNA gene sequencing technology employed in this study, in conjunction with OTU clustering and ITS sequencing, has established itself as a fundamental method for

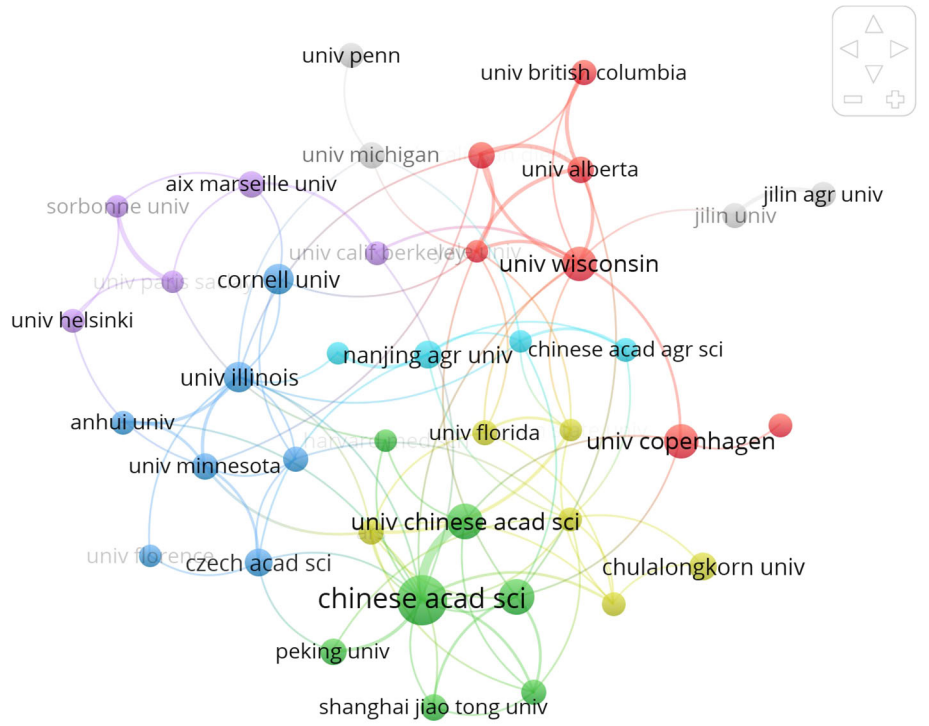

FIGURE 4
International collaboration network of the top 40 institutions in gut fungi. All institutions have been categorized into eight distinct clusters of different colors, with institutions of the same color exhibiting more profound collaboration ties.

TABLE 4 The academic contributions of the top ten scholars in the comprehensive evaluation score.

Element	Articles Fractionalized	h_index	g_index	m_index	TC	NP	PY_start
ILIEV ID	1.48	13	13	1	2092	13	2012
POULSEN M	1.99	11	14	1	588	14	2014
ZHANG F	2.41	11	17	1.1	665	17	2015
LEELAHAVANICHKUL A	1.75	9	12	1.125	334	12	2017
SAPOUNTZIS P	1.83	9	10	0.9	296	10	2015
BOOMSMA JJ	1.54	8	9	0.727	534	9	2014
HUBE B	0.86	8	10	1.143	626	10	2018
UNDERHILL DM	0.75	8	8	0.615	1617	8	2012
WANG Y	1.73	8	13	1.6	182	17	2020
ZHANG H	1.17	8	9	0.889	234	9	2016

Articles Fractionalized (AF) quantifies collaborative research in an author's work. The h-index measures productivity and influence, tallying papers cited at least as often as they were published. The g-index evaluates impact through citation distribution. The m-index combines productivity and publication longevity. PY_start marks the start of an author's scholarly contributions. These metrics, rooted in citation patterns, aid in evaluating scholarly impact and collaboration.

subsequent research on gut fungal communities. This approach has also facilitated the advancement of interdisciplinary studies involving gut fungi (16). Another significant study, authored by Teresa Zelante and featured in Immunity, explores the relationship between diet and fungal and archaeal populations. It reveals the influence of dietary tryptophan on host-fungal symbiosis mediated by the microbiota (17). Subsequent entries in the studies explore various aspects of fungal involvement across different biological contexts, ranging from inflammatory bowel disease to neonatal intestinal fungal microbiota and immunity, shedding light on the intricate interplay between diet, fungi, and archaea, while also evaluating the advantages and disadvantages of culturomics and metagenomic sequencing methodologies.

Co-citation refers to the phenomenon where two or more documents are cited together by a third document, indicating their relatedness or relevance in a specific research context. The concept of "co-citation of references" emerges as a valuable tool for understanding the interconnectedness of scholarly discourse, indicating instances where multiple papers are cited together in subsequent works (26). Figure 6 illustrates six papers with particularly strong co-citation relationships, authored by Caporaso JG 2010, Sokol H 2017, Nash AK 2017, Iliev ID 2012, Callahan BJ 2016, and McMurdie PJ 2013. These papers hold prominent positions within the top ten list, highlighting the symbiotic relationship between co-citation and scholarly influence. From the network relationships, it is also clear that research on gut fungi is divided into two schools of thought: one focusing on the connection between gut fungi and the immune system, and the other dedicated to expanding the diversity of gut fungal communities.

3.5 Keyword visualization and evolutionary trend analysis

In this study, keywords extracted from titles and abstracts served as proxies for authors' primary themes and research. A total of 3782 keywords were collected from the reviewed papers. The top 50 most recurrent keywords were then visualized using word clouds and treemaps, with the word cloud presented in Figure 7A, the treemap in Figure 7B, and the heat map in Figure 7C, facilitated by the R tool's Bibliometrix packages. The analysis revealed prevalent terms such as "gut microbiota," "diversity," "microbiota," "bacteria," "identification," "fungi," "health," and "Candida albicans," among others, highlighting their frequent occurrence as both search terms and research emphases. This observation carries significant implications. For instance, the concurrent appearance of keywords such as "gut microbiota" and "diversity" likely reflects the current research emphasis on enhancing the diversity of gut microbiota. This focus has, in turn, facilitated advancements in the study of gut fungi.

A clustering analysis was conducted using VOSviewer to assess the interconnections among identified keywords, leveraging both the frequency of co-occurrence in publications and the robustness of their associations. Keywords were clustered into distinct categories, each denoted by a unified color scheme. As shown in Figure 7D, keywords were segregated into five clusters. Cluster 1, the "red topic" with 27 items, highlights the diversity within the gut microbiota, emphasizing the interplay between gut fungi and bacteria. Cluster 2, the "green topic" with 8 items, explores the relationship between intestinal fungi and diseases such as inflammation and obesity, alongside biochemical processes like fermentation and metabolism. Cluster 3, marked by an azure hue with 7 items, focuses on fungal microbiota colonization, particularly organisms like Candida albicans. Cluster 4, colored yellow with 5 items, examines immune cell responses influenced by fungi. Cluster 5, the "purple topic" with 3 items, investigates gene degradation within gut fungi. The connections observed between nodes within these clusters highlight a significant level of keyword co-occurrence, illustrating the complex web of interactions that characterizes this research field.

The evolution and trajectory of keywords serve as a gauge, providing insights into the frontiers of knowledge progression. Figure 8A meticulously delineates the fluctuations in citation

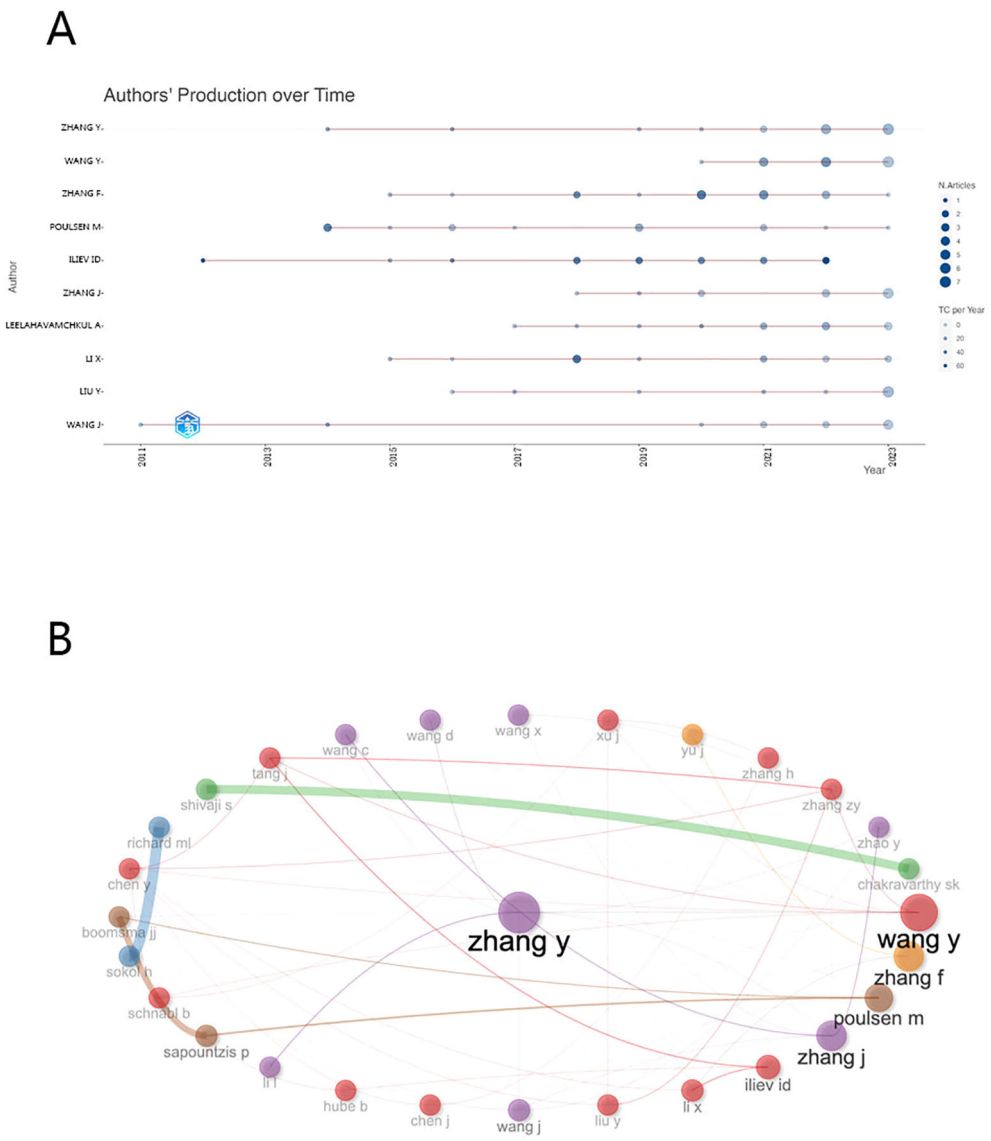

FIGURE 5
(A) The top 10 authors' annual publications over time in gut fungi **(B)** The top 30 authors' Cooperative network in gut fungal microbiome.

frequencies of the top ten frequently used keywords spanning from 2000 to 2023. Notably, the simultaneous surge in occurrences of "gut microbiota" and "diversity" highlights a parallel and rapid ascent, indicative of a close interconnection between them. The presence of keywords such as "health," "bacteria," and "infection" underscores the broader interdisciplinary integration of gut fungal microbiome research with fields such as bacteriology and clinical medicine.

Utilizing Citespace, we imported and analyzed keyword trend data, resulting in the generation of a comprehensive Sankey diagram, as illustrated in Figure 8B. This visual representation intricately traces the evolutionary path of various keywords from 2000 to 2023. Initially, scholarly inquiries focused on unraveling the complexities of the structure and diversity within the intestinal fungal community. Over time, attention shifted towards exploring biochemical substances and reactions, illustrating the dynamic nature of scientific inquiry. Currently, research emphasis has shifted towards investigations centered around the intestinal microbiota, emerging as a focal point in this field. Our anticipation suggests that this growing interest will persist and guide research directions in the foreseeable future.

In Figure 8C, a bubble chart visually presents the temporal occurrences of keyword eruptions. The size of each bubble reflects the level of attention it received during specific time intervals. The emergence of keywords in 2011 can be linked to the advancement of gradient gel electrophoresis technology, enabling more cost-effective and convenient extraction of microorganisms. In 2012, a multinational, multi-laboratory consortium assessed six DNA regions and subsequently recommended the Internal Transcribed Spacer (ITS) region as the standard barcode for fungi. This recommendation established a crucial foundation for future fungal research (27). Moreover, the remarkable increase in scholarly interest in the relationship between gut microbiota and

TABLE 5 The top 10 most cited articles in the field of gut fungi research from 2000-2023.

Paper	DOI	Total Citations	TC per Year	Normalized TC	56Journal IF
DAVID LA, (16), NATURE	10.1038/nature12820	6047	549.73	26.25	64.80
ZELANTE T, (17), IMMUNITY	10.1016/j.immuni.2013.08.003	1422	118.50	8.86	32.40
SOKOL H, (18), GUT	10.1136/gutjnl-2015-310746	752	94.00	9.30	24.50
ILIEV ID, (19), SCIENCE	10.1126/science.1221789	750	57.69	5.28	56.90
FUJIMURA KE, (20), NAT MED	10.1038/nm.4176	676	75.11	10.18	82.90
LAGIER JC, (21), CLIN MICROBIOL INFECT	10.1111/1469-0691.12023	673	51.77	4.74	14.20
HOFFMANN C, (22), PLOS ONE	10.1371/journal.pone.0066019	513	42.75	3.20	3.70
LEWIS JD, (23), CELL HOST MICROBE	10.1016/j.chom.2015.09.008	509	50.90	6.97	30.30
SARTOR RB, (24), GASTROENTEROLOGY	10.1053/j.gastro.2016.10.012	503	62.88	6.22	29.40
NASH AK, (25), MICROBIOME	10.1186/s40168-017-0373-4	485	60.63	6.00	15.50

diseases like cancer and Crohn's disease led to the peak of keyword explosion in 2021.

4 Discussion

4.1 General information

In our study, we examined 1434 articles from the Web of Science Core Collection (WoSCC) database, focusing on the intersection of fungi and gut microbiota. We employed R language, VOSviewer, and CiteSpace for data processing and visualization. The analysis revealed a significant increase in annual publication output and citation counts, particularly from 2014 onwards. China, France, and the United States emerged as prominent contributors in this field. Among the top 10 institutions by publication volume, 5 are from the USA, including the University of California System, Weill Cornell Medicine, Cornell University, Harvard University, and the University of Wisconsin-Madison, highlighting the leading position of the United States. While only one Chinese institution, the Chinese Academy of Sciences, is listed, its second-ranking in publication output underscores China's growing attention and investment in this area. These findings may be associated with the increased funding from sources such as the National Natural Science Foundation of China, the "973" program, the "863" program (28), and initiatives supported by the Chinese Academy of Sciences.

Professor Zhang F, a prominent Chinese scholar from the Department of Food Science and Engineering at Jinan University, focuses on the relationship among food nutrition, intestinal microbiota, and human health (29). During the Covid-19 pandemic, Dr. Zhang F's research explores changes in the gut microbiota of infected patients (30). On the Western side, Professor Iliev ID, based at Cornell University's Mycobiota and Mucosal Immunology Laboratory, stands out for his significant study. His research reveals the complex interaction between gut fungi and the immune system through the Dectin-1 receptor, influencing susceptibility to colitis (19). This study also clarifies the association between Dectin-1 gene variations and severe ulcerative colitis, enhancing our understanding of the implications of the gut microbiome.

From a journal perspective, Frontiers in Microbiology and PLOS ONE are the leading publications, each having published over 2000 papers. Frontiers in Microbiology, renowned for its pioneering research dissemination in microbiology, maintains a distinguished reputation within the Frontiers journal series for its excellence and innovation in scientific publishing. Encompassing diverse microbiological domains such as microbial ecology, virology, bacteriology, mycology, and microbial genetics, it serves as a comprehensive platform for scholarly exchange. Meanwhile, MICROBIOME, with its prolific publication output, boasts the highest impact factor and is widely recognized as the foremost microbiology journal. Both MICROBIOME and Frontiers in Microbiology advocate for open access and scholarly collaboration, yet MICROBIOME's focused approach distinguishes it within the field.

4.2 Historical evolution in the research

Through the analysis of influential papers and historical citation networks, we have gained insights into the trajectory of gut fungal community research. It began with a mouse experiment by Iliyan D. Iliev in June 2012, revealing a diverse fungal community in the mammalian gut interacting with the immune system via the Dectin-1 receptor. This study unveiled a new eukaryotic fungal community in the gut, sparking further research. Over the following five years, research on gut fungi gradually increased as scholars delved into their associations with critical life and health issues. Zelante and colleagues (17) revealed a metabolic pathway of endogenous tryptophan metabolites that resist Candida albicans colonization and shield the mucosa from inflammation. Hoffmann (22) identified associations between fungi and diet, noting positive correlations between Methanobrevibacter and Candida in high-

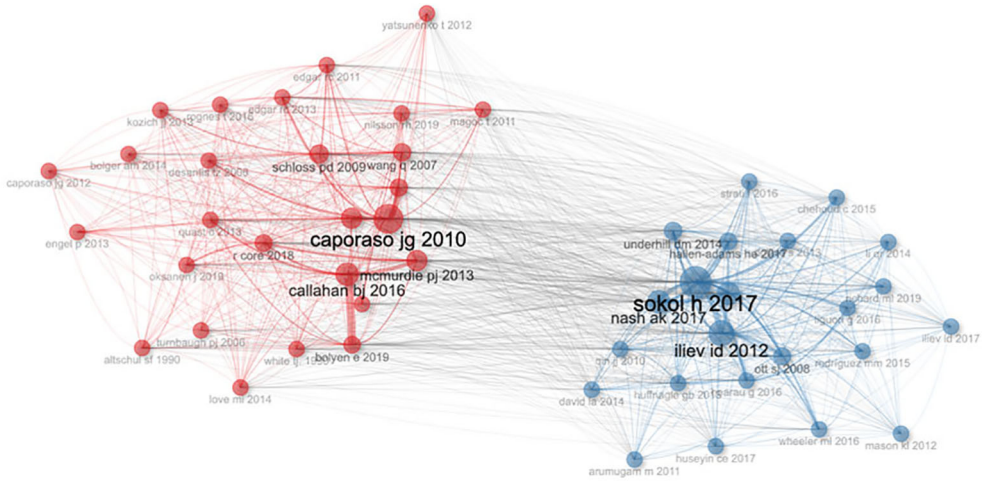

FIGURE 6
Co-citation Network of gut fungal microbiome.

carbohydrate diets, and negative correlations with diets rich in amino acids, proteins, and fatty acids. This supported Lewis's milestone work (23) indicating the gut microbiota's rapid response to dietary changes but without establishing the precise link between gut fungi and diet. The focus of gut fungal community research shifted towards inflammatory bowel disease (IBD) in 2017 due to increased recognition of the microbiome's role in IBD, advancements in sequencing technologies, emerging evidence of fungal involvement, and heightened clinical relevance. Sokol et al. (18) conducted a large cohort bioinformatics analysis, revealing a distinct dysbiosis of fungal microbiota in IBD, characterized by an altered Basidiomycota/Ascomycota ratio, reduced Saccharomyces cerevisiae, and increased Candida albicans. This dysbiosis could be attributed to the specific intestinal milieu of Crohn's disease, favoring fungi while hindering bacteria (8). Towards the year-end, "The Gut Mycobiome of the Human Microbiome Project Healthy Cohort" was published in the esteemed journal MICROBIOME (25), revealing ITS2 sequencing results. These indicated lower diversity in the human gut mycobiome compared to the bacterial community, primarily comprising yeasts like Saccharomyces, Malassezia, and Candida. Amid the COVID-19 pandemic, some researchers connected gut fungal research with the virus. In 2020, a study observed sustained changes in 53% (16 out of 30 patients) of fecal fungal communities during the disease process, with elevated proportions of Candida albicans, Aspergillus, and Penicillium in fecal samples throughout COVID-19 patients' hospitalization (31). Despite the pandemic's conclusion, research on the gut fungal community remains active. For instance, Liang, S.H et al. discovered that Candida albicans has developed hyphal-specific factors, such as candidalysin, which could potentially restrict bacterial metabolic output, thereby enhancing its competitive edge against bacterial species within the intestinal niche (32). Szóstak et al. elucidated that fungal composition and specific fungal species are linked to an elevated risk of melanoma progression and influence the response to Anti–PD-1 therapy, underscoring the pivotal involvement of gut fungi in cancer immunotherapy (33). In essence, as research advances, we anticipate a proliferation of knowledge concerning gut fungi, unraveling further complexities in this evolving field.

4.3 Analysis of research hotspots

Through diverse techniques including keyword WordCloud, TreeMap, trend bubble chart, fluorescence chart, and highly cited literature, we have identified the predominant research focuses on intestinal fungal communities.

4.3.1 Methods for studying the human gut fungi

Historically, scientists have typically relied on culture-dependent methods to survey fungal populations within complex microbial ecosystems (34–36). These methods involve traditional microbiological techniques such as microscopy (37), biochemical assays (38), and growth on selective media (39), often using widely available media like Sabouraud dextrose and potato dextrose. However, these techniques have inherent biases and limitations, favoring rapidly proliferating species and potentially missing rare fungal diversity. Additionally, discrepancies between culture-dependent and culture-independent data further complicate analysis of microbial communities.

Advancements in molecular methodologies (40–44), including PCR, Sanger sequencing (45), and next-generation sequencing (NGS) technologies (46, 47), have liberated scientists from traditional culturing techniques in ecological surveys. These technologies offer a more cost-effective means to investigate and identify microbial communities, eliminating the dependence on culturomics (48, 49). Nevertheless, it's crucial to acknowledge the absence of a standardized approach for culture-independent gut fungi analysis. Researchers are tackling this issue through the development of standardized protocols, the establishment of reference databases, advancements in sequencing technologies, and collaborative efforts to formulate unified guidelines.

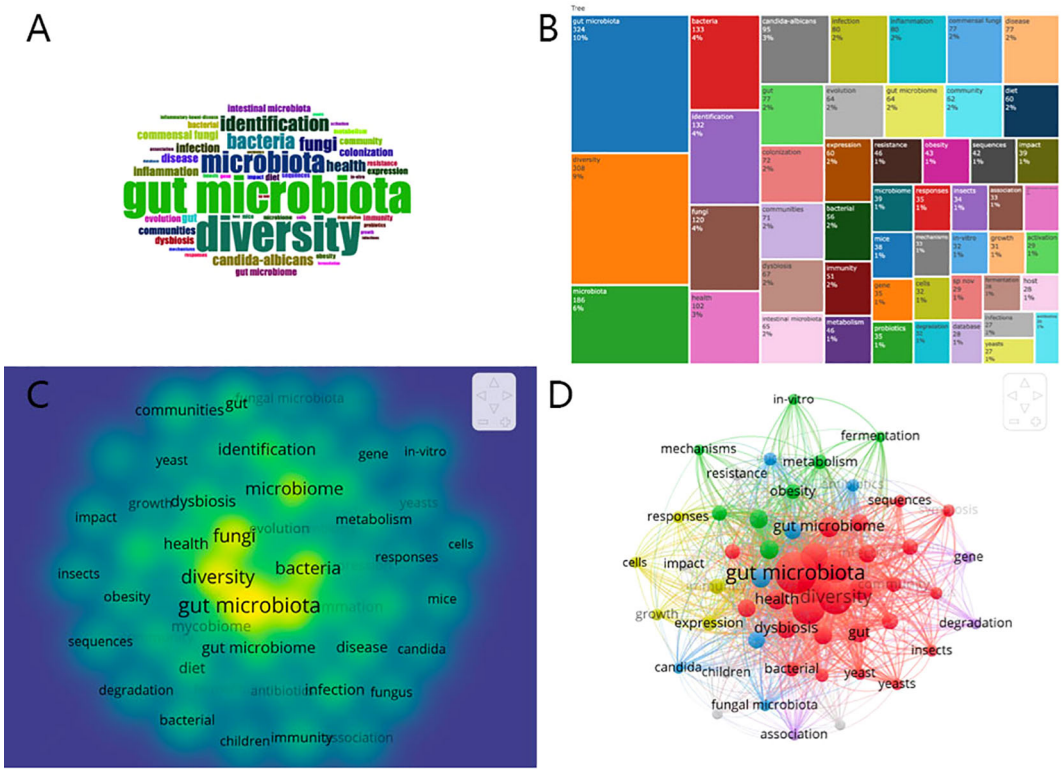

FIGURE 7
(A) Distribution of top 50 Keywords in the form of WordCloud. **(B)** Treemap chart showing the proportion of the top 50 keywords. **(C)** Fluorescence heat map of top 50 Keywords. **(D)** Cluster analysis of high-frequency keywords (frequency ≥15) based on all keywords of publications in gut fungal microbiota (different colors represent different clusters, the size of the circle represents the frequency the keywords appear, and the thickness of the line represents the total link strength between keywords).

4.3.2 Fungal interactions

4.3.2.1 Gut fungus and Immunity

Recent research highlights the pivotal role of Dectin-1, a crucial pattern recognition receptor (PRR), in fungal immunity. Its structure, featuring a single extracellular C-type lectin domain within a type II transmembrane architecture, enables recognition of the β-1,3 glucan motif present in fungal cell walls, eliciting a robust immune response against these pathogens. Moreover, insights from Moyes and Naglik (50) suggest Dectin-1's involvement in colitis development via commensal fungi interaction. Recent discoveries elucidate Dectin-1's capacity to bind β-glucans (BG), pivotal fungal cell wall components, promoting fungal cell phagocytosis and reactive oxygen species generation within macrophages. Additionally, Dectin-1 synergizes with Toll-like receptors (TLRs), notably TLR2, while independently orchestrating immune responses, emphasizing its multifaceted and indispensable role in host-fungal interactions (51). In summary, further research into the functions and interactions of these pattern recognition receptors (PRRs) is anticipated to provide valuable insights that could inform the development of immunotherapies. These therapies would aim to enhance antifungal host defenses and address the rising mortality rates associated with invasive fungal infections.

4.3.2.2 Gut fungus and gut-brain axis

While much attention has been devoted to studying the gut-brain axis with a focus on intestinal bacteria, the potential impact of the gut fungal community may have been overlooked. Recent clinical and experimental research indicates that fungi could indeed play a crucial role in brain-gut communication, utilizing neuro-immuno-endocrine mediators, akin to the microbiome-gut-brain axis (11). Tao Ye's study exemplifies mycobiome-gut-brain communication, showcasing how S. boulardii modulates the TLRs pathway and inhibits neuroinflammation via the gut-brain axis (52). Research has found that probiotic SB treatment significantly alleviates cognitive deficits, Aβ aggregation, synaptic dysfunction, neuroinflammation, intestinal barrier impairment, and fungal microbiome abnormalities in APP/PS1 mice. The potential mechanism by which probiotic SB modulates AD-related neuroinflammation may be associated with the TLRs pathway. This study lays a foundation for further investigation into the involvement and manipulation of intestinal fungi within the gut-brain axis.

4.3.2.3 Fungal-bacteria Interactions

Research exploring the interaction between fungi and bacteria often involves inducing gut dysbiosis, followed by assessing outcomes via antifungal or antibacterial treatments (53). The sensitivity of Candida species to anaerobic-specific or broad-spectrum antibiotics has garnered attention in these studies (54). Jiang et al. demonstrated that commensal fungi like Candida albicans or Staphylococcus aureus can effectively substitute for intestinal bacteria during antibiotic-induced bacterial dysbiosis (55). Conversely, Mason et al. found that Candida albicans colonization during antibiotic recovery might

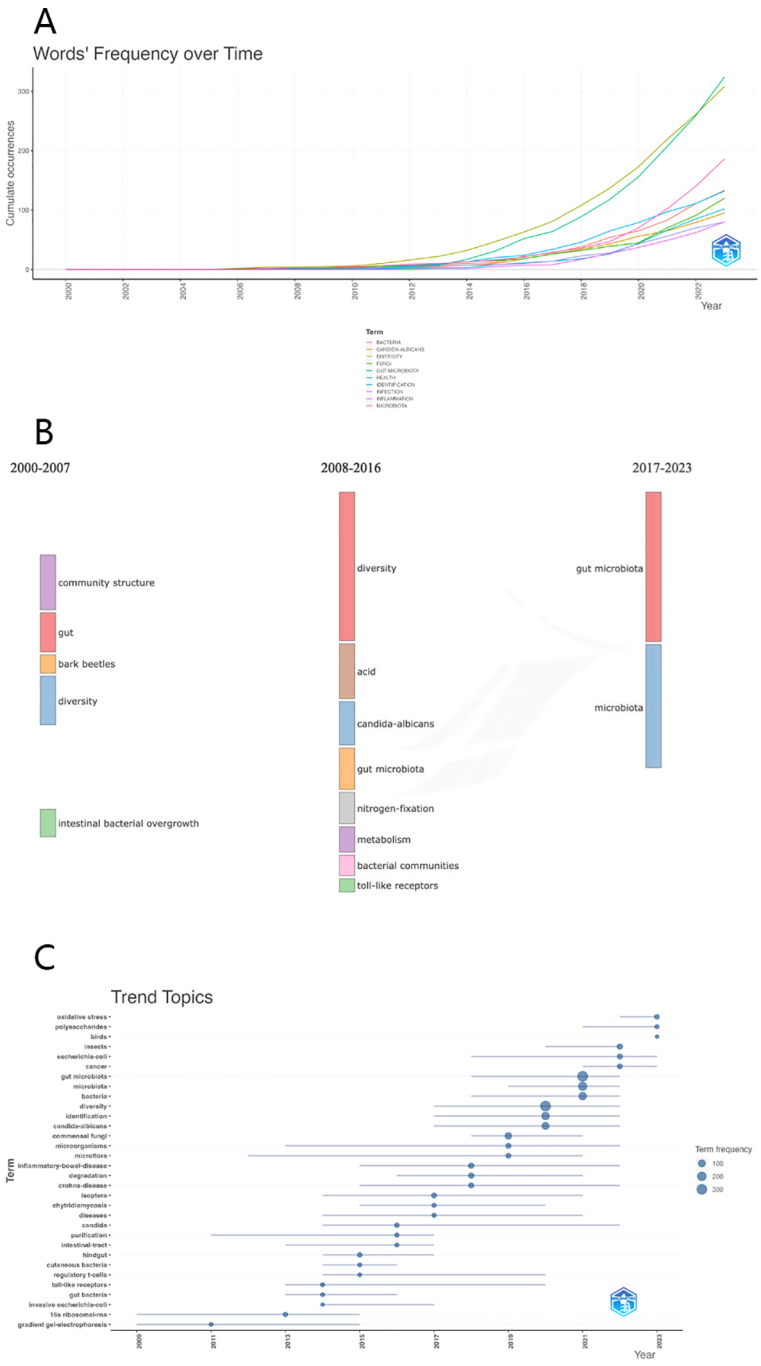

FIGURE 8
(A) The heat map illustrating the fluctuation in popularity of the top ten keywords from the year 2000 to 2023. **(B)** The Sankey diagram showing the key terms across three developmental phases in the field of intestinal fungi: **(C)** Bubble chart indicating the change of keywords over time and the keyword explosion.

increase lactobacilli prevention and enterococcal colonization (56). The presence of specific bacteria reducing fungal colonization opportunities is notable. Tampakakis et al. showed that Salmonella enterica serovar Typhimurium reduces both viability and colonization of Candida albicans in planktonic *in vitro* cultures (57). Additionally, certain fatty acid metabolites from bacterial communities appear to modulate Candida albicans germination. Recent studies, like García et al., suggest that gut microbial metabolites inhibit the invasion of human intestinal cells and the filamentous growth of Candida albicans via the rapamycin (TOR) signaling pathway (58).

In addition, current research underscores complex interactions in biofilm habitats, known as "mixed-species biofilms," between fungal and bacterial cells. Fungi, like Candida species, can boost invasiveness via hyphae induction and extracellular enzyme production, including aspartic proteases. Concurrently, bacteria may gain antibiotic resistance advantages in this shared habitat. Consequently,

TABLE 6 Substantial implication of fungi in human disease progression.

Disease	Disease subtypes	Significant findings that involved fungi
Inflammatory bowel disease	Crohn's disease (CD)	(i) Fungal dysbiosis is closely related to CD in most of the conducted studies
		(ii) Interkingdom interaction between fungal and bacteria was observed
Inflammatory bowel syndrome (IBS)	—	(i) Fungal dybiosis, predominant by *Saccharomyces cerevisiae* and *Candida albicans* in IBS patients
Cancers	Colorectal cancer	(i) Fungal dysbiosis is observed in most of the reported studies
Infectious diseases	Hepatitis B	(i) High levels of *Aspergillus*, *Candida*, *Galactomyces*, *Saccharomyces*, and *Chaetomium* were identified
		(ii) Richness and diversity of fungal species is associated with chronic HBV infection
	HIV	(i) *C. parvum*, *C. difficile*, and *C. albicans* are significantly present in HIV-seropositive patients
		(ii) *C. albicans*, *C. krusei*, and *C. tropicalis* were associated with diarrhea in HIV patients
		(iii) Fungal dysbiosis and high prevalence of *Candid*a species were observed in HIV patients
		(iv) Prevalence of *Candida* in HIV patients without antiretroviral treatment was higher than HIV patients with active antiretroviral treatment
Noncommunicable diseases	Obesity	(i) *Candida*, *Nakaseomyces*, and *Penicillium* genera were commonly identified in obese subjects
		(ii) *Mucor racemosus* and *M. fuscus* were identified in nonobese patients.
		(iii) Specific fungal composition could be potentially used to distinguish between obese and nonobese patients
	Diabetes	(i) *C. albicans* is more prevalence in type 1 diabetes
		(ii) *C. albicans* is more prevalence in type 1 and type 2 diabetes
		(iii) No difference is found between *C. albicans* colonization in type 1 and type 2 diabetes.
		(iv) Isolated fungal species from type 1 diabetes patient is more resistant towards antifungal treatment
Atherosclerosis	—	(i) Phylum *Zygomycota*, which consists of family *Mucoraceae* and genus *Mucor*, was negatively correlated with the risk of cardiovascular disease development through carotid intima-media thickness (cIMT) method
Alcoholic liver disease	—	(i) Decreased in fungal diversity along with *Candida* overgrowth in alcohol-dependent patients
Central nervous system diseases	Rett syndrome	(i) High abundance of *Candida* genus were detected
	Autism Spectrum disorder	(i) *Candida*, *Malassezia*, *Aspergillus*, and *Penicillium* genera were identified
	Schizophrenia	(i) Increased levels of *S. cerevisiae* and *C. albicans* species
		(ii) Close association of gastrointestinal tract disturbance with elevation of *C. albicans* species and lower cognitive score

characterized fungal-bacterial interactions can be fundamentally synergistic, antagonistic, commensal, or mutualistic (59–61).

4.3.3 Gut mycobiome and disease susceptibility

The human mycobiome plays a pivotal role in gastrointestinal-related ailments such as IBD (62–64), fungal infections (15), colorectal adenomas (65), pouchitis post ileal pouch-anal anastomosis (66), and diarrhea (67), significantly impacting host health status (19, 68). For instance, fungal colon infections arise when impaired immune responses, including deficiencies in IL-22, IL-23, and Th1/Th17 cell functions, fail to effectively control fungal growth and dissemination (69). Table 6 outlines the substantial contribution of fungi to human diseases.

4.3.4 Human microbiome project healthy cohort

In a study led by Nash et al., the gut fungal community was assessed via analysis of the ITS2 region and the 18S rRNA gene within

the Human Microbiome Project (25). Results indicated lower diversity in fecal fungi compared to bacteria, with yeasts being prevalent, especially among the top 15 genera. Notably, Malassezia and Candida were prominent within the fungal community. Various OTUs, including Saccharomyces cerevisiae and Candida albicans, were widely present. Unlike bacteria, fungal diversity showed significant variability, albeit with some species consistently observed across samples from the same subjects.

4.4 Limitations

This study has several limitations. Firstly, we adhered to bibliometric analysis guidelines by utilizing only the Web of Science (WoS) database, a major biomedical database (70). However, this approach may have resulted in the exclusion of relevant publications not indexed in WoS. Secondly, our analysis is limited to English-language publications, which may overlook significant non-English research. Thirdly, the limitations of search keywords could introduce bias, potentially leading us to miss emerging areas of focus; in the future, we might incorporate artificial intelligence to address keyword drift issues. Additionally, the ambiguous nomenclature of gut mycobiome communities means that some articles might instead address gut bacterial communities. Lastly, the selected search terms may not capture all relevant documents.

5 Conclusions

Utilizing the R language's Bibiometrix package, VOSviewer, and CiteSpace software, we conducted a bibliometric analysis of gut microbiome fungal research. Visual results depict a steady increase in research volume since 2000, with a notable surge post-2014, indicating a growing interest in gut fungi. China leads in publication volume, while the United States produces high-quality articles. Despite being a research hotspot, the molecular mechanisms linking gut fungal communities with bacterial communities, the immune system, the gut-brain axis, and diseases remain unclear and require further elucidation. Remarkably, the recent advent of third-generation sequencing technologies and artificial intelligence is poised to substantially advance research on gut fungi. We anticipate that future research will shed light on the overlooked significance of the gut fungal community within the gut microbiome.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

This study did not involve human participants, human data, or animal subjects. No ethical approval was required as the research was conducted solely using secondary data and computational analyses. All data used in this study were publicly available and did not require ethical review or consent.

Author contributions

WZ: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization. JC: Investigation, Methodology, Writing – review & editing. YZ: Funding acquisition, Writing – review & editing. DW: Funding acquisition, Writing – review & editing. XX: Funding acquisition, Writing – review & editing. XL: Project administration, Writing – review & editing. KX: Resources, Writing – review & editing. WS: Software, Writing – review & editing. HH: Supervision, Writing – review & editing. WW: Validation, Writing – review & editing. ZL: Visualization, Writing – review & editing. ZZ: Conceptualization, Writing – review & editing. YL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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PANoptosis in intestinal epithelium: its significance in inflammatory bowel disease and a potential novel therapeutic target for natural products

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The intestinal epithelium, beyond its role in absorption and digestion, serves as a critical protective mechanical barrier that delineates the luminal contents and the gut microbiota from the lamina propria within resident mucosal immune cells to maintain intestinal homeostasis. The barrier is manifested as a contiguous monolayer of specialized intestinal epithelial cells (IEC), interconnected through tight junctions (TJs). The integrity of this epithelial barrier is of paramount. Consequently, excessive IEC death advances intestinal permeability and as a consequence thereof the translocation of bacteria into the lamina propria, subsequently triggering an inflammatory response, which underpins the clinical disease trajectory of inflammatory bowel disease (IBD). A burgeoning body of evidence illustrates a landscape where IEC undergoes several the model of programmed cell death (PCD) in the pathophysiology and pathogenesis of IBD. Apoptosis, necroptosis, and pyroptosis represent the principal modalities of PCD with intricate specific pathways and molecules. Ample evidence has revealed substantial mechanistic convergence and intricate crosstalk among these three aforementioned forms of cell death, expanding the conceptualization of PANoptosis orchestrated by the PNAoptosome complex. This review provides a concise overview of the molecular mechanisms of apoptosis, necroptosis, and pyroptosis. Furthermore, based on the crosstalk between three cell deaths in IEC, this review details the current knowledge regarding PANoptosis in IEC and its regulation by natural products. Our objective is to broaden the comprehension of innovative molecular mechanisms underlying the pathogenesis of IBD and to furnish a foundation for developing more natural drugs in the treatment of IBD, benefiting both clinical practitioners and research workers.

KEYWORDS

inflammatory bowel disease, intestinal epithelium, cell death, PANoptosis, natural products

1 Introduction

Inflammatory bowel disease (IBD) is a chronic idiopathic inflammation disease of the gastrointestinal tract, encompassing ulcerative colitis (UC) and Crohn's disease (CD) (1). Characterized by inflammation and recurrent ulceration, UC dominates the colonic mucosa, whereas CD manifests any parts of the entire gastrointestinal tract (2). A global epidemiological survey has demonstrated the rise prevalence not only in Western nations but also in emerging countries (3). Despite the ongoing ambiguity surrounding its etiology and pathogenesis, recent studies have elucidated that the intricate interplay between genetic, environmental and immune factors are indispensable trigger for progression to intestinal epithelial barrier damage (4). Consequently, the concept of "mucosal healing" that necessitate the complete regeneration of the intestinal mucosa has been raised as the therapeutic benchmark of IBD (5, 6). Mounting evidence underscores that mismanaged intestinal epithelial cells (IEC) death compromised barrier breach, which underlies instances of widespread epithelial erosion (7, 8). So undoubtedly, a more profound grasp of the IEC cell death paradigm is imperative.

Apoptosis, necroptosis, and pyroptosis are extensively studied forms of cellular demise, each featuring unique morphological and biochemical changes. These processes are meticulously choreographed by tightly-structured signaling cascades of reactions and molecules in response to a certain signal or stimuli, aiming at eliminating unwanted or damaged cells to maintain tissue homeostasis (9). Apoptosis is a non-lytic cell death with an integral cellular membrane and is considered immunologically silent. In contrast, necroptosis and pyroptosis are lytic and inflammatory form of unregulated and accidental cell death (9). Historically, it has been viewed that apoptosis, necroptosis, and pyroptosis act in parallel without overlap, but the three PCDs have recently shown to be tightly interconnected and interact with each other, laying a theoretical foundation for a novel form of PCD known as PANoptosis (10, 11). Comprehensive research has demonstrated abnormal apoptosis, necroptosis, and pyroptosis of IEC during the onset and progression of IBD as well as the complex crosstalk among them (7). Therefore, PANoptosis may represent an innovative therapeutic target for the effective treatment of IBD.

In this review, we present a concise summary of apoptosis, necroptosis, and pyroptosis, subsequently introducing a more elaborate understanding about the intricate interplay among them within IEC to investigate their potential relationship with IBD. Building on this foundation, we further detail the recent advance of PANoptosis in IEC and its regulation by natural products. We aims to offer theoretical basis and reference for targeting PANoptosis in IEC, thereby fostering the development of more effective therapeutic regimens and pharmacological interventions to improve the efficacy of IBD therapy in clinical practice.

2 The overview of apoptosis, necroptosis, and pyroptosis

2.1 Apoptosis

Apoptosis, the first discovered form of programmed cell death, is a physiological and proactive "conscious suicide" behavior (12). Under specific physiological or pathological conditions, apoptosis is initiated through either receptor-mediated (extrinsic) or mitochondria (intrinsic) pathways, marked initially by the cellular shrinkage and rounding, nuclear fragmentation and chromatin condensation (12). Subsequently, the apoptotic cell features the plasma membrane blistering, tightly encapsulating the cellular debris, culminating in forming the apoptotic bodies. Apoptotic bodies are engulfed by adjacent parenchymal cells and macrophages, and thus this process does not elicit an inflammation response in surrounding tissues (9, 12).

The extrinsic apoptotic pathway is initiated by the binding of extracellular death ligands (TNF family members or Fas ligand) to their corresponding death receptors (DR) on the plasma membrane. Following, the cytoplasmic death domain of death receptors recruits adapter proteins (FADD or TRADD), and then the precursor of caspase-8 is recruited to form the death-inducing signaling complex (DISC), activating the initiator caspase-8 and further activating effector caspases-3/7, ultimately leading to apoptosis (9). The intrinsic (mitochondria) apoptotic pathway is induced by internal apoptotic stimuli, such as oxidative stress, hypoxia, toxic substances, or cytokine deprivation. These stimuli cause the B-cell lymphoma-2 (Bcl-2) protein family to alter the permeability of the mitochondrial membrane, releasing cytochrome c within the mitochondria into the cytoplasm. Subsequently, cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1), facilitating apoptosome assembly, which ignites pro-caspase-9 (13, 14). Then, the activated initiator caspase-9 further activates the effector proteins caspase-3/7, amplifying downstream signals and culminating in apoptosis (14).

2.2 Necroptosis

Necroptosis is a lytic and inflammatory form of PCD independent of caspases, typically occurring when pathogens or chemical mediators inhibit apoptosis (15). Morphologically, necroptotic cells feature with necrotic cells, including swollen mitochondria, the explosive rupture of plasma membrane and cell lysis with the leakage of cytosolic constituent into the surrounding tissues (9, 15).

Caspase-8 determines whether the cell undergoes apoptosis or necroptosis. When caspase-8 is inactivated or inhibited by pathogens or chemical mediators, the activated RIPK3-mediated necrosome is formed (16). Primarily, the external stimulus (such as TNF-α, Fas ligand and TLR ligands) binds to death receptors (such as TNFR1 and Fas) and pattern recognition receptors (PRRs, such as Toll-like receptor), which then recruits and activates receptor-interacting kinase 1 (RIPK1). Following, the activated RIPK1 recruits and phosphorylates receptor-interacting kinase 3 (RIPK3) to form the RIPK1-RIPK3 complex (necrosome), then recruiting and phosphorylating mixed lineage kinase domain-like (MLKL) (9). The phosphorylated MLKL translocates to cellular membranes and lyses the cell by forming membrane pores (17). The consequent membrane rupture results in the release of DAMPs, inevitably triggering an inflammation response.

2.3 Pyroptosis

Pyroptosis is a lytic and inflammatory form of PCD dependent of a series of caspase families to induce the assembly and activation of inflammasome in response to bacterial or pathogen infections (18). Pyroptotic cells exhibit the distinct and characterized morphology with cell swelling, DNA fragmentation within the intact nucleus and plasma membrane rupture, ultimately leading to cell lysis with the release of inflammatory factors (9).

Pyroptosis is induced by two primary mechanisms: the canonical (caspase-1 dependent inflammasome activation) and the non-canonical (caspase-1 independent inflammasome activation) pathways. In the canonical pathway, pattern recognition receptors (PRR), such as TLRs and Nod-like receptors (NLRs), sense pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) to initiate inflammasome sensors (19). The sensors generally comprise Nod-like receptor protein 3 (NLRP3), NLR family pyrin domain-containing 1 (NLRP1), NLR family CARD domain containing 4 (NLRC4), absent in melanoma 2 (AIM2), and pyrin proteins, with NLRP3 being the most extensively studied. The activated inflammasome sensor then enlists the adapter protein apoptosis-related speck-like protein (ASC) and pro-caspase-1 to form inflammasome (9). Then, pro-caspase-1 is hydrolyzed and converted into the catalytically active form caspase-1, which further cleaves gasdermin D (GSDMD), pro-IL-1β and pro-IL-18. The processed GSDMD releases the N-terminal fragment of GSDMD (GSDMD-N), which inserts into the cell membrane to form pores to leaking the mature IL-1β and IL-18 as well as other DAMPs, thereby amplify the inflammatory response (20).

The non-canonical pathway is launched by lipopolysaccharide (LPS) from gram-negative bacterial. LPS directly interacts with and activates human caspase-4/5 and its murine ortholog caspase-11 to cleave GSDMD, thus inducing inflammation (21, 22). Beyond forming membrane pores, GSDMD-N also facilitates the activation of the non-canonical NLRP3 inflammasome and caspase-1, which cleaves pro-IL-1β and pro-IL-18 in a cell-intrinsic manner (23). With in-depth investigates, the caspase-3/8-dependent pyroptotic pathway and the granzyme-mediated GSDMD- independent pyroptosis pathway have recently been revealed (24). When cell is treated with partial chemical inducers, pyroptosis is induced by caspase-3-mediated cleavage of GSDME, yielding a GSDME-N fragment with the pore-forming activity, while caspase-8 specifically cleaves GSDMC to trigger cell death pathway (24, 25). In addition, granzyme A/B involves in extensive pyroptosis by cleaving GSDMB and GSDME, respectively (26, 27).

3 The crosstalk among apoptosis, necroptosis, and pyroptosis in IEC involving in IBD

The appropriate model of IEC death is crucial for maintaining intestine homeostasis. However, excessive and abnormal IEC death programmers can have catastrophic consequences, such as the onset of inflammatory bowel diseases. The epithelium of patients with UC or CD manifests high level of cell deaths (7, 8). Substantial evidence have well-established the involvement of apoptosis, necroptosis, and pyroptosis of IEC in the onset of IBD. Simultaneously, the three models of cell death described above are interconnected and superimposed at multiple levels, which have been mostly described as 'crosstalk'. Caspase-8 and its adapter FADD are the core molecular bridging apoptosis, necroptosis and pyroptosis in IEC (7). In response to TNF and TLR, caspase-8 is activated, which not only ignites the downstream executioner caspase-3/7 in the apoptotic pathway, but also cleaves RIPK1 and RIPK3 in the necroptotic pathway (28–31). Additionally, caspase-8 and FADD process caspase-1, inflammasome assembly, and GSDMD activation to launch pyroptosis (31–33).

Caspase-8 switches apoptosis and necroptosis (Figure 1A). An early study demonstrated that TLR stimulation induced apoptosis and increased shedding of IEC under inflammatory conditions, which was related to the activated caspase-8 (34). The activated caspase-8 also processed gasdermin-D-mediated pyroptosis-like death of epithelial cells and ensuing ileitis (31). In situation where caspase-1 was deleted in IEC, the inflammasome sensors NLRP1b and NLRC4 triggered apoptosis by ASC-dependent caspase 8 activation (35). Under steady state conditions, caspase-8 curbs RIPK1 and RIPK3 activity by proteolytic cleavage in IEC, thereby impeding necroptosis (31). Thus, IEC lacking caspase-8 or FADD due to epithelial cell-specific deletion underwent RIPK3-dependent necroptosis instead of apoptosis in response to TLR or TNF stimulation, leading to a complete absence of Paneth cell, serious tissue damage, enteritis and severe erosive colitis *in vivo* (36–38). Similarly, the deficiency of intestinal epithelial caspase-8 signaling induced necroptosis-mediated enteritis and high lethality after *Salmonella* Typhimurium infection (39). Regarding RIPK1, mice suffering from the deficiencies in both RIPK1 and FADD in IECs displayed RIPK3-dependent IEC necroptosis, Paneth cell loss and focal erosive inflammatory lesions in the colon (40). In line with the results observed in mice, patients with a biallelic 710A > G mutation in the caspase-8 gene presented the increased necroptosis instead of apoptosis in the gut with non-resolving inflammation (41). The imaging *in vivo* showed that IEC necroptosis is considered the basis for the micro erosions and epithelial gaps observed in mice and humans, which is consistent with the significantly high expression of RIPK3 in the terminal ileum of CD patients (38, 42). Indeed, the specific cell type of IEC necroptosis may be Paneth cell. Paneth cells in humans and mice represented a high level of RIPK3 expression (38). Interestingly, Paneth cells without caspase-8 do develop but undergo necroptosis *in vivo* (38). As is well-known, Paneth cells are predominantly rich in the ileum. This may provide an explanation for why mice deficient in caspase-8 bear ileitis but no colitis (38). So far, however, no study has illustrated RIPK3 as a susceptibility gene for IBD in genetic research. This evidence hints that necroptosis of Paneth cells is not a causative factor, but rather a contributing factor supporting intestinal inflammation. In a word, necroptosis is frequently encountered in Paneth cells when apoptosis fails to be induced due to caspase-8 deficiency in IEC.

Although blocking apoptosis through restricting caspase-8 in IEC and switching their death to necroptosis cause overt intestinal

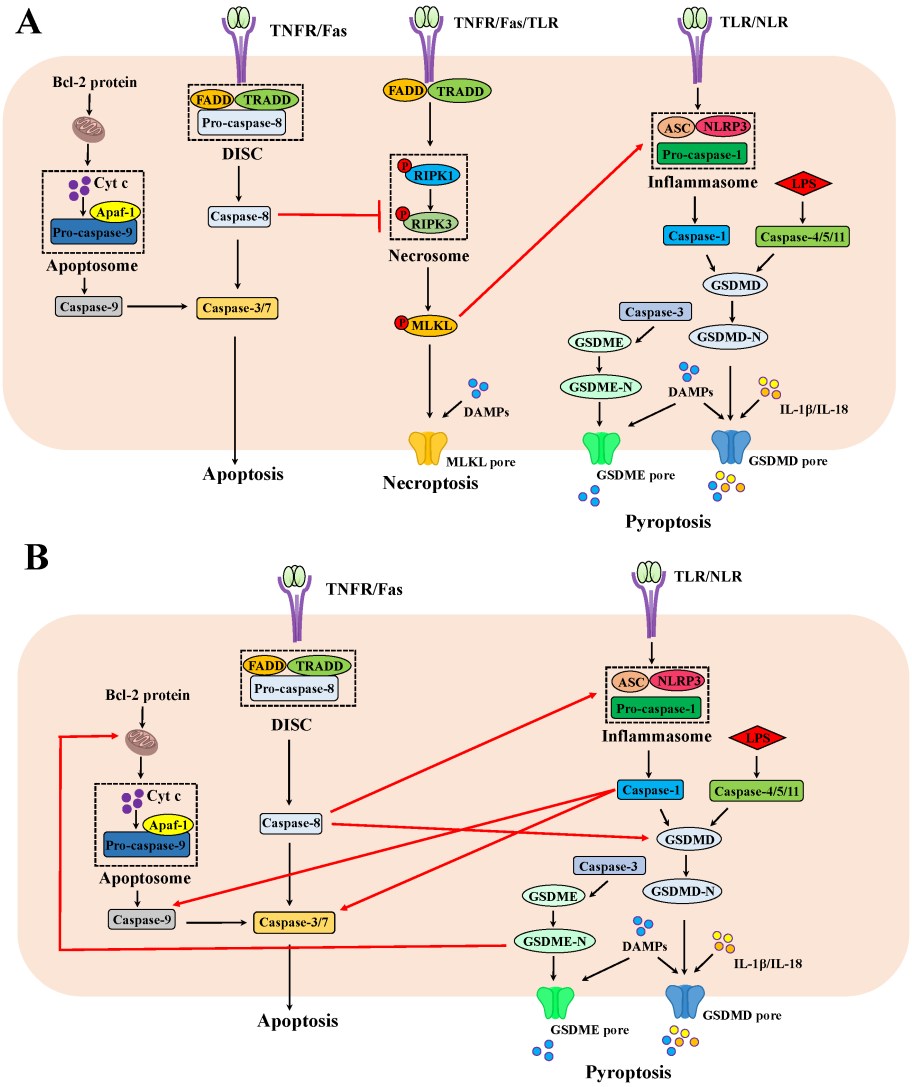

FIGURE 1
The mechanism of apoptosis, necroptosis and pyroptosis as well as the crosstalk among them in IEC. **(A)** The crosstalk between apoptosis and necroptosis as well as necroptosis and pyroptosis in IEC. Beyond cleaving the effector caspases-3/7 in the apoptotic pathway, the apoptotic molecule caspase-8 could cleave RIPK1, thus inhibiting necroptosis. In addition, the phosphorylated MLKL could foster ASC polymerization and following lead to caspase-1 activation to trigger pyroptosis. **(B)** The crosstalk between apoptosis and pyroptosis in IEC. The apoptotic molecule caspase-8 could process GSDMD activation and interact with ASC to launch pyroptosis. The apoptotic effector caspase-3 is indispensable for GSDME-mediated pyroptosis. When GSDMD is absent, the pyroptotic molecule caspase-1 proceeds to activate caspase-3/7 and caspase-9. The pyroptotic molecule GSDME-N fragment permeabilizes the mitochondrial membrane to induce cytochrome c release and ensuing activate the apoptosome, fueling the mitochondrial apoptotic pathway.

inflammation, it may be a self-protection mechanism evolved by the body when the caspase-8-dependent apoptotic pathway has been hijacked by microbes or viral infection. CrmA from the cowpox virus or B13R from the vaccinia virus encodes caspase-8 inhibitor, thus preventing infected cells from apoptosis; however, RIPK3-dependent necroptosis serves as a backup mechanism for killing infected cells and ensures host survival (43, 44). Nevertheless, some pathogens prevent epithelial cell death by delivering effectors via the type III secretion system to inhibit both apoptosis and necroptosis, which can maintain their replicative niche and multiply within cells. For example, *Shigella flexneri* hijacked colonic epithelium via a dual mechanism: it yielded OspC1 to impede caspase-8, thus blocking apoptosis; simultaneously, it also yielded OspD3 to degrade RIPK1 and RIPK3, thereby preventing necroptosis (45).

Collectively, the afore-mentioned studies hint that co-inhibition of caspase-8 and RIPK3 seemingly protects IEC from cell death. Bewilderingly, mice with deletion of RIPK3 with the FLIP proteins-induced caspase-8 inhibition in IEC protected from neither cell death nor inflammation (46). However, the co-ablation of caspase-8 and MLKL downstream of RIPK3 or FADD and RIPK3 in IEC fully protected against cell death and prevented ileitis in mice (31). These evidence imply the involvement of additional molecules in inducing cell apoptosis or necroptosis beyond caspase-8 or RIPK3, respectively. Alternatively, the involvement of another form of

cell death in this process might provide another explanation for this observation.

Although no knowledge about additional molecules to induce cell necroptosis independent of RIPK3 has been established, some evidence point out the regulation of pyroptotic molecules on apoptosis beyond caspase-8 (Figure 1B). For instances, the canonical molecule of pyroptosis, caspase-1, could cleave the conventional aspartate activation sites (Asp^{23} and Asp^{198}) of caspase-7 in macrophages in response to *Salmonella* Typhimurium infection combined with LPS and ATP (47). When GSDMD is absent, caspase-1 also proceeds to activate caspase-3 and caspase-9 as well as trigger mitochondrial damage in macrophages, thereby triggering the apoptotic signaling (48). In addition, GSDMD-N and GSDME-N fragments permeabilized the mitochondrial membrane to induce cytochrome c release and ensuing activate the apoptosome in HEK293T cells, fueling the mitochondrial apoptotic pathway (49). Further studies are required to address whether the regulation of pyroptotic molecules on apoptosis also exists in IEC and whether they affect IBD pathogenesis.

As for the involvement of another cell death, mounting evidence now point out significant contributions of pyroptosis in IECs to the onset of IBD, which could be regulated by apoptotic or necroptotic molecules (Figure 1). Elevated levels of GSDMB, GSDMD and GSDME were obviously detected in the inflamed colonic mucosa of IBD patients, predominantly localizing to the intestinal mucosal epithelium (50–52). The apoptotic effector caspase-3 is indispensable for TNBS-induced and GSDME-mediated pyroptosis in IEC (52). Studies with mice carrying enzymatically inactive caspase-8 revealed that the CASP8-RIPK1 platform shared by apoptosis and necroptosis was genetically associated with ASC, the adaptor protein of inflammasomes. The DED domain of caspase-8 in cells from these mice interacted with ASC, triggering pyroptosis and severe inflammation in the intestine (53, 54). Additionally, knockdown of caspase-8 or inhibition of its function promoted RIPK3-mediated inflammasome NLRP3 activation in macrophage, independent of MLKL (55). Similarly, MLKL could foster ASC polymerization and following lead to caspase-1 activation in response to combined treatment of a TLR3 agonist poly(I:C) and zVAD in macrophage (56). The effector molecule of necroptosis MLKL activated NLRP3 inflammasome (57, 58). Therefore, the IEC death in mice with co-ablating RIPK3 and caspase-8 could be rescued when the pyroptotic mediator was deleted (53, 54).

In summary, the crosstalk of events underlying apoptosis, necroptosis and pyroptosis in IEC can be succinctly outlined as follows: caspase-8 switches apoptosis and necroptosis in IEC (Figure 1). TLR or TNF immoderate stimulation induced the activation of caspase-8 in IEC, thus initiating an apoptotic fate. Concurrently, caspase-8 fosters gasdermin-D-mediated pyroptosis-like death of epithelial cells. When caspase-8 is deleted or dysfunctional due to pathogen-mediated or pharmacological inhibition, caspase-8 mediated-apoptosis and pyroptosis could not be triggered, resulting in RIPK3-mediated necroptosis of IEC or additional molecules igniting apoptotic/pyroptosis signals in IEC. For example, caspase-1, GSDMD and GSDME induced apoptosis, while caspase-3 or MLKL orchestrate pyroptosis. These excessive cell death modalities, based on the crosstalk of molecules, collectively cause catastrophic intestinal inflammation.

4 The role of PANoptosis in IEC and its regulation by nature products

4.1 The role of PANoptosis in IEC

Based on the extensive cross-talk between PCD pathways, the conceptualization of a united cell death modality, named PANoptosis, was proposed in 2019 (59). PANoptosis ("P", pyroptosis; "A", apoptosis; "N", necroptosis) is induced by multifaceted PANoptosome complexes with key features of pyroptosis, apoptosis, and/or necroptosis, which could not be fully accounted for by any other PCD pathway alone. The PANoptosome components are tripartite, consisting of: 1) PAMPs or DAMPs sensors like ZBP1, AIM2, and NLRP3; 2) scaffolding proteins such as ASC and FADD; 3) catalytic effectors including RIPK1, RIPK3, CASP-1 and CASP-8 (59). To date, several distinct PANoptosome complexes have been identified, featuring unique sensors and regulators, such as the ZBP1-, AIM2-, RIPK1-, and NLRP12-PANoptosomes (10).

Given the crosstalk of apoptosis, pyroptosis and necroptosis in IEC, the researches has demonstrated the important regulator of PANoptosis in the pathogen-induced intestinal inflammation (Figure 2). For example, *S. Typhimurium* effector SopF regulated PANoptosis in IEC to attenuate intestinal inflammation. Specifically, SopF inactivated caspase-8 through the PDK1-RSK signaling, thereby inhibiting apoptosis and pyroptosis of IEC with the promotion of necroptosis. Thus, SopF restricted the dislodging of IECs to promote bacterial dissemination, which exacerbates systemic infection (60). In addition, ventilator-induced lung injury (VILI) substantially promoted the expression of caspase-3, N-GSDMD and p-RIPK3 in the gut due to systemic cytokines, suggesting that PANoptosis involved in VILI-induced gut injury and inflammation in the mice (61).

Recently, PANoptosis has been implicated in the onset and progress of IBD. Based on multiple transcriptome profiles of intestinal mucosal biopsies from the GEO database, bioinformatics analysis identified that four pivotal PANoptosis-related gene (ZBP1, AIM2, CASP1/8) were significantly activated in UC patients, which regulated specific immune cells and interacted with key signaling pathways contributing to the pathogenesis of UC. These findings were validated in the DSS-induced mouse colitis model (62–64). Another comprehensive study combining bioinformatics, machine learning, and experimentation confirmed that PANoptosis played an undeniable role in CD by regulating the immune system and interacting with CD-related genes (65). The abnormal activation of ZBP1 caused embryonic lethality and intestinal cell death. In mouse models, the knockdown of key sensor molecules of PANoptosis has been shown to rescue the death fate of innate immune-induced epithelial cells (66). Researches indicated the crucial role of IFN-γ in inducing the intestinal epithelial barriers (67). The result obtained from human

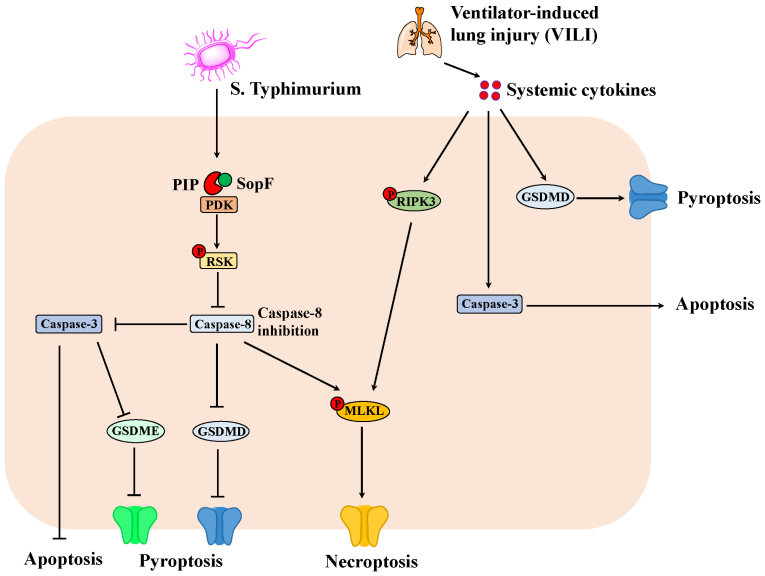

FIGURE 2
The PANoptosis in IEC involving in the intestinal inflammation. *S. Typhimurium* effector SopF inactivates caspase-8 through the PDK1-RSK signaling, thereby inhibiting apoptosis and pyroptosis of IEC with the promotion of necroptosis. Consequently, SopF attenuates intestinal inflammation, however, promotes bacterial dissemination, which exacerbates systemic infection. Moreover, ventilator-induced lung injury (VILI) results in systemic cytokines, which promotes the expression of Caspase-3, N-GSDMD and p-RIPK3 in the gut. Thus, apoptosis, necroptosis and pyroptosis can lead to intestinal injury and inflammation in mice.

intestinal organoid (enteorid) model has shown that PANoptosis is the major mechanism of IFN-γ-induced IEC damage. Furthermore, bulk and single-cell RNA sequencing (RNA-seq) found that PCD-associated gene expression was upregulated in enterocytes and goblet cells, but not in intstinal stem cells and Paneth cells (68). This finding demonstrates IFN-γ-induced intestinal epithelial cell type-specific PANoptosis. Mechanistically, NLRC5 could function as an innate immune sensor in inflammatory conditions and interact with NLRP12 to form a PANoptosome in response to NAD^+ depletion. Thus, deletion of NLRC5 protect mice from colitis (69). However, this study did not illuminate whether NLRC5-mediated PANoptosome formation bears intestinal epithelial cell type specificity.

IFN regulatory factor 1 (IRF1) is a transcription factor of the PANoptosome sensors ZBP1 and NLRP3, and knocking out IRF1 will reduce the expression of PANoptosome core molecules such as NLRP3, CSAP1/3/8, and MLKL in the innate immune response (70, 71). In a UC mouse model, the knockdown of IRF1 also significantly inhibited the expression of colonic apoptosis, pyroptosis, and necroptosis executioner proteins CASP3/7, GSDMD, and MLKL, thus inhibiting PANoptosis (72). Colitis-associated cancer (CAC) is the most serious complication of inflammatory bowel disease, which is driven by long-term inflammatory damage. In the case of CAC, loss of IRF1 suppressed PANoptosis of colon, thereby increasing the susceptibility of $Irf1^{-/-}$ mice to CRC (72). Hence, IRF1 functions as a key upstream mediator of PANoptosis, which could potentially be a therapeutic target for IBD. More in-deep and systematic investigation is warranted to uncover the substantial insights into the essential role of IRF1 in the PANoptosis process of IEC, which is beneficial for a momentously advanced understanding of the etiology and pathogenesis of IBD.

4.2 Potential nature compounds targeting IEC PANoptosis in IBD

It is widely acknowledged that in pathological contexts, three distinct modalities of cell demise can coexist, exhibiting overlapping mechanisms and functioning as complementary death strategies. For instance, the inhibition of caspase-8 can effectively mitigate the process of apoptosis; however, this intervention may precipitate alternative forms of cell death, such as necroptosis or pyroptosis. Notably, PANapoptosis encompasses all three types of cell death, and thus, targeting PANapoptosis presents a strategic approach to concurrently inhibit these three distinct modes of cell demise.

It's well-established that natural products stemmed from multifarious medicinal plants, vegetables and fruits orchestrate different cell death modalities for IBD treatment with lower costs, flexible dosage adjustments, fewer side effects and long-term application (73, 74). However, no reports delineate natural products regulating PANoptosis of IEC for IBD treatment. Nevertheless, numerous natural products are currently being investigated as PCD regulators for IBD therapy. Natural compounds that modulate both apoptosis and necroptosis or apoptosis and pyroptosis in IEC are listed in Table 1, setting a precursor for research into PANoptosis of IEC in IBD.

HuanglianGanjiang Tang (HGT) is a renowned prescription of traditional Chinese medicine (TCM). HGT obstructed necroptosis in IEC by activating vitamin D receptor (VDR) signaling pathway,

TABLE 1 Compounds that induce cell.

Natural compounds	Cell death	*In Vivo*/Vitro	Cell lines/Animals	Mechanisms	References
HuanglianGanjiang Tang (HGT) and the compound berberine	Necroptosis	*In Vitro*	Mice with DSS-induced colitis	Activating vitamin D receptor (VDR) signaling pathway	(75)
berberine	Apoptosis	*In Vivo* and *In Vitro*	Caco-2 Cells/ Mice with DSS-induced colitis	Lowering JNK phosphorylation	(76, 77)
	Pyroptosis	*In Vivo* and *In Vitro*	Caco-2 Cells/ Mice with DSS-induced colitis	Activating the Wnt/β-catenin pathway via modulating the miR-103a-3p/BRD4 axis	(78)
L. edodes polysaccharides	Necroptosis	*In Vivo* and *In Vitro*	Caco-2 Cells/ Mice with DSS-induced colitis	Inhibiting pMLKL-mediated necroptosis	(79)
	Pyroptosis	*In Vitro*	Human umbilical vein endothelial cells	Regulating the LncRNA MALAT1/miR-199b/mTOR axis and the NLRP3/Caspase-1/GSDMD pathway	(80)
Traditional herbal formula Wu-Mei-Wan (WMW) and the compound ginsenoside Rb1	Necroptosis	*In Vivo*	Mice with TNBS-induced colitis	Promoting RIPK3 O-GlcNAcylation and suppressing the binding of RIPK3 and MLKL	(81)
Ginsenoside Rb1	Apoptosis	*In Vivo* and *In Vitro*	IEC-6 Cells/ Mice with DSS- and TNBS-induced colitis	Activating Hrd1 signaling pathway	(82)
	Pyroptosis	*In Vitro*	Non-IEC (astrocytes, hepatocytes and renal cells)	Activating mitophagy	(83–85)
Cucurbitacin E	PANoptosis	*In Vitro*	Non-IEC	Regulating ZBP1	(86)

thereby attenuating DSS-induced colitis. Furthermore, molecular docking analysis has successfully proved the binding affinity of the five compounds to VDR, including berberine, phellodendrine, 6-Gingerol, ferulic acid and citric acid (75). Previous studies uncovered that berberine could evidently lessen cytokine-induced Caco-2 apoptosis *in vitro* by lowering JNK phosphorylation, thus in turn promoting the recovery of colon epithelium in DSS-treated mice (76, 77). Recently, a research found that berberine weakened colitis-induced pyroptosis and intestinal mucosal barrier defects by activating the Wnt/β-catenin pathway via modulating the miR-103a-3p/BRD4 axis (78).

The natural compound polysaccharides from the edible mushrooms *Lentinus edodes* showed therapeutic properties on DSS-induced colitis. The carbohydrate-rich component of *L. edodes* polysaccharides suppressed TNF-induced cell death of Caco-2 cells in a dose-dependent manner by inhibiting pMLKL-mediated necroptotic cell death, thus counteracting DSS-induced colitis in mice (79). This study also revealed that L. edodes polysaccharides prevented apoptotic cell death in Caco-2 cells (79). The effect of *L. edodes* polysaccharides on pyroptosis is reported in non-IEC. In human umbilical vein endothelial cells, *L. edodes* polysaccharides dampened advanced glycation end products (AGEs)-induced pyroptosis via regulating the LncRNA MALAT1/miR-199b/mTOR axis and the NLRP3/Caspase-1/GSDMD pathway (80).

Traditional herbal formula Wu-Mei-Wan (WMW) could augment colonic O-GlcNAc transferase (OGT) activity and inhibit O-GlcNAcase (OGA) activity, which may be regulated by the compounds hesperidin, coptisine and ginsenoside Rb1 found in WMW. As a result, WMW could prevent necroptosis through promoting RIPK3 O-GlcNAcylation and suppressing the binding of RIPK3 and MLKL, ultimately alleviating TNBS-induced colitis in mice (81). A previous study demonstrated that ginsenoside Rb1 abated LPS-induced apoptosis via activating Hrd1 signaling pathway in intestinal cell line IEC-6, thus alleviating colitis symptoms in DSS- and TNBS-treated mice (82). Additionally, ginsenoside Rb1 diminished pyroptosis by activating mitophagy in non-IEC, such as astrocytes, hepatocytes and renal cells (83–85). Importantly, forthcoming research endeavors about the effect of ginsenoside Rb1 on the IEC and colitis are necessary.

Cucurbitacin E (CurE), a natural product extracted from plants in the *Cucurbitaceae* family. In the adrenocortical carcinoma cells, is a CDK1 inhibitor. CDK1 regulated the PANoptosis of adrenocortical carcinoma cells through binding with the PANoptosome in a ZBP1–dependent way (86). Machine learning and integrated bioinformatics identified possible hub genes (AURKB, CDK1, and CCNA2) between bladder cancer and inflammatory bowel disease (87). Further investigation is warranted to confirm whether cucurbitacin E regulates PANoptosis in a ZBP1–dependent way in the context of IBD specifically.

In a word, berberine, *L. edodes* polysaccharides, ginsenoside Rb1 and cucurbitacin E are potential natural compounds that regulate PANoptosis of IEC for the treatment of IBD. These evidence pave the way for future pharmacological research.

5 Conclusion and discussion

Over the past decade, a wealth of evidence has established the basic knowledge on apoptosis, necroptosis and pyroptosis and their

roles in the pathogenesis of IBD. This review followed with interest in the current understanding of the crosstalk between these cell deaths modalities in IEC, particularly in the context of inflammatory bowel disease. However, IEC constitute a heterogeneous population along the gut, embodying absorptive cells, goblet cells, enteroendocrine cells, Paneth cells, M cells, cup cells, and Tuft cells. The significance of location-specific and cell type-specific cell death along the length of the intestine has been highlighted by various transgenic mouse models. Further investigation is needful to unravel the cell death and their crosstalk at single-cell resolution in different IEC types from the intestine, which will potentially illuminate the underlying nature of IBD.

Within the landscape about the crosstalk between apoptosis, necroptosis and pyroptosis in IEC, this review further provided an overview about the IEC PANoptosis in the context of IBD. However, the body of literatures addressing this topic is scant. The study of PANoptosis in IBD remains limited to in preliminary experimental phases. As such, there is an imperative need to probe more intricately into the underlying mechanisms governing PANoptosis and the upstream modulators in IEC through both foundational research and clinical trials. This endeavor will undoubtedly foster the emergence of innovative and more efficacious treatment strategies for IBD.

Given the well-established role of natural products in the prevention and treatment of IBD, coupled with their minimal adverse effects, this review also highlights potential nature compounds targeting IEC PANoptosis for IBD treatment based on the limited literatures. This merits further attention and contemplation. We hope to provide a solid groundwork for researchers in this field to explore the potential drugs for IBD treatment in the foreseeable future.

Author contributions

CZ: Conceptualization, Funding acquisition, Writing – original draft. SL: Investigation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A potential therapeutic approach for ulcerative colitis: targeted regulation of mitochondrial dynamics and mitophagy through phytochemicals

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Mitochondria are important organelles that regulate cellular energy and biosynthesis, as well as maintain the body's response to environmental stress. Their dynamics and autophagy influence occurrence of cellular function, particularly under stressful conditions. They can generate reactive oxygen species (ROS) which is a major contributor to inflammatory diseases such as ulcerative colitis (UC). In this review, we discuss the key effects of mitochondrial dynamics and mitophagy on the pathogenesis of UC, with a particular focus on the cellular energy metabolism, oxidative stress, apoptosis, and immunoinflammatory activities. The therapeutic efficacy of existing drugs and phytochemicals targeting the mitochondrial pathway are discussed to reveal important insights for developing therapeutic strategies for treating UC. In addition, new molecular checkpoints with therapeutic potential are identified. We show that the integration of mitochondrial biology with the clinical aspects of UC may generate ideas for enhancing the clinical management of UC.

KEYWORDS

UC, mitochondrial dynamics, mitophagy, targeted therapy, nature products

1 Introduction

Ulcerative Colitis (UC) is an inflammatory bowel disease (IBD) affecting the rectum and extending to the proximal colon parts (1). In 2023, the global prevalence of UC was estimated to be 5 million cases, with over 400 diagnoses per 100,000 people reported in North America (2–4). The development of UC involves multiple pathways including changes in genetic mutations, environmental influences, impaired gut microbiota, and imbalance in the mucosal immune system (5). The primary clinical symptoms of UC patients are blood in the stool, diarrhea, and abdominal pain, fever, dehydration, weight

loss, and possibly depression, anxiety, sleep disorders, and sexual dysfunction (6–9). If untreated, UC can potentially increase the risk of colon cancer (10). Several treatments have been proposed for UC, which include 5-aminosalicylic acid (5-ASA), corticosteroids, immunosuppressants, biologics, and even surgical procedures. However, the maximum response to these treatments is estimated at 30% to 60% (11). Moreover, for patients with UC, the rate of colon resection are 12%-19% at 12 months despite treatment with sequential therapy (12). In recent year, UC has become an intractable clinical challenge due to the lack of safe and long-lasting treatment options.

The available treatments focus on alleviating symptoms, preventing complications and improving the patients' quality of life (13). Evidence from previous studies has shown that the intestinal epithelial barrier is impaired in UC patients, accompanied with dysbiosis of gut microbiota, and a dysregulated immune response (4). Furthermore, mucosal destruction and oxidative-antioxidant imbalance have been recognized as the primary factors influencing the recurrence of UC (14). Oxidative stress (OS), mediated by ROS, plays a crucial role in the initiation of inflammatory response in the colon through positive feedback mechanisms (15). The mitochondria facilitate the production of ROS, which can be extremely harmful to cells at excessive levels. Uncontrolled ROS production from damaged mitochondria increases inflammatory reactions. Studies have demonstrated that mitochondria are cellular hubs for infection (16).

Roediger et al. reported that the UC can be classified as a metabolic disorder arising from mitochondrial dysfunction. For instance, impaired mitochondrial dynamics and mitophagy were detected in DSS- or TNBS-induced mouse models of enterocolitis and in UC patients (17–19). Moreover, the mitochondrial dynamics and mitophagy have been extensively investigated as important sources of disease biomarkers, including UC. Research has uncovered that, besides the environmental factors, cytokines changes in antimicrobial pathways, and autophagy form part of the pathomechanisms of UC, driven by numerous pathways (20–22). Currently, drugs such as mitochondrial fission antagonist P110 and Mdivi-1 are being investigated for their potential to treat UC, but their therapeutic efficacy is unknown and some side effects have been reported (18, 23). Phytochemicals are extracts from natural products with numerous advantages such as multi-targeting properties, few side effects and less costly (24). Several agents targeting mitochondrial dynamics and mitophagy have been explored for the management of degenerative neurological diseases, tumors, and osteoarthritis (25–27). Therefore, we aimed to discuss the alterations in mitophagy and mitochondrial dynamics in UC as reported the available studies. The treatment of UC using natural ingredients is discussed to provide new ideas for better management of UC in the future.

2 Mitochondrial dynamics

Mitochondrial dynamics comprises fusion, fission, and transport processes (28). Mitochondrial fission and fusion, along with their distribution along cytoskeletal trajectories, are highly coordinated mechanisms involved in the regulation of the mitochondrial network (Figure 1). Proper mitochondrial dynamics are driven by the biogenesis, turnover, distribution of mitochondrial DNA(mtDNA), and metabolic status (29). Studies have demonstrated that mitochondrial fission and fusion are essential components of cell survival, playing important roles in the maintenance of cellular health and disease development.

2.1 Mechanisms of mitochondrial fission

Mitochondrial division predominantly occurs during the S, G2, and M phases of the cell cycle (30). This process is initiated by stress stimuli and is aimed at eliminating damaged mitochondria from the cell through mitophagy. Therefore, mitochondrial division prevents the buildup of damaged mitochondria, alleviating the excessive production of ROS and the subsequent cellular stress (31).

On the other hand, mitochondrial fission is driven by the dynamin-related protein 1 (Drp1), which functions at the contractile site established by the interaction of actin and the endoplasmic reticulum (ER) (32). Phosphorylation-activated Drp1 translocates to the outer mitochondrial membrane (OMM) where it

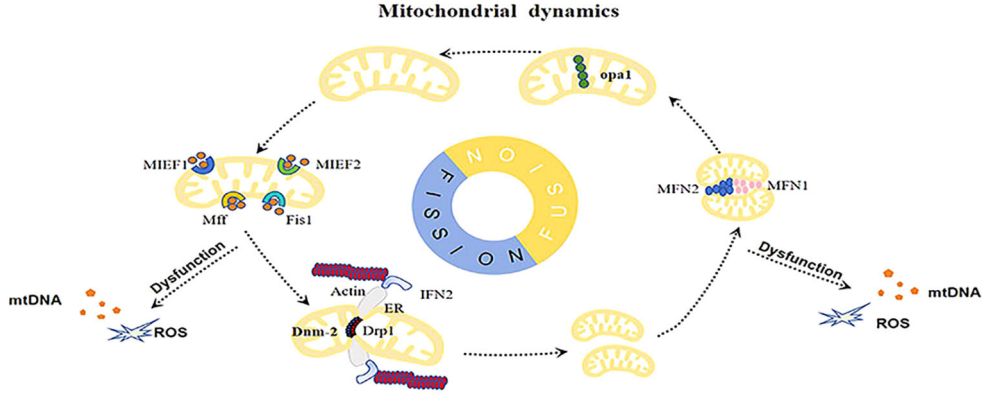

FIGURE 1
Abnormal mitochondrial dynamics increase the accumulation of damaged mitochondria, resulting in the buildup of mtDNA, ROS, and other damage-associated molecular patterns (DAMPs), inducing inflammation and cellular stress.

oligomerizes to form helices that drive OMM contraction and cleavage, thereby facilitating mitochondrial fission (33, 34). Given the Drp1 lacks the pleckstrin homology (PH) structural domain required for the direct interaction with the phospholipid membrane, its activity requires adaptor proteins. In mammals, four proteins regulate the recruitment of Drp1 to the OMM: fission protein 1 (Fis1), mitochondrial fission factor (Mff), mitochondrial elongation factor 1 (MIEF1), and mitochondrial elongation factor 2 (MIEF2), that serve as Drp1 receptors to improve the fission process (35). activation of fission following the enclosement of mitochondria by the ER leads to the Mff-dependent assembly of Drp1 (36). Inactivation of the Mff gene then inhibits mitochondrial recruitment of Drp1, mitochondrial and peroxisomal elongation, while Mff overexpression can fragment the network (37, 38). It has been shown that MIEF1 binds to the adenosine diphosphate (ADP) cofactor, whereas MIEF2 recruits Drp1 to the mitochondrial surface (39). Mff and MIEF1/MIEF2 function are important receptors of Drp1, which mediate it role on mitochondrial fission. Researchers have demonstrated that overexpression of Mff or MIEF is overexpressed, they significantly attract cytoplasmic Drp1 to the mitochondria, independent of hFis1, which enhances the process of mitochondrial fission (40). These receptors work together to help Drp1 divide mitochondria. In mammals, Fis1 usually plays a minor role in recruiting Drp1. However, during cell death or autophagy, Fis1 becomes more important, significantly boosting mitochondrial division. The role of Fis1 in humans is still unclear (41). Finally, the GTPase dynamin 2 (Dnm2) is transiently recruited to the ER contact site downstream of Drp1 where it facilitates the fission process (42, 43). Subsequently, Drp1 is transported back to the cytoplasm, creating a dynamic cycle between the cytoplasmic space and the mitochondrial membrane. The Drp1's activation, movement, and oscillation between cellular compartments are tightly regulated by several post-translational modifications. These include phosphorylation, ubiquitination, sumoylation, and glycosylation. The mechanisms by which these modifications alter the functionality of Drp1 are described in details in later sections of this review (44–47).

2.2 Mechanisms of mitochondrial fusion

Mitochondrial fusion is commonly detected in the early S and G1 phases, and is essential to the generation of sufficient ATP needed to sustain respiration and protein synthesis (48). Moreover, the fusion modulates the exchange of materials such as mtDNA, proteins, and metabolites, enhancing the repair of damaged molecules, inhibiting phagocytosis of elongated mitochondria, which may result from site-blocking during nutrient deprivation and the induction of autophagy (48, 49).

The process of mitochondrial fusion involves the fusion of the inner mitochondrial membrane (IMM) and the OMM, occurring in multiple steps beginning with the activation of dynamin-associated GTPases, including mitofusins (MFN1 and MFN2) on the OMM, and optic atrophic protein 1 (Opa1) on the IMM. This is followed by the GTP hydrolysis-induced fusion of the OMM (50). Structurally, the MFNs are embedded in the OMM via two transmembrane regions, divided by a short loop. This structure allows the N-terminal region, containing the GTPase domain, the coiled-coil heptad repeat 1 (HR1), and the C-terminal region that harbors the HR2 domain, to orient towards the cytoplasmic side (51). This is followed by the IMM fusion, orchestrated by Opa1 in the IMM and endosomal lipid components (52). During fusion, two Opa1 proteins in the IMM interact to form oligomers that assemble into flexible helices, causing membrane swelling and bringing the two IMMs into close proximity. The nucleotide binding organizes and tightens the helix assembly, pulling the IMM closer together to initiate fusion. At the end of the fusion, the helical structure of the Opa1 oligomerization is uncoiled (53). This mechanism is crucial for Opa1 to form a helical structure by dimerizing the GTPase domain. Furthermore, the membrane-bending oligomers of Opa1 undergoes conformational changes, which retract the membrane insertion loop from the outer leaflet, causing remodeling (54). OMM fusion is usually coordinated with inner membrane fusion, but can sometimes occur independently. This phenomenon may be triggered by occurrence of mutations or reduced membrane potential which impairs the fusion of the IMM, but the OMM fusion is not affected (55).

2.3 Factors regulating mitochondrial dynamics

2.3.1 Drp1

The function of Drp1, a key regulator of mitochondrial fission, is influenced by multiple post-translational modifications, such as phosphorylation, ubiquitination, and sumoylation. Its activity is modulated through phosphorylation at three main sites: ser616, ser637, and ser693 (56). The ser616 site is phosphorylated by protein kinase Cδ (PKCδ), Rock kinase, or Pink1, which then activates fission and promotes the binding to other fission proteins (57, 58). On the other hand, the ser637 site is phosphorylated by protein kinase A, which leads to its inactivation (59). Phosphorylation of the ser693 site by GSK3β serves to inhibit mitochondrial division (60). Furthermore, the membrane-associated E3 ligase March5 can regulate Drp1 through ubiquitination (61). Likewise, the E3 ligase Mulan, which is also membrane-bound, was reported to influence Drp1 by promoting sumoylation (62). Functionally, Drp1 that functions without involvement in nitrosylation is thought to trigger mitochondrial fission (63, 64). Ubiquitination, particularly by March5, is crucial for regulating mitochondrial fission. However, while March5 ubiquitinates Drp1, it does not affect its stability. Instead, the process of ubiquitination may affect the dynamics of the Drp1's interaction through the mitochondrial membrane. In this context, the attachment of ubiquitin alters the subcellular transport, promotes Drp1 assembly, and modulates the mitochondrial fission (61).

2.3.2 MFN1/2

MFN1/2 undergoes multiple post-translational modifications, including oxidation, ubiquitination, and phosphorylation. The redox state of specific residues in MFN can regulate its activity and promote membrane fusion by triggering oligomerization (65).

During stress conditions, March5 activates the ubiquitination of acetylated MFN1, which marks it for degradation via the proteasome (66). In contrast, the histone deacetylase 6 augments MFN1-mediated mitochondrial fusion, particularly in response to oxidative stress (67). March5 specifically targets mitochondrial MFN2 for ubiquitination, promoting its oligomerization and strengthening mitochondria-ER tethering (68). In the context of cell apoptotic, the E3 ubiquitin ligase Parkin targets MFN1 for ubiquitination (69) and the activity of the deubiquitinating enzyme USP30 can reverse this process, which reactivates the mitochondrial fission process (70). Phosphorylation of MFNs can either promote or inhibit mitochondrial fusion, depending on the specific site and kinase involved. For example, ERK-mediated phosphorylation of the HR1 domain in MFN1 can suppress fusion and promote its interaction with Bak, triggering apoptosis (71). Cellular stress stimulates Jnk to phosphorylate MFN2 and the subsequent recruitment ofHuwe1 to MFN2, a critical step required for its proteasomal degradation and subsequent apoptosis activation (72).

2.3.3 Opa1

Studies have shown that the Opa1 is the only dynamin-like GTPase detected within the IMM. The MFN1 is by Opa1 to enhance mitochondrial fusion (52). Similarly, the Opa1's activity is influenced by several post-translational modifications. However, the exact mechanisms need to be clarified through further studies.

The balance between mitochondrial fusion and fission benefits cells by regulating mitochondrial shape, enabling content exchange, ensuring fair mitochondrial inheritance, preserving healthy mtDNA, and eliminating damaged mitochondria (73). These structural changes in mitochondria may lead to the development of diseases by impairing the expression levels of proteins involved in mitochondrial dynamics. Moreover, abnormal activation of the signaling pathways can alter the mitochondrial dynamics (74). Previous studied investigations have shown that bacterial, viral, and parasitic pathogens can also modify host mitochondrial dynamics upon cell infection, facilitating their proliferation, significantly influencing disease outcomes (75). The subsequent mitochondrial dysfunction may activate intracellular inflammatory signaling pathways triggering the release of inflammatory factors (76). Mitochondrial dynamics contribute to the activation of immune cells (77). Specifically, the Drp1-mediated mitochondrial fission alters the T-cell activity, making it an important regulator of diverse autoimmune inflammatory diseases (78).

3 Mitophagy

Mitophagy is a selective autophagy mechanism known to maintain cellular homeostasis by eliminating damaged mitochondria. This process is initiated by mitochondrial depolarization and is especially critical for highly differentiated post-mitotic cells, which are largely dependent on aerobic metabolism (79). This process is a major regulator of mitochondrial quality control, which prevents the accumulation of potentially harmful mitochondria that initiates excessive inflammatory responses (80). Defective mitophagy can lead to inflammatory and autoimmune diseases by disrupting inflammatory cytokine secretion and immune cell function. Currently, there are two types of mitophagy pathways: the ubiquitin-dependent pathway and the non-ubiquitin-dependent pathway (Figure 2).

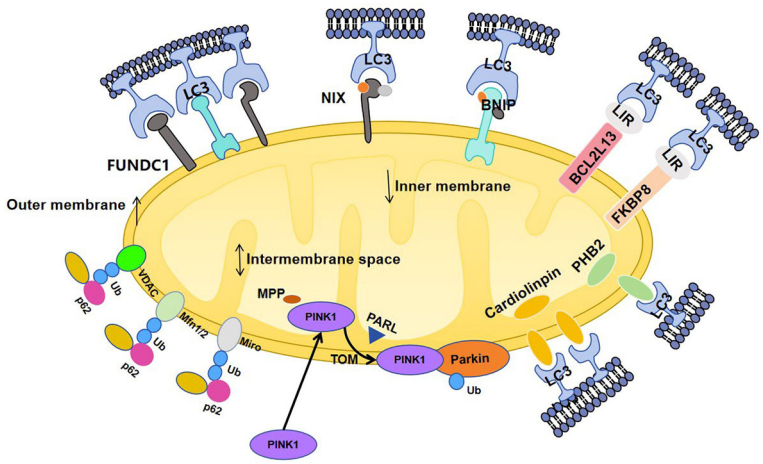

FIGURE 2
1) Pink1 is sequentially targeted to the mitochondria via a targeting sequence and is degraded by the matrix processing peptidase (MPP) and subsequently cleaved by the IMM protease progerin-associated rhodopsin-like enzyme (PARL). Pink1 accumulates on the OMM via the regulation of the enzyme translocase of the outer membrane (TOM). Accumulated pink1 is autophosphorylated and activated through autophosphorylation which then phosphorylates ubiquitin on serine 65 (Ser65), triggering the recruitment of Parkin to the mitochondrial membrane. PINK1 and its substrate, ubiquitinates, phosphorylates and activates Parkin. Specifically, polyubiquitination of Parkin substrates, such as voltage-dependent anion channel-1 (VDAC1), mfn1/2, and Miro 1, induces their degradation by the proteasome. 2) Bcl2 like 13 (BCL2L13) is the mammalian homolog of atg32. In mammalian cells, BCL2L13 facilitates mitosis independently of Parkin. Like other LC3 receptors, BCL2L13 is located on the outer mitochondrial membrane and binds to LC3 via the LIR motif. Specifically, phosphorylation of the Ser272 site enhances the binding of BCL2L13 to the LC3.FK506-binding protein 8 (FKBP8). FKBP8 is located on the outer mitochondrial membrane and stimulates mitochondrial autophagy by interacting with LC3A; 3) Cardiolipin is an inner mitochondrial membrane lipid, and PHB2 are IMM proteins, which bind to the LC3 receptor to initiate mitophagy.

3.1 Pink1-Parkin

Autophagy receptors involved in the mitochondrial protein ubiquitination function through the ubiquitin-dependent mitophagy pathway, specifically the serine/threonine kinase Pink1/Parkin pathway (81). Once Pink1 is exposed on damaged mitochondria, it initiates Pink1/Parkin-dependent ubiquitination, which is often triggered by a loss of mitochondrial membrane potential (82). The expression of Pink1 is kept at low levels under physiological conditions, however, during mitochondrial damage, including mtDNA mutations, elevated mitochondria ROS (mtROS), and the buildup of misfolded proteins, the Pink1 accumulates at the OMM. This is followed by autophosphorylation and activation, as well as phosphorylation of ubiquitin on serine 65, promoting the recruiting Parkin from the cytoplasm to the mitochondrial membrane (83). Next, the Parkin is activated inducing the ubiquitination of mitochondrial proteins and triggering mitophagy (84). Even being absence in Pink1, Parkin could still be attracted to depolarized mitochondria, aiding in mitophagy. For example, HtrA2/Omi or LRRK2 can phosphorylate mitochondrial proteins or parkin itself to promote mitophagy under conditions of pink1 deficiency (85, 86). Alternatively, overexpressed or hyperactive FUNDC1 can recruit parkin to initiate autophagy (87). Among the known targets of Parkin ligase on the OMM are MFN1/2, voltage-dependent anion channel protein 1 (VDAC1), and mitochondrial GTPases (88–90). Proteomics analyses on the degradation of OMM components and the reorganization of the OMM proteome are highly advocated to increase our understanding of the process of mitophagy. Being the most abundant OMM protein, VDAC1 forms the mitochondrial pore and is a key modulator of metabolites, ions, and water transport under physiological conditions and influences mitochondrial homeostasis (91–93). The VDAC1 participates in the regulation of mitophagy, interacting with Parkin to facilitate polyubiquitination and recruitment of Parkin to induce mitophagy (94). By energizing the Pink1/Parkin mitophagy pathway, VDAC1 in conjunction with BNIP3 alters the mitophagic flux process (95). Hypoxia-induced GPCPD1 depalmitoylation has been reported to initiate mitophagy by regulating the PRKN-mediated VDAC1 ubiquitination (96). Application of the antidepressant drug sertraline was found to alter the VDAC1 protein, decrease ATP levels, activate AMPK, and inhibit the MTOR signaling pathway to induce autophagy (97).

3.2 NIX/BNIP3/FUNDC1 receptor-mediated

NIX and BNIP3 belong to the Bcl-2 protein family which is located on the OMM. The Bcl-2 family members on the OMM participate in the initiation of cell apoptosis, influencing the mitophagy process (98, 99). One of the mechanisms by which autophagy activates mitophagy is through the direct binding to the light chain 3 (LC3) via the BH3 structural motif. This motif functions as a molecular effector of mitochondrial membrane depolarization-induced hypoxia which promotes occurrence of mitophagy (100). NIX was reported to act as an adaptor protein that transports the components of the autophagy machinery to the mitochondria to trigger mitophagy. Another proposed model suggests that BNIP3 or NIX competes with Beclin-1 for binding to Bcl-xl. During erythropoiesis, the expression of NIX is upregulated, disrupting the Bcl-xl-Beclin-1 interaction and freeing Beclin-1 to trigger autophagy (101). Notably, the BNIP3 was demonstrated to lower the mTOR activity and regulate autophagy by increasing LC3 expression. Similar to BNIP3 and NIX, FUNDC1 is a mitochondrial junction protein induced by hypoxia that bind with LC3 via its LIR motif. Under hypoxic environments, the ULK1 kinase relocates to mitochondria, phosphorylating FUNDC1, enhancing its binding with LC3 to promote mitophagy (102).

Most researchers agree that mitophagy is closely linked to mitochondrial dynamics. Inhibiting fission with DRP1K38A or FIS1 RNAi reduces mitophagy, while overexpressing Opa1 also inhibits autophagy (103). Impaired fusion results in a reduction in IMM potential, triggering pink1 accumulation and parkin activation. However, the inverse dependence of fusion and autophagy on membrane potential makes them complementary rather than competing processes for daughter mitochondria after a fission event (104). Therefore, we suggest that mitochondrial fission is a prerequisite for mitophagy and that fusion will inhibit autophagy. However, inhibiting mitochondrial hyperfusion by silencing Drp1 or Mff does not affect mitophagy induced by Fis1 loss (105). In future, researchers should aim to explore the crosstalk between mitochondrial dynamics and mitochondrial autophagy. Moreover, several proteins involved in mitochondrial dynamics also participate in mitochondrial autophagy process. MFN1/2 is extracted from the OMM using ubiquitin-dependent chaperones and is subsequently degraded by the proteasome (106). The deletion of ubiquitination of MFN1/2 prevents the fusion of damaged mitochondria, while enhancing the fission, thereby promoting mitophagy (107). Additionally, Pink1 phosphorylates MFN2, which acts as a Parkin receptor to eliminate impaired mitochondria (108). However, it is unclear whether the OMM proteins coordinate both mitochondrial fission and mitophagy. The precise interplay between mitochondrial dynamics and autophagy requires further investigation.

4 Relationship between mitochondrial dynamics and UC

Previous studies have demonstrated changes in GTPase mRNA expression, such as Drp1, Opa1, and mitophagy in UC. Mancini et al. reported that in DSS-versus DNBS-treated mice with intestinal inflammation, the mRNA levels of Drp1 and Fis1 were elevated,

triggering excessive mitochondrial fission (18). The expression levels of MFN1, MFN2, and Opa1 proteins were found to be downregulated in the intestinal epithelial cells of UC patients and DSS-induced mice, which weakens the mitochondrial fusion capacity and triggers mitochondrial dysfunction (109). Restoring the expression of these proteins may improve mitochondrial function in UC. In UC mice, mitochondria in the subnuclear region of inflammatory cells appear swollen and fragmented. A strong correlation exists between inflammation and the mitochondrial network disruption in colitis. The mitochondrial network is also disrupted in non-inflamed colonic regions, suggesting that the mitochondrial structure may be an early event in UC (17). Excessive mitochondrial fission and reduced fusion can stimulate the development of UC by altering energy metabolism, oxidative stress, and apoptosis (Figure 3).

4.1 Changes in energy metabolism

As early as 1980, Roediger et al. postulated that IBD, particularly UC, may be an energy-deficient disease (19). Since then, several studies have demonstrated that patients with UC exhibited reduced mitochondrial function, including structural abnormalities, mtDNA mutations, reduced electron transport chain activity, decreased oxidative phosphorylation, and lower ATP concentrations (110–113). Mitochondrial dysfunction and imbalance in dynamics are often accompanied with impaired energy metabolism, which triggers inflammation and gastrointestinal symptoms (114). The colonic epithelial cells consume a high amount of energy to execute various processes, including the electrolyte exchange in the intestinal epithelium, glycoprotein synthesis in the mucus layer, lipid synthesis in cell membranes, structural protein synthesis, and detoxification. Therefore, energy deprivation may impair these processes, causing epithelial cell atrophy in the short term and colonic mucosal barrier damage in the long-run, leading to the development of UC (115, 116). Compared to healthy donors, oxidative phosphorylation metabolism in UC patients shifts towards glycolysis as a strategy to compensate for ATP production defects. This increases the cellular levels of lactate acid within colonic cells, whereas bacteria in the intestinal lumen consume epithelial oxygen, inducing ecological dysregulation which promotes inflammation (117). Cells with inflammatory colitis may use glycolysis to produce ATP as an adaptive response to butyrate transport and oxidative stress. A reduction in ATP levels induce detrimental effects on the actin cytoskeleton, whereas actin irregularities may impair the localization and function of cellular junctions, increased gaps between cells (118, 119). Lan A et al. demonstrated that in DSS-induced enterocolitis mouse model, the ATP activity was enhanced, and the expression of energy-dependent differentiation markers was upregulated. This was accompanied by enhanced epithelial repair, requiring large amounts of energy to

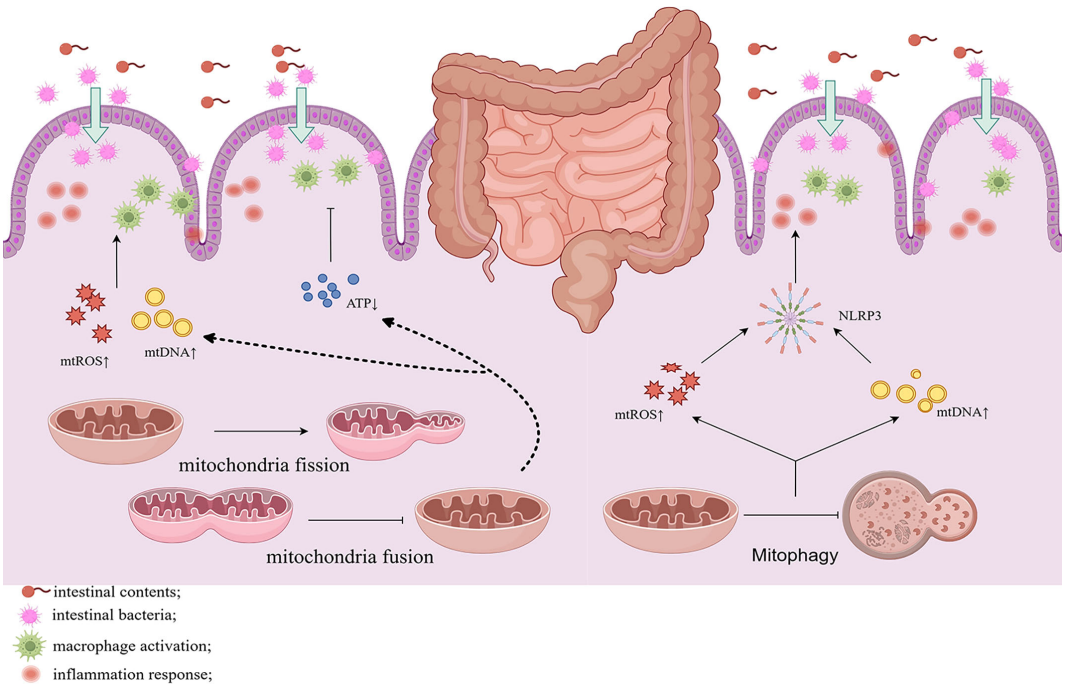

FIGURE 3
Altered mitochondrial dynamics of the intestinal cells in UC induces mitochondrial fission and inhibits fusion while attenuating mitochondrial autophagy. This results in the reduction of ATP production and promotes the release of mtDNA, mtROS, and activation of NLRP3 inflammasome, leading to inflammation and disruption of the intestinal barrier. Chronic inflammation and gut barrier disruption also activate mitochondrial fission and reduces autophagy, establishing a vicious cycle.

enhance the structural and functional recovery of the colonic mucosa. This process has been linked to the increase in expression levels of regulators of mitochondrial mass and biogenesis, such as Sirt3, FoxO3, and PGCα. It is also characterized by enhanced fusion, as evidenced by the upregulated Mfn2 gene expression and good mitochondrial morphology (120). In previous studies, creatine supplementation or administration of energy sensors improved intestinal epithelial repair. Therefore, strategies for promoting cellular energy metabolism via modulating mitochondrial dynamics and intestinal barrier are potential treatments for UC (121, 122).

4.2 Increased oxidative stress

Impaired mitochondrial dynamics have been linked to the development of oxidative stress in UC patients. Mitochondrial aerobic metabolism promotes the proliferation and differentiation of intestinal stem cells within colonic crypts and supports the synthesis of intestinal epithelial cells (123). However, excessive production of ROS may occur in damaged mitochondria. Under normal conditions, mitochondria exhibit a well-coordinated self-repair mechanism. However, in cases of a dynamics imbalance, oxidative stress levels increase within the cells, triggering an inflammatory response (124). Excessive mitochondrial fission reduces energy production and leads to the generation of oxidative stress, increasing mitochondrial fission, and reducing cell viability (125). Mitochondrial fusion may trigger the mixing of mitochondrial contents, including DNA and respiratory chain complexes, which are involved in the repair of damaged mitochondria and reduce localized oxidative stress levels. However, a decrease in mitochondrial fusion may increase the development of oxidative stress (126). Studies have shown that gut microbiota and their metabolites participate in this process. Colic acid, a major biofilm component of *Escherichia coli* (*E. coli*), increases mitochondrial fission in enterocytes in a Drp1-dependent manner, enhancing the stress-activated transcription factor-mediated unfolded protein response (UPR) in response to mitochondrial stress (127). DSS can cause damage to the intestinal epithelial respiration and stimulate the mitochondrial complexes I, II, and IV, leading to the accumulation of damaged mitochondria and ROS, both of which cause cellular damage (128).

The mitochondrial chaperone protein Prohibitin 1 (PHB1), an endomembrane protein, regulates Opa1-mediated fusion within the IMM (129). Mitochondrial dysfunction in intestinal epithelial cells, induced by PHB1 deletion was found to trigger spontaneous ileitis in mice (130, 131). In the study, it was observed that epithelial cells showed mitochondrial dysfunction, crypt cell death, and Paneth cell abnormalities, which are also observed in the intestinal epithelium of patients with IBD. Treatment with the mitochondria-targeted antioxidant Mito-Tempo ameliorated the Paneth cell defects and reduced intestinal epithelial inflammation in mice lacking PHB1. The role of Paneth cell mitochondrial impairment in the development of ileitis has been documented in multiple studies on PHB1-deficient mice (131).

4.3 Cell apoptosis

The mitochondrial dynamics have been implicated in the regulation of apoptosis. Excessive mitochondrial fission can induce morpho-functional changes in mitochondria, which triggers the production of intrinsic apoptotic signals. Studies have demonstrated that inhibition of mitochondrial fusion stimulates that intracellular stress response and enhances apoptosis (132). The pathogenic *E. coli* secreted effector proteins, mitochondria-associated protein(Map), and *E. coli* secreted protein (EapF) are within the mitochondrial matrix. Map induces mitochondrial fission, while EspF promotes the permeabilization of the mitochondrial outer membrane (MOMP) in the OMM (133). Changes in mitochondrial membrane permeabilization may be accompanied by the release of pro-apoptotic factors, such as cytochrome c, which activate the apoptotic signaling pathways (134). Mitochondrial fission resulting from MFN deficiency in HeLa cells protects against *Lactobacillus monocytogenes*, suggesting that *Listeria hemolysin O* can disrupt mitochondrial dynamics and promote apoptosis via disrupting calcium influx. In multiple cell types, enhanced mitochondrial fusion inhibits the propagation of apoptotic signals, delaying cellular apoptosis (135). This phenomenon provides a potential alternative approach for treating UC.

In neurological diseases, dysregulation of Drp1 disrupts mitochondrial dynamics, stimulating inflammation and exacerbating disease severity (136). In pancreatic islet inflammatory cells, NF-κB activation was reported to increase Opa1 expression, while interleukin-6 (IL-6) was found to increase Fis1 and downregulate MFN2, enhancing disease progression (137). In colitis, abnormal mitochondrial dynamics stimulate multiple inflammatory pathways, such as the NF-κB pathway, which activated the inflammatory response. Mitochondrial fission can also trigger apoptosis, compromising intestinal barrier function and promoting cellular inflammation. In future, researchers should explore the role of mitochondrial dynamics in UC to identify robust therapeutic disease.

5 Relationship between mitophagy and UC

Mitophagy provides an important mechanism for eliminating damaged mitochondria to maintain normal mitochondrial function. Excessive mitochondrial damage and mitophagy levels have been detected in the intestinal tissues of UC patients, which are correlated with high disease severity (138). In epithelial tissues of DSS-induced enterocolitis mice, the number of damaged mitochondria, autophagy and mtROS were recorded, which further activated the NLRP3 inflammasomes to promote inflammation (139). This indicates that the role of mitophagy in apoptosis is bidirectional. Excessive mitophagy can trigger energy depletion, thereby inducing apoptosis, whereas, suppression of

mitophagy may potentially prevent the clearance of damaged mitochondria, increasing the generation of ROS levels and inflammation.

5.1 Autophagy gene and development of UC

Genome-wide association studies (GWAS) have identified several autophagy genes, including Atg16L1, Lrrk2, and Irgm, which influence the genetic susceptibility of IBD (140–142). Atg16L1 is a critical component of the mitophagy machinery involved in the regulation of immune responses and inflammation. It is part of the complex that cleaves the ubiquitin-like protein LC3 via a lipolytic mechanism, promoting autophagosome formation and activity. The Atg16L1 risk variant (Atg16L1T300A), was found to influence the risk of IBD, creating a caspase cleavage site that weakens the stability of the protein, thereby diminishing autophagy, particularly in the presence of TNF-α (143). Irgm, a human immune-associated GTPase, translocates to mitochondria, where it regulates mitochondrial division and induces mitophagy (144). Irgm1, a homologue of Irgm, participated in the development of intestinal inflammation in mice. Irgm1 knockout mice exhibited increased severity of inflammation following exposure to DSS. Mice with DSS knockout exhibited significant disruptions in Paneth cell positioning and granule structure, which contributed to mitophagy and autophagy impairment within the Irgm1-deficient enterocytes, including Paneth cells. These findings demonstrate that Irgm1 regulates acute inflammatory response in mouse intestines, probably by regulating autophagy, which modulates the normal Paneth cell function (145). Lrrk2 is a multifunctional protein with kinase and GTPase activity; with mutations in the Lrrk2 gene found to decrease mitochondrial numbers, impaired mitochondrial dynamics, and inhibition of the mitophagy pathway (146). In individuals with UC, the expression level of Lrrk2 were upregulated in peripheral blood samples. In preclinical animal models of UC, Lrrk2 knockout ameliorated the progression of DSS-induced enterocolitis, which was accompanied with the transition of the intestinal macrophages (MΦs) to alternatively activated macrophages, promoting probiotic colonization and attenuating the onset and progression of colitis (147). This indicates that Lrrk2 plays a crucial role in the development of UC.

5.2 Mitophagy and immune inflammation response in UC

Mitophagy influences the innate and adaptive immune responses, thereby maintaining intestinal homeostasis (148). In previous studies, most studies focused on the intestinal immunity of macrophages and T cells. In UC patients and mice with DSS-induced colitis, the mitophagy of intestinal macrophages was significantly decreased. It was found that the expression of proteins associated with the Pink1/Parkin and NIX-mediated autophagy pathways was decreased. This was accompanied by the accumulation of mtROS and mtDNA, which were then released into the cytoplasm, activating the NLRP3 inflammasome and enhancing the maturation of IL-18 and IL-1β (149, 150). In a mouse model of T-cell metastatic colitis, the expression the Th1 transcription factor TBX21 and Th1 cells in the lamina propria of Pink1 knockout (Pink1KO) mice was higher compared to the levels of naïve T cells. Treatment with the Urolithin A (UA), a mitophagy agonist, inhibited the Th1 differentiation, reducing the formation of IFN-γ and IL-17, which suppresses the levels of the associated inflammatory response (151). This finding suggests that inhibiting inflammation via enhancing mitophagy may be an attractive strategy for alleviating enteritis symptoms. Several cytokines have been reported to modulate mitophagy. IL-10, a key anti-inflammatory factor, inhibits the metabolic shifts associated with inflammatory stimuli in macrophages, while maintaining mitochondrial integrity and function by inhibiting mTOR (152).

Mitochondria are important regulators of the innate immune signaling, with several immune effectors clustered on the OMM (153). Among then, MAVS which targets the OMM through its C-terminal transmembrane domain was demonstrated to activate the downstream signaling pathways via NF-κB to regulate IFN production, and its activation is modulated by mitochondrial dynamics (154, 155). Furthermore, studies have shown that depletion of autophagy proteins, such as LC3B and Beclin 1, results in the accumulation of damaged mitochondria and the translocation of mtDNA into the cytoplasm, activating inflammatory gene transcription. The activities of TOMM20 and HSP60, proteins forming part of the OMM and mitochondrial matrix, respectively, negatively correlate with autophagy. In LPS-treated UC mouse models, MODE-K cells and colonic tissues displayed increased expression of p62, TOMM20, and HSP60, causing autophagy inhibition and enhancing inflammation (156). The absence of Nix/BNIP3L-mediated mitophagy during PHB1 protein deficiency causing excessive production of ROS and activation of inflammation, which suggests that mitochondrial dysfunction may impair mitophagy (157). Studies investigating the effect of polystyrene nanoplastics (PS-NPs) on enterocolitis development revealed that the buildup of these plastics in mitochondria induced mitochondrial stress, initiating Pink1/Parkin-mediated mitophagy. Additionally, LC3 dots in Caco-2 cells co-localized with mitotracker-labeled mitochondria, indicating the initiation of mitophagy (158).

6 Current studies of potential therapeutic approaches to modulate both mitochondrial dynamics and mitophagy in UC

The recent advancements in research investigating potential strategies for modulating mitochondrial dynamics and mitophagy

in UC have achieved significant progress. Despite the advances in UC treatment, many patients show poor response to biological therapies (159). Proper mitochondrial fission, fusion, and mitophagy are essential for mitochondrial health. Inhibition of excessive fission has been shown to enhance mitophagy with beneficial effects in animal models of myocardial infarction, pulmonary hypertension, ischemia-reperfusion injury, multiple sclerosis, and Huntington's disease (160–164). Modulating this mechanism may help to control mitochondrial dysfunction in colitis. Pharmacological or natural products have been proposed to restore mitophagy homeostasis, promoting the clearance of irreversibly dysfunctional mitochondria, making them promising therapeutic approaches for UC.

6.1 Mitochondria-targeted therapy for UC

Suppressing excessive mitochondrial fission has been shown to accelerate mucosal healing in mice. Patients with UC exhibit increased mitochondrial fission, along with higher butyrate exposure in the environment surrounding colonic stem cells. Excessive mitochondrial fission inhibits stem cell proliferation by disrupting butyrate metabolism in colonic organoids. Mechanistically, results from various enzyme activity assays in colonoids have shown that excessive fission delays mucosal repair by increasing ROS leading to the inhibition of mitochondrial acetoacetyl CoA thiolase activity, which impairs butyrate metabolism. Therefore, the failure of antibiotics to promote mucosal healing in mice was reversed following the treatment of the mitochondrial fission antagonist P110 and exogenous butyrate (165). Further findings show that P110 suppresses excessive fission by blocking Drp1 binding to Fis1, significantly alleviating intestinal inflammation and reducing UC symptoms (18). Unfortunately, few drugs effectively inhibit mitochondrial fission *in vivo*. Mdivi-1 is one such drug, targeting Drp1, but it may induce unintended side effects on oxidative phosphorylation and ROS levels (23). Leflunomide, an antirheumatic drug that inhibits pyrimidine synthesis has been found to mitigate mitochondrial fission by enhancing fusion to alleviate the associated symptoms (166). In addition, Chen W and colleagues described several chemotherapeutic agents that target different mechanisms of mitochondrial fission and fusion. However, evidence-based data to support the therapeutic benefits of these agents in the treatment of UC (50).

6.2 Natural products on UC-related tissues through mitophagy

Natural products, such as curcumin, can treat diseases by targeting mitochondrial function. For instance, curcumin protects cartilage in osteoarthritis by stimulating AMPK/PINK1/Parkin-mediated mitophagy (167). Berberine protects glomerular podocytes by inhibiting Drp1-mediated mitochondrial fission and dysfunction (168). Natural products can regulate mitophagy making them potential treatments for UC-associated mitochondrial dysfunction (Table 1). To assess the therapeutic efficacy of these natural products and their potential side effects on tissues or cells, UC-associated models and *in vitro* experiments need to be performed to accelerate the clinical translation of these findings. Dipak Kumar Sahoodeng et al. described the effects of various natural antioxidant components on IBD (169). In the following section, we provide a summary of natural products that regulate UC by modulating mitochondrial dynamics and mitophagy.

6.2.1 Curcumin with mitophagy on UC

Curcumin is the main active ingredient in dried, powdered rhizome of turmeric and has been found to exert beneficial effects against the development and progression of UC in animal and human trials (185, 186). A meta-analysis on the use of curcumin to treat UC comprising nine randomized controlled trials found that compared with the control group, the curcumin treatment increased the clinical remission rates (RR=2.28, 95%CI[1.43, 3.62], P=0.0005) and endoscopic remission rates (RR=1.66, 95%CI[1.07, 2.60], P=0.03), without inducing significant adverse events (187). Another study showed that it enhanced G2/M cell cycle arrest and autophagy and low doses of curcumin may activate adaptive stress responses, while high doses trigger acute responses (188). Shuting Cao et al. demonstrated that H_2O_2-induced epithelial barrier disruption and mitochondrial dysfunction models of UC in IPEC-J2 cells, curcumin treatment upregulated Pink1 and Parkin genes and proteins, but did not affect the expression of Nix, BNIP3, and FUNDC-1, necessitating the hypothesis that curcumin may activate mitophagy primarily via the Pink1-Parkin pathway (171). Other studies have demonstrated that curcumin abolished the diquat-induced oxidative stress and jejunal injury in piglets, enhanced the activity of complexes I-IV and suppressed the expression level of phosphorylated-PERK/PERK and phosphorylated-eIF2α/eIF2α, to improve the expression of mitochondrial function (172).

6.2.2 Resveratrol with mitophagy on UC

The RES is a natural polyphenol found in various plants and fruits (189). Dietary supplementation of RES improved intestinal barrier integrity, oxidative stress, and intestinal inflammation in a colitis model (190). The study by Shuting Cao et al. concluded that RES activated mitophagy which was indicated by the upregulated expression of Pink1, Parkin, and LC3-II/LC3-I relative to the piglets injected with diquat (176).

6.2.3 Berberine with mitophagy on UC

Berberine is a quaternary ammonium alkaloid primarily extracted from Coptis chinensis and Phellodendron amurense (191). It has the potential to treat various diseases such as cardiac aging and acute kidney injury via enhancing mitophagy (192, 193). Approximately, 211 potential targets of Berberine and 210 UC genes were predicted on the PharmMapper database whereas UC genes were determined on the GeneCards database and the OMIM database (194). Berberine targets IRGM1 to inhibit the PI3K/AKT/mTOR pathway, suppressing inflammatory response in UC (179). Similarly, Berberine was found to stimulate autophagy via the AMPK/MTOR/ULK1 pathway, as

TABLE 1 Effects of natural products on UC-related tissues through mitophagy.

Nature products	Species	Experimental models	Signaling pathway	Main results	Ref.
curcumin (50 mg/kg)	BALB/c mice	3.5%DSS induced N=80/4groups	SIRT1/mTOR	mTOR&SIRT1 ↑ Atg12, Beclin-1&LC3-II ↓	(170)
curcumin (200 mg/kg)	IPEC-J2	H2O2 induced N=24/4groups	AMPK/TFEB/ pink1/parkin	Pink-1&and Parkin ↑ LC3-II&Beclin1 ↑ BNIP3L, BNIP3and FUNDC-1	(171)
curcumin (200 mg/kg)	piglets	diquat induced N=24/4groups	P-ERK	PGC-αand NRF-1 ↑ P-PERK/PERK and MFN2 ↓	(172)
curcumin (15、30、60mg/kg)	BALB/c mice	5%DSSinduced&2.5% ethanol IP. N=60/6groups	p38MAPK	p-p38MAPK ↑	(173)
curcumin (200 mg/kg)	BALB/c mice	3%DSS induced N=45/3groups	NS	LC3-II/LC3-I and Beclin-1 ↑ p62 ↓	(174)
curcumin (15、30、60mg/kg)	BALB/c mice	3%DSS induced N=40/5groups	NS	Bcl-2 ↑ Atg5, LC-3IIand Beclin-1 ↓	(175)
RES (80 mg/kg)	BALB/c mice	3.5%DSS induced N=80/4groups	SIRT1/mTOR	mTOR&SIRT1 ↑ Atg12, Beclin-1and LC3II ↓	(170)
RES (100 mg/kg)	piglets	diquat induced N=24/4groups	pink1/parkin	Pink1and Parkin ↑ LC3II and LC3- II/LC3- I ↑	(176)
RES (100 mg/kg)	C57BL/6 mice	3%DSS induced N=48/4groups	NS	LC3B, Beclin-1and LC3-II/ I ↑	(177)
RES (100 mg/kg)	C57BL/6 mice	3%DSS induced N=50/5group	AMPK	CDX2, p-AMPKand SIRT1 ↑ p- NF-κB ↓	(178)
Berberine (25、50、100mg/kg)	C57BL/6 mice	DSS induced and LPS-induced RAW264.7 cells N=105/7groups	IRGM1/PI3K/ AKT/mTOR	IRGM1 ↑ p-mTOR, p-AKT&p-PI3K ↓	(179)
Berberine (25、50、100mg/kg)	KuNSing mice	3%DSS induced N=120/6groups	AMPK/mTOR/ULK1 ATG16L1/NOD1/RIPK2	p-ampk and p-ulk1 ↑ LC3B, ATG12and ATG16L1 ↑ p- mtor, NOD1and RIPK2 ↓	(180)
Baicalin (1、5 、10μ g/mL)	HT-29 (Human)	LPS induced	NS	LC3, Atg5 and BECN1 ↑	(181)
Ginsenoside Rd (10、20、40mg/kg)	C57BL/6 mice THP-1	DSS induced LPS + ATP induced	AMPK/ULK1	p62, AMPK and p-ULK1 ↑	(182)
Ginsenoside Rh2 (50mg/kg)	C57BL/6 mice	3%DSS induced N=24/4groups	STAT3/miR-214	PTEN ↑ p-STAT3and miR- 214↓	(183)
Ginsenoside Rk2 (5、10、20μM)	THP-1	LPS induced	SIRT1/ERK/MEK	SIRT1 ↑ P- ERK, MEK and SIRT1 ↑	(184)

NS, Not specified; HT-29, The human colonic epithelial cell line;THP-1, human intestinal epithelial THP-1 cells.
↑, up; ↓, down.

well as inhibit lysozyme and its secretion, to accelerate lysosomal maturation and expression, implying that it has the potential to treat inflammation (180).

6.2.4 Ginsenoside with mitophagy on UC

Ginsenoside is the most abundant active ingredient in the traditional Chinese medicine ginseng, with diverse structures. Evidence for its anti-fatigue, immunomodulation, and anti-tumor properties have been documented (195). In a meta-analysis comprising 15 studies with 300 animals, it was observed that ginsenosides significantly reduced the levels of pro-inflammatory factors (IL-β, IL-6, TNF-α) and upregulated the expression of the anti-inflammatory factors IL-10 and tight junction proteins (Zonula Occludens-1, occludin) (196). Elsewhere, oral administration of ginsenoside Rd alleviated the DSS-induced enteritis symptoms in a dose dependent-manner. *In vitro*, ginsenoside Rd significantly inhibited the NLRP3 inflammasome, enhancing the p62-dependent mitochondrial translocation and mitophagy predominantly through the AMPK/ULK1 signaling pathway (182).

These natural compounds have shown promising potential in treating UC. The cellular and animal models discussed in the above sections have uncovered the pivotal roles of natural products in enhancing mitophagy via pink1/parkin, AMPK/mTOR/ULK1, and SIRT1/ERK/MEK pathways, to prevent inflammation and enhance the repair of intestinal barrier. However, overstimulation or inhibition of mitochondrial function may result in detrimental effects, and thus, it is imperative to balance between mitochondrial dynamics and mitophagy to achieve UC treatment. Several animal model experiments have explored the mechanisms of chronic UC, identifying new targets for drug development. Compared with pharmaceutical drugs, natural ingredients have the advantages of being multi-target and eliciting fewer side effects. However, the efficacy of such drugs is based on findings from preclinical investigations, necessitating further well-designed clinical trials to verify their clinical efficacy and address the current limitations.

7 Concluding remarks and future perspectives

In conclusion, a strong link exists between intestinal barrier damage, chronic inflammation, and mitochondrial dysfunction in ulcerative colitis. Natural products targeting these processes show promise as potential treatments. However, several challenges remain to be solved. 1) Although the roles of mitochondrial dynamics and autophagy in UC have been recognized, the precise regulation of the balance between mitochondrial fission and autophagy is poorly understood. In addition, several differences exist mitochondrial function and autophagic response across different species, making it difficult to formulate appropriate targeting strategies. Most of the current studies are based on animal models, with few studies conducted in actual UC patients. 2) Although several natural products have been found to modulate mitochondrial autophagy, most of such studies involved *in vitro* experiments and small-scale animal models. Therefore, the clinical efficacy of natural products need to be validated because data from preclinical trials may not accurately reflect the clinical settings, in terms of the dosage, safety, and side effects. 3) Current treatments for UC often target mitochondrial fission or autophagy, but these approaches may not be specific to individual patient needs. How to select appropriate therapeutic targets and avoid over-regulation of mitochondrial function remains to be clarified.

In the future, clinical trials for various mitochondria-targeted therapies should be conducted to confirm the therapeutic efficacy of natural products discussed in this review. The trials should integrate a wide spectrum of data, including clinical profiles, gene mutations, and gut molecular signatures, to identify patient subgroups that are most likely to benefit. Moreover, an integrated approach is essential to address the inherent heterogeneity of UC and enhance the application of personalized treatment. This primary focus of this review was to enhance the current understanding of the complexity of UC and discuss the available treatments for specific patient subgroups.

Author contributions

JZ: Writing – original draft, Writing – review & editing. YX: Writing – review & editing. TW: Writing – review & editing. XZ: Writing – review & editing. JY: Writing – review & editing. LP: Writing – review & editing. HF: Writing – original draft, Writing – review & editing. CZ: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The role of the esophageal and intestinal microbiome in gastroesophageal reflux disease: past, present, and future

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Gastroesophageal reflux disease (GERD) is one of the common diseases of the digestive system, and its incidence is increasing year by year, in addition to its typical symptoms of acid reflux and heartburn affecting the quality of patients' survival. The pathogenesis of GERD has not yet been clarified. With the development of detection technology, microbiome have been studied in depth. Normal microbiome are symbiotic with the host and can assist the host to fulfill the roles of digestion and absorption, and promote the development of the host. Dysbiosis of the microbiome forms a new internal environment, under which it may affect the development of GERD from the perspectives of molecular mechanisms: microbial activation of Toll-like receptors, microbial stimulation of cyclooxygenase-2 expression, microbial stimulation of inducible nitrous oxide synthase, and activation of the NLRP3 inflammatory vesicle; immune mechanisms; and impact on the dynamics of the lower gastrointestinal tract. This review will explore the esophageal microbiome and intestinal microbiome characteristics of GERD and the mechanisms by which dysbiotic microbiome induces GERD.

KEYWORDS

gastroesophageal reflux disease, microbiome, microbial dysbiosis, inflammation, barrier, motility

1 Introduction

Gastroesophageal reflux disease (GERD) is a chronic condition characterized by reflux of gastric contents into the esophagus, which can cause uncomfortable symptoms and potential complications (1). The prevalence of GERD is estimated to be 10-20% of the adult population in Western countries and ranges from 2.5 - 7.8% in Asia (2). The chronic nature of GERD leads to psychological distress, including anxiety and depression, which not only affects the quality of life of the patient symptomatically, but is also associated with a number

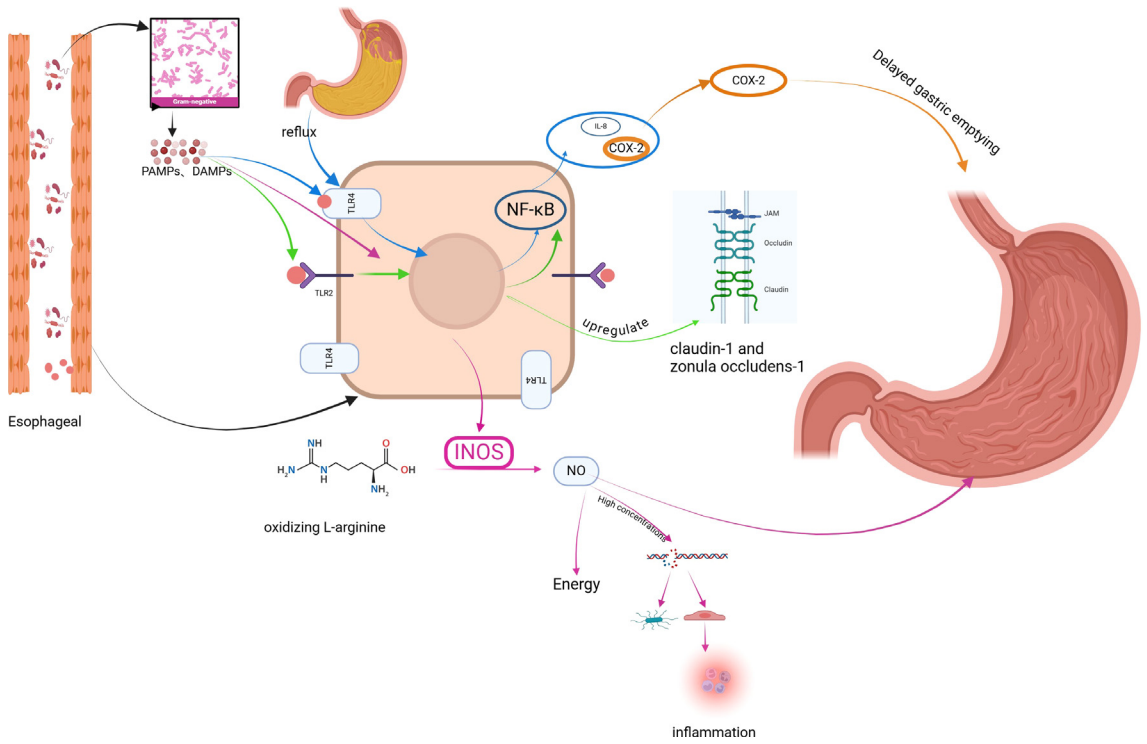

FIGURE 1
Mechanism of GERD induced by dysbiosis of esophageal microbiome. Figure created with BioRender.com.

of serious complications (3). Routine diagnostic techniques of GERD mainly include endoscopy and 24-hour pH monitoring, and the diagnosis is usually made by symptomatic and empirical qualitative examination of the patient's condition. The initial diagnosis is often made by symptoms and empirical proton pump inhibitor (PPI) testing. Traditional therapeutic strategies include lifestyle modifications (e.g., dietary changes and weight loss), pharmacologic interventions (primarily PPIs and histamine 2 {H2} receptor antagonists), and surgical options for refractory cases, such as fundoplication (4).

Therapeutic aspects of the combined use of probiotics have a positive effect on GERD. In a clinical trial, patients with esophagitis (RE) were randomized into a test or control group, the test group received rabeprazole tablets + Streptococcus lactis MH-02, and the treatment group received rabeprazole tablets + placebo, and the results of the trial showed that patients in the test group experienced earlier symptom relief, significantly lower Gastrointestinal Symptom Rating Scale (GSRS) scores, and a longer mean time to relapse (5). Another study also examined patients with RE, with the test group taking esomeprazole + Bacillus subtilis and Enterococcus faecalis enteric capsules, and the control group taking esomeprazole + placebo, and its results showed that the time to relapse was significantly shorter in the control group than in the test group, and that the risk of relapse was lower in the test group than in the treatment group at any point in time during the 12-week follow-up period (6). The above trial results suggest that microbiomes may have a positive impact on the treatment of GERD from a more profound perspective.

This paper will summarize the structural characteristics of the esophageal and intestinal microbiome of GERD and the known possible triggering mechanisms and potential future triggers are detailed.

2 Esophageal microbiome

Traditionally, the esophagus was thought to have no significant microbiota, and the microbiota in the normal esophagus was thought to be derived from the oral cavity and to be variable (7–10). In 1998, Gagliardi et al. isolated the microorganism *Streptococcus viridans* from oropharyngeal cultures by traditional esophageal culture (9). With the development of next-generation sequencing technologies such as 16S rRNA gene sequencing, internal transcribed spacer region (ITS) sequencing, Polymerase Chain Reaction (PCR), Birdshot Macro-genomics, Macro-transcriptomics, Metabolomics, and Macro-proteomics Mass Spectrometry, etc., the study of esophageal microecology has been gradually improved (11). It has been found that some members of the phylum *Thick-walled Bacteria*, including *Clostridium* spp, *Fusobacterium* spp, *Megalococcus* spp, *Morgillus* spp, and *Moriella* spp, are unique microbiota of the esophageal mucosa, which are found only in the esophagus and not in the oral cavity (12–14).

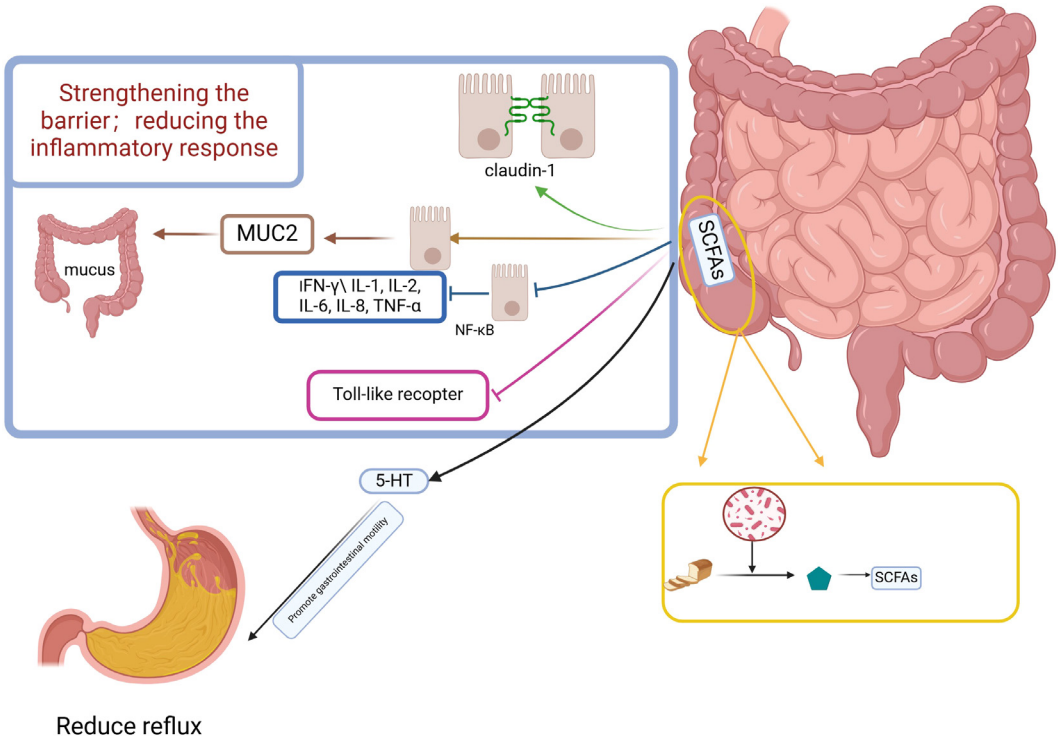

FIGURE 2
Potential mechanism of esophageal protection against reflux reduction by short-chain fatty acids. Figure created with BioRender.com.

2.1 Dominant bacterial families in the esophagus of healthy individuals

Esophageal microbiota is the dynamic community of microorganisms inhabiting the esophagus (15). Since the early 2000s, many scholars have begun to study sesophageal microbiomes using new techniques. In 2004 Pei et al. examined biopsy tissues from the normal esophagus of four adults by using wide-range 16S rDNA PCR and showed that members of six phyla: *Thick-walled Bacteria, Anaplastic Bacteria, Actinobacteria, Aspergillus, Clostridium*, and *TM7* were all represented, with *Streptococcus* spp. *(39%), Prevotella* spp. *(17%)* and *Veronella* spp. *(14%)* were the most prevalent (11). In 2012, Fillon et al. identified the esophageal microbiota in 15 individuals from children with normal esophageal mucosa, and investigated the bacterial composition by using 16S r RNA gene sequencing to identify 31 genera, of which *Streptococcus, Prevotella*, and *Veronella* spp. were the three most common (16). Macrogenome sequencing of human populations has shown that the gastroesophageal (GE) microbiome is broadly controlled by six major phyla (ibid), and *Streptococcacae, Veilonellacae*, and *Prevotellacae* have been described by other authors as the dominant bacterial phyla in the healthy esophagus by 16Sr RNA sequencing technology (12, 16, 17).

2.2 Esophageal microbiome in reflux

The microbiome changed with the spatial structure of the esophagus. The relative abundance of *Streptococcus* spp. increased proximally to the middle of the esophagus and then decreased significantly in the distal esophagus, with gram-negative(G-) microorganisms concentrated in the distal esophagus (18).

2.2.1 Changes in esophageal microbiome by age, medicine, and diet which potentially affecting GERD

Esophageal microbiome changes with age. A study also showed that the composition of the microbiome was more stable with age, with more gram-positive (G+) bacteria and fewer G- bacteria, regardless of disease state (19). The change in microbiome toward a G+ microbiome may have a supportive effect on esophageal function.

Esophageal microbiome changes with medication. A study recruiting healthy subjects partially treated with Proton Pump Inhibitors (PPIs) showed that short-term PPI treatment increased the microbial abundance of *Streptococcaceae, Leuconostacaceae*, and *Pasteurellaceae* at the family level and at the corresponding genus level. PPIs may enhance the colonization of some probiotic species such as *Streptococcus thermophilus* and other species present in the multi-strain probiotic (20). Another study in patients with non-erosive reflux disease (NERD), esophagitis (RE) and Barrett's esophagus (BE) found that PPIs use was associated with a reduction in Bacteroidetes in NERD and RE (19). In another study, esophageal biopsies performed before and after 8 weeks of PPIs treatment showed a significant decrease in G- *Clostridium* spp. species and an increase in G+ Clostridia (*Clostridiaceae* and *Lacertidae* species) and *Actinobacteriaceae* (*Micrococcaceae* and *Actinobacteriaceae* species)

(18). This implies that acid suppression by PPIs alters the survival environment of the microbiome in favor of G+ bacteria that prefer high pH environments, thereby altering the esophageal microbiota. Therefore, PPIs may have a positive impact on the treatment of GERD in terms of directly reducing chemical damage and improving the esophageal microenvironment to reduce the inflammatory response.

Potassium-Competitive Acid Blocker (P-CAB): have an effect on oral microbiome. In a randomized trial, patients with laryngopharyngeal reflux disease (LPRD) were enrolled were given oral vonoprazan and saliva specimens were collected before and after treatment, and *Neisseria, Burkholderia*, and *Leptospira* were found to be more prevalent in the LPRD group than in the post-LRPD group. In contrast, the LPRD group had a lower abundance of Fulminant *Prevotella* and unidentified negative bacteria compared to the post-LPRD group (21).

Esophageal microbiome changes with diet. In a study where subjects underwent esophageal sampling along with a validated food frequency questionnaire to quantify dietary fiber and fat intake, findings showed that increased fiber intake was significantly associated with increased relative abundance of thick-walled phyla and decreased relative abundance of overall G- bacteria, including *Prevotella, Neisseria*, and *Eikenella* (22). Therefore, in the treatment of GERD while requiring dietary modifications makes sense for the treatment of GERD.

2.2.2 Altered esophageal microbiome is strongly associated with GERD

In 2009, Yang et al. proposed that esophageal microbiome can be divided into two categories: type I microbiome for normal people is dominated by G+ taxonomic units, with Streptococcus spp. as the main bacterial taxa; type II microbiome for patients with GERD and Barrett's esophagus is dominated by G- taxonomic units, including *Weyoungerella* spp., *Prevotella, Haemophilus, Campylobacter, Clostridium*, and *Actinomyces, etc.*, and his study found high exposure to type II microbiota in GERD (23). Similarly, Park found that esophageal microbiome of NERD patients was most commonly dominated by type II microbiome at the phylum level in the phyla *Thick-walled Bacteria, Aspergillus* and *Mycobacterium* (24). *Fusobacterium, Neisseria* and *Veilonella* were commonly detected in patients with RE and BE. Blackett et al. found increased abundance of *Campylobacter* in GERD patients (25).

The ratio of G+ to G- changes in the GERD esophagus. Liu et al. By comparing the esophageal microbiome of patients in 3 groups: normal esophagus, RE, and Barrett, they found that Streptococcus spp. had a slightly higher proportion in the normal group than in the RE or BE groups, and that the esophageal microbiome in the RE/BE state was highlighted by an increased proportion of G- bacteria (26). In the Zhou trial, the composition of the microbiome of NERD patients was characterized by higher levels of Proteobacteria and Bacteroidetes, and reduced levels of the microorganisms Clostridium and Actinobacteria (27).

Overall, the dysbiosis in GERD patients is characterized by an increase in G- bacteria.

3 Intestinal microbiome

Intestinal microbiome are normal microorganisms in the gut that synthesize a variety of vitamins essential for human growth and development, participate in glucose metabolism and protein metabolism, maintain normal intestinal physiological functions, and antagonize the colonization of pathogenic microorganisms (28). The intestinal microbiome affects the systemic metabolism by influencing intestinal nutrient absorption and catabolism, etc. (29), which in turn affects the systemic immune and inflammatory status, and has a significant impact on the progression of disease (30). Blackett et al. (25) found that the intestinal microbiome has 128 phylotypes, which belong to 8 phyla, of which the dominant microbiome accounts for 5 phyla, which are *Thick-walled phyla*, *Actinobacteria phylum*, *Anaplasmomycetes, Clostridium* and *Methanobacteria phylum*. Based on their association with the host, the intestinal microbiome are divided into commensal, conditionally pathogenic and pathogenic bacteria (31). Several experiments have shown that *bifidobacteria, lactobacilli*, and *Streptococcus pepticus* predominate in the intestinal microbiome of healthy subjects.

3.1 GERD affects intestinal microbiome

GERD patients have a decreased number of commensal bacteria and an increased number of conditionally pathogenic and pathogenic bacteria in their intestinal microbiome. A retrospectively analyzed study, by comparing the intestinal microbiome between patients with GERD and healthy subjects, found that the abundance of microorganisms such as *Desulfovibrioides*, *Halobacterium* species, and *Sphingobacterium* was higher in patients with GERD, and that microorganisms such as *Lactobacillus* intestinalis and *Streptococcus pepticus* were in higher abundance in a control group made up of healthy subjects (32). Another randomized trial, comparing LPRD patients with healthy subjects, found that there were significant differences in the structure of the intestinal microbiome between the two groups. Not only was the relative abundance of *Actinobacteria* phylum in the LPRD group significantly higher than that of the healthy control group, but also the genera of *Rhodobacteriaceae*, and *Collins'* spp. which belong to the same phylum of *Actinobacteria*, were enriched in the LPRD, and it was also found that *Streptococcus* spp., *Prevotella* species, and *Clostridium* spp. were enriched in the LPRD group (33). Using GERD patients as the study group and selecting healthy volunteers in the same period as the control group, it was found that the number of fecal *E. coli* and *Enterococcus* spp. in the test group was higher than that in the control group, and the number of *Lactobacillus* spp., *Bacteroidetes* spp. and *Bifidobacterium* spp. in the test group was lower than the control group (34). *Bifidobacteria* and *Lactobacillus* counts were significantly higher in non-GERD patients than in GERD patients, while *Staphylococcus* and *E. coli* counts were significantly lower than in GERD patients (35).

3.2 Changes in intestinal microbiome with GERD treatment

In patients with GERD, the relief of clinical symptoms is accompanied by changes in the intestinal microbiome, the number of commensal bacteria rises significantly after treatment. In a randomized controlled study, GERD patients were randomly grouped, the test group was given PPI+Moxabilis, and the control group was given PPI, and the within-group comparison revealed that the number of *Escherichia coli* and *Staphylococcus* spp. decreased, and the number of *bifidobacteria* and *Lactobacillus* spp. increased in the two groups, which also revealed that the clinical remission rate of the test group was higher than that of the control group, and the number of *bifidobacteria* and *Lactobacillus* spp. was higher than that of the control group, and the number of *Escherichia* and *Staphylococcus* counts were lower than the control group (36). Another randomized controlled study also divided GERD patients into 2 groups, the control group was treated with western medicine, and the treatment group was given western medicine + Chinese herbal medicine compound, and the results of the treatment showed that the symptomatic relief of the 2 groups was accompanied by an increase in the number of *Lactobacillus* and *Bifidobacterium* in the intestinal tract and a decrease in the number of *Staphylococcus* in the intestinal tract compared with that of the control group before the treatment (37). The phenomenon of improvement of symptoms and change of microbiome was also found in another trial, the results of this study showed that the clinical symptoms of patients in both groups improved significantly after treatment, and the numbers of *Enterobacteriaceae* and *Enterococcus* were lower than before treatment, and the numbers of *Lactobacillus* and *Bifidobacterium* microbiome were higher than before treatment (38).

4 Mechanisms of dysbiosis on GERD

Ecological dysbiosis is an abnormal state of the microbial ecosystem in the host (39). Dysbiosis may be one of the environmental factors contributing to the etiology of GERD (23) (Figure 1).

4.1 Activation of Toll-like receptors by dysbiotic microbiome affects esophageal barrier structure, epithelial repair, and triggers inflammatory responses involved in GERD

G- bacteria are key producers of lipopolysaccharide (LPS), which abnormally activates a variety of Toll-like receptors (TLRs) leading to epithelial barrier dysfunction and inflammatory responses leading to GERD.

TLRs are pattern recognition receptors expressed by immune cells and epithelial cells that assist the host in differentiating between pathogenic and commensal microorganisms by recognizing pathogen-associated molecular patterns (PAMPs), conserved structures specific to pathogenic and non-pathogenic microorganisms, or damage-associated molecular patterns (DAMPs) (40). Activation of TLRs exerts phagocytosis, inflammatory cytokine release, and complement activation (41, 42). When TLRs recognize PAMPs expressed by LPS, there is activation of downstream transcription factors that regulate cytokine gene expression of NF-κB, generating an inflammatory response leading to GERD.

TLR2 is widely activated in GERD patients, and activated TLR2 regulates the epithelial barrier, epithelial cell proliferation, and inflammatory responses of the body, which in turn are involved in the development of GERD. Compared with normal esophageal epithelium, TLR2 mRNA expression was increased in inflammatory cells and epithelial cells in biopsies from patients with GERD (43), and TLR2 also recognized a variety of PAMPs expressed by dysbiotic colonies (44, 45). In normal esophageal epithelium, moderately activated TLR2 up-regulated the tight junction complexes kelotanin-1 and occluding zona pellucida-1, which enhanced the function of the esophageal epithelial barrier (46). Normal G-stimulation of TLR2 is beneficial to the enhancement of esophageal barrier function, but activation of the NF-κB pathway through other pathways leads to esophageal barrier dysfunction when the microbiome is dysbiotic. Experiments have found that overexpression of the NF-κB subunit and NF-κB target genes in esophageal tissues of mice with GERD and down-regulation of the tight junction complexes, claudin1 and claudin4, resulted in esophageal barrier dysfunction in the mice. Esophageal barrier dysfunction (47). TLR2 agonists significantly increase the proliferation of epithelial cell lines through multiple protein kinase pathways, and such experimental results were further confirmed by experiments in TLR2-deficient mice, where epithelial value-added was reduced in TLR2-deficient mice (46), and this effect would potentially impact on esophageal mucosal injury and repair.

A prospective study analyzing esophageal microbiology in patients with GER found that patients with GER symptoms exhibited significantly higher TLR2 expression, reduced claudin-1 expression, and dilated intercellular spaces (DIS). *In vitro*, exposure of human esophageal epithelial cells to LES significantly upregulated TLR2 expression and downregulated claudin-1 and DIS expression. These effects can be mediated by blocking TLR2. Thus, enriched G- in patients with GER symptoms may induce esophageal barrier dysfunction via the LPS-TLR2-IL-6-claudin-1-DIS pathway (48).

TLR4 expression is increased in patients with GERD, triggering an inflammatory response involved in the further development of GERD.TLR4 expression in normal squamous epithelial samples is mainly confined to the basal layer of the squamous epithelium, which routinely may not come into contact with PAMPs expressed by the esophageal microbiota, and reflux increases TLR4 expression (49). TLR4 expression is increased (1.9-fold) in the squamous epithelium of patients with RE compared to normal esophageal squamous epithelium (50). Epithelial myofibroblasts from GERD patients were found to activate TLR4 upon acid and LPS stimulation, which in turn activates the downstream NF-κB inflammatory pathway and promotes the secretion of inflammatory factors IL-6 and IL-8 (51).

4.2 Dysbiotic microbiome stimulates cyclooxygenase-2 expression, affects gastric emptying, and induces GERD

Cyclooxygenase-2 (COX-2) is the rate-limiting enzyme that catalyzes the initiating step in the metabolism of arachidonic acid to prostaglandin H2 and is a precursor to prostaglandins such as prostaglandins, thromboxanes, and prostacyclins, which act as autocrine and paracrine lipid mediators in the maintenance of local homeostasis by mediating vascular function, wound healing, and inflammation (52). Dysbiotic colonies with their LPS-induced COX - 2 expression may mediate the development of GERD, a study treated rats with LPS as well as different prostaglandins and COX - 2 inhibitors and showed that the use of COX - 2 inhibitors blocked LPS-induced delayed gastric emptying, which is a possible risk factor for GERD (53, 54). Delayed gastric emptying dilates the stomach, making more food available for reflux into the esophagus. This also produces transient lower esophageal sphincter relaxation, which facilitates GERD (55).

4.3 Dysbiosis leads to overexpression of inducible nitric oxide synthase affecting LES function and inducing GERD

Inducible Nitric Oxide Synthase (iNOS) is an enzyme that produces nitric oxide (NO) by oxidizing L -arginine. Under the action of appropriate stimulating factors, almost any type of cell can be induced and thus express iNOS, and LPS is one of the inducing factors. Compared to normal esophagus, iNOS is overexpressed in BE (56, 57). iNOS-produced NO induces LES relaxation leading to the development of GERD, and in a mouse model of sepsis infected with lipopolysaccharides, the LES releases INOS causing impaired LES contraction, which can be blocked by utilizing NOS inhibitors (58). In addition, NO can damage pathogenic and host cells by affecting cellular energy production (59), forming inflammatory free radicals and causing DNA rupture (56, 60), leading to cell necrosis, dysfunction, and inflammatory responses that lead to GERD.

4.4 Activation of NLRP3 inflammatory vesicles by dysbiotic microbiome triggers inflammatory response and cell necrosis, inducing GERD

Inflammatory vesicles are expressed by epithelial and immune cells. LPS expressed by G- dysbiotic colonies both initiates and activates the NLRP3 inflammasome and leads to downstream production of IL-1 β and IL-18. The NLRP3 inflammasome triggers an immune response in due course, causing cellular pyroptosis (61). Inflammatory response, cellular pyropoiesis is a potential mechanism for the development of GERD.

4.5 Dysbiosis leads to abnormal immune function of the body and inflammatory response triggers GERD

NF-κB is an important transcriptional regulator associated with inflammation in cells and is usually inactivated, and colony dysbiosis can activate NF-KB, which in turn encodes many pro-inflammatory genes (62). In RE rats, activation of the NF-κB pathway was found to result in the release of large amounts of TNF-α and IL-6 (63). Activation of NF-κB pathway signaling initiates the transcriptional release of IL-6, TNF-α, etc., and promotes the polarization of M1 macrophages (64, 65). M1 macrophages also release a large amount of pro-inflammatory factors, such as TNF-α, IL-1β and other pro-inflammatory factors, which can cause peripheral and central sensitization, and then enhance the esophagus's susceptibility to a variety of reflux stimuli. sensitization of the esophagus to various reflux stimuli, and symptoms such as reflux and heartburn are manifested.

Inflammatory response induced by microbiome is a possible cause of GERD. Many studies have found that reflux does not directly damage the esophageal epithelium, but stimulates the secretion of inflammatory chemokines by the epithelial cells, which induces proliferative changes in the epithelium and recruitment of inflammatory cells such as T lymphocytes, which in turn leads to the damage of the esophageal epithelium. A study reported that histological abnormalities were first observed in the esophageal tissues of rats with reflux esophagitis, and found that the recruitment of inflammatory cells and the expression of inflammatory chemokines within the esophageal mucosal epithelium were significantly earlier than the macroscopically or microscopically visible esophageal epithelial cell injury (66). In *in vitro* studies, transient exposure of human esophageal squamous epithelial cells to acidic bile salt solution did not result in epithelial cell necrosis, but rather promoted cellular secretion of IL-8 and IL-1β, which induced recruitment of lymphocytes and neutrophils further leading to epithelial cell necrosis (66). In addition, it has been found that inflammation can induce GERD by decreasing the function of the upper laryngeal-esophageal sphincter and pharyngeal-esophageal sphincter receptors (67).

Bacterial colonies stimulate the production of pro-inflammatory cytokines through multiple effector pathways, including the formation of inflammatory vesicles and nuclear factor-κB (NF-κB), stress kinases, and interferon regulatory factor (IRF); genotoxins released by bacteria, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and hydrogen sulfide (H2S), may have a direct cytotoxic effect (68); and microbial metabolites, such as short chain fatty acids (SCFAs) or lipopolysaccharides (LPS), can modulate immune cells.

4.6 Bacterial microbiome affecting lower GI tract dynamics may indirectly trigger GERD

The microbiome may induce reflux by affecting the lower GI tract dynamics, which in turn affects emptying. Lower GI pressure plays an important role in the pathogenesis of GERD. It has been shown that lower GI pressure is significantly elevated in patients

with GERD compared to healthy subjects, which may be a major factor in the development of GERD (69, 70). It has also been suggested that delayed emptying of the lower GI tract in one-half of GERD patients leads to an increase in intragastric pressure, and that a decrease in barrier pressure (the difference between the lower sphincter pressure and the intragastric pressure) is one of the factors contributing to the pathogenesis of GERD (71). A study found a higher prevalence of small intestinal bacterial overgrowth (SIBO) in patients with RE (72) and SIOB is associated with intestinal motility failure (73, 74). One trial found that intestinal microbiome promotes circulation and distal spread of migrating motility complexes (MMCs) in the interdigestive phase, as well as intestinal transport during and after feeding (75, 76). There are also trials demonstrating the important role of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in promoting the migration of MMCs and facilitating small intestinal transit, lower GI motility (77).

4.7 The products of microbiome (SCFAs) indirectly protect the esophagus from multiple pathways and have potential for the treatment of GERD

Microorganisms in the colon are capable of fermenting indigestible food components and consuming prebiotics to produce short-chain fatty acids (SCFAs) (78, 79) The relationship between SCFAs and GERD is not yet clear, but known studies have found that SCFAs have some potential for treating GERD (Figure 2).

4.7.1 SCFAs regulate gastrointestinal motility

Bowel motility influences GERD, and SCFAs have been suggested in several studies to influence bowel motility. Fecal SCFAs concentrations are low in patients with constipated IBS (IBS-C) and high in patients with diarrheal IBS (IBS-D) (80). SCFAs promote the production of 5-Hydroxytryptamine affecting gastrointestinal motility, enterochromaffin (EC) cells sense SCFAs and produce 5-HT, which promotes gastrointestinal motility through activation of 5-HT4 expressed on enteric neuron receptors to promote gastrointestinal motility (81); 5-HT signaling system is very important in visceral hypersensitivity, and many scholars believe that 5-HT ergic neurons are the underlying structure for the regulation of visceral sensorimotor and autonomic functions (71, 82).A randomized trial found that serum levels of 5-HT were higher in GERD patients than in healthy subjects. SCFA cells sense SCFAs and produce 5-HT subjects (83). SCFAs may affect ileal motor function after colo-ileal reflux by causing long-duration contractions and discrete clusters of contractions (84), which can directly stimulate ileal and colonic smooth muscle contractility (85). SCFAs affect emptying by influencing gastrointestinal motility, which can have an impact on reflux symptoms in GERD.

4.7.2 Increase in SCFAs enhances barrier function and mucus secretion of epithelial cells

The three defense barriers of the esophageal mucosa (86), among them are the pre-epithelial barrier consisting of the mucus layer and bicarbonate, etc., and the epithelial barrier and post-epithelial barrier of the second layer of the intercellular and apical linking complexes constituting the tight junction (TJ).

SCFAs induce redistribution of the tight junction protein occludin and the closed-loop mini-loop protein ZO - 1 in the cell membrane (87–89). In monolayer differentiated intestinal epithelial cells (cdx2-ECE), butyrate enhances intestinal barrier function by increasing the expression of the TJ protein claudin- 1 (90). An experiment in an *in vitro* model of a porcine intestinal epithelial cell line (IPEC-J2) found that butyrate attenuated the negative effects of LPS on epithelial integrity while selectively up-regulating TJ protein expression (91). Another animal experiment caused changes in the colony structure of mice by altering their feeding regimen, resulting in increased production of SCFAs, and found that multiple aspects of mucus and epithelial barrier integrity were enhanced (92). SCFAs also stimulated the expression of the MUC2 gene, which resulted in an increase in mucus volume (93, 94).

There is no clear evidence that SCFAs can affect esophageal barrier function, but production of SCFAs in the colon is completely and rapidly absorbed by colonic cells and circulates systemically through the portal vein (95).

The modulation of esophageal defenses after the absorption of SCFAs into the bloodstream needs to be further studied and explored.

4.7.3 SCFAs may alleviate GERD symptoms by supporting immune homeostasis and reducing inflammation

SCFA, especially butyrate, can alter the secretion of pro-inflammatory mediators (e.g., interferon (IFN) - γ (IL-1, IL-2, IL-6, IL-8, tumor necrosis factor (TNF) - α) by a possible mechanism through inhibition of NF-κB in intestinal epithelial cells (96, 97). Activation of G protein-coupled receptors (GPRs) on immune cell membranes by butyric acid leads to an increase in cytoplasmic calcium levels, and an increase in calcium concentration leads to the activation of NLRP3 inflammatory vesicles and subsequent activation of caspase - 1. Activated caspase - 1 converts pro - IL - 18 to IL - 18 which promotes epithelial repair (98), but in there are also data to support that IL-18 contributes to intestinal inflammation (98). SCFAs also inhibit the release of pro-inflammatory cytokines from intestinal epithelial cells induced by TLR activators such as LPS (99). SCFAs alleviate the symptoms of GERD by reducing the inflammatory response through their role in supporting immune homeostasis (100, 101).

4.8 Bacteriocins and polysaccharides protect the esophagus from multiple perspectives

Bacteriocins are ribosome-derived peptides produced by microorganisms that reside in the gastrointestinal tract and are thought to inhibit competitive microbiome. Bacteriocins in humans

are thought to maintain barrier function, participate in immunomodulation, and have direct anti-microbial activity (102). Recent studies have reported that the size and nature of bacteriocins allow them to cross this intestinal blood barrier (103). Size and charge help bacteriocins to cross cell membranes and barriers and play important roles in different physiological mechanisms. Bacteriocins in the gastrointestinal tract also have specific and potent antimicrobial properties (104, 105). This antimicrobial property makes it essential in maintaining and influencing the composition of the local microbiome. It has been found in Anabaena fragilis and has been associated with the synthesis of metabolites with immunomodulatory properties. Polysaccharide A (PSA) produced by this bacterium has been shown to modulate the immune response by promoting the production of anti-inflammatory cytokines (106).

Polysaccharides (PSs) have a wide range of pharmacological activities, including modulation of immune function (107), antioxidant effects (108), anti-inflammatory properties (109), and gastrointestinal health benefits (110). PSs modulate the intestinal microbiota and have anti-inflammatory and antioxidant potential (111, 112). PSs have a positive effect on Gastroesophageal reflux (GR), and dibasic sodium alginate has emerged as a promising therapeutic option, because in addition to protecting the esophageal mucosa and limiting gastric reflux into the esophagus, they also adhere to the gastric mucosa, protecting it and promoting its repair (113). PSs regulate gastric juice secretion, increase mucus production, enhance antioxidant capacity, reduce inflammation (114, 115), and act as probiotics in the gut microbiota and are involved in protein regulation, which ensures the maintenance of barrier function and mucin production.

5 Discussion

Normal microbiome and the human body to live in harmony, through the microbiome of digestion, decomposition, promote the absorption of the role of the host better, the host can also provide a good environment for its habitat. Bacterial microbiome is an intelligent living organism, its existence for the organism in the end is symbiotic beneficial bacteria or harmful bacteria with toxicity, such a delineation of the boundaries should take into account not only the characteristics of the bacteria themselves, the composition of the microbial ratio, but also should take into account the host's own functional conditions, assuming that the purpose of our medicine is to guide the adjustment of the bacterial microbiome, the use of the bacterial microbiome, so as to let it give play to its own complex, multi-faceted and subtle functions, through the treatment of disease in this way may be more comprehensive. treating the disease in this way may be more comprehensive. Understanding the microbiome as much as possible is only the first step, and the ultimate goal is to harmonize the symbiosis of good people and microorganisms.

The effects of microbiome and its metabolites on body functions are complex, and in many studies we have found that the improvement of symptoms before and after treatment is accompanied by a certain change in the microbiome structure, a phenomenon that leads us to think further about the question of whether a drug is effective by targeting a certain stage of the physiological/pathological response directly, or whether a drug is effective by affecting certain signals through changes in the internal environment affecting the structure of the microbiome, or both. Or both. Current basic research in pharmacology is mostly devoted to the effects of pathways activated by specific drugs, but the role played by drugs in the human microbiome is multifaceted. If the human microbiome can be transplanted in experimental animals, in addition to the study of the direct effect of drugs can play, but also to further observe the effect of drugs on the microbiome, the microbiome on the body.

Based on the existing research, we can clearly see the interaction between microbiome and GERD, and there is a close relationship between microbiome and the pathogenesis of GERD. However, due to the complexity of the composition of the microbiome and its effect on the organism, future research needs to gain a deeper understanding of the esophageal and intestinal microbiome and their metabolites in GERD through basic research to understand how the microbiome and their metabolites influence the occurrence of GERD, and also in the pathogenesis of GERD, abnormalities in digestive tract dynamics are gradually being emphasized by researchers. In addition, in the pathogenesis of GERD, the abnormalities of digestive tract dynamics have gradually been emphasized by researchers, and it has been further found that the brain-gut peptide (neurotransmitter) in the "brain-gut axis" is not only related to visceral hypersensitivity, but also plays an important role in regulating gastrointestinal dynamics, and changes in the bacterial microbiome and its structure can stimulate the secretion of brain-gut peptide, so the emphasis on the bacterial microbiome in the future research will also provide therapeutic opportunities for the treatment of GERD. likewise provides new therapeutic ideas for the treatment of GERD.

Author contributions

YG: Writing – original draft, Writing – review & editing. HC: Supervision, Writing – review & editing. NZ: Supervision, Writing – review & editing. YC: Investigation, Writing – review & editing. QZ: Investigation, Writing – review & editing. XJ: Investigation, Writing – review & editing. AW: Writing – original draft. HZ: Writing – original draft. BJ: Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Natural products for the treatment of ulcerative colitis: focus on the JAK/STAT pathway

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Ulcerative colitis (UC) is an autoimmune disease with an incompletely understood pathogenesis. The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway plays a key role in immune response and inflammation. More and more studies demonstrated that JAK/STAT signaling pathway is associated with the pathogenesis of UC. The JAK/STAT pathway affects UC in multiple ways by regulating intestinal inflammatory response, affecting intestinal mucosal barrier, modulating T cell homeostasis, and regulating macrophages. Encouragingly, natural products are promising candidates for the treatment of UC. Natural products have the advantage of being multi-targeted and rich in therapeutic modalities. This review summarized the research progress of JAK/STAT pathway-mediated UC. Furthermore, the latest studies on natural products targeting the JAK/STAT pathway for the treatment of UC were systematically summarized, including active ingredients such as arbutin, aloe polysaccharide, berberine, matrine, curcumin, Ginsenoside Rh2, and so on. The aim of this paper is to provide new ideas for drug development to regulate JAK/STAT signaling for treating UC.

KEYWORDS

Janus kinase (JAK), signal transducer and activator of transcription (STAT), ulcerative colitis, natural products, traditional herbal medicine

1 Introduction

Ulcerative colitis (UC) is a refractory digestive disease defined by recurring and remitting mucosal inflammation. Common clinical signs of UC include recurring stomach pain, diarrhea, and hematochezia. Typical medications used clinically as the primary treatment option for UC include aminosalicylates, corticosteroids, immunosuppressants, biological agents, and microecologics (1). Despite the large number of drugs available for the treatment of UC, its treatment remains complex and challenging due to a variety of side effects, medication tolerance, and high relapse rates (2). Therefore, further development of more effective treatments for UC has become urgent.

The Janus tyrosine protein kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway has been identified as a classical inflammatory pathway. The JAK/STAT signaling is involved in biological processes such as cell proliferation, differentiation, and apoptosis. The JAK/STAT pathway plays a key role in the immune response and has become a focus of research in autoimmune and inflammatory diseases (3). Notably, the JAK/STAT pathway is associated with damage induced by exaggerated an innate immune system response stimulated by immune checkpoint inhibitors (4). JAK/STAT signaling is frequently dysregulated in UC patients, indicating the importance of JAK/STAT regulation in UC (5, 6). Furthermore, in the colitis rat model, the severity of intestinal illness was positively associated to the expression of JAK2 and STAT3 (7). Theoretically, intervening in the JAK/STAT signaling pathway using safe and effective drugs may be an effective way to alleviate or treat UC. Currently, several JAK inhibitors have achieved efficacy in numerous clinical settings. The non-selective JAK inhibitor tofacitinib has been approved for moderate and severe UC (8). Encouragingly, natural products shows potential for the treatment of UC (9, 10). However, the existing studies are scattered and unsystematic. To our knowledge, this is the first thorough review that elaborates on recent advances of active ingredients in treating UC by modulating the JAK/STAT signaling pathway.

In this review, the current knowledge of the composition, activation, and regulation of the JAK/STAT pathway was discussed. Secondly, the role and mechanism of the JAK/STAT pathway in UC were particularly emphasized. Finally, we also systematically summarized the application of natural products targeting JAK/STAT signaling against UC. This review aims to provide new research ideas for traditional Chinese medicine (TCM) in the prevention and treatment of UC.

2 JAK/STAT pathway

2.1 Composition and activation of the JAK/STAT pathway

JAK is a non-receptor tyrosine protein kinase that is activated by numerous cytokines and initiates downstream target genes via STAT, which in turn regulates a variety of cellular functions (3).The JAK/STAT pathway consists of three main components, including tyrosine kinase-associated receptors, JAKs, and STATs. Four types of JAKs have been identified, including JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2). Among them, JAK3 is expressed only in bone marrow and lymphocytes, while other members are widely found in various tissues and organs in the body (11). The JAK proteins are made up of FERM (the complex of four point one, ezrin, radixin, and moesin), Src homology domain (SH2), pseudokinase, and kinase domains. STAT proteins are downstream signaling molecules of JAK. STATs consist of seven members, namely STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6, which are widely distributed in various tissues. STATs proteins mainly contain five structural domains, including N-terminal conserved sequences, DNA-binding region, Src homology domain 3 (SH3) structural region, SH2 structural region, and C-terminal transcriptional activation sequence. The SH2 structural area of STATs is identical to the analogous core sequence in JAKs, which is in charge of recognizing individual JAKs. Cytokines attach to cell-surface receptors, which dimerize and stimulate the polymerization and phosphorylation of JAKs. Activated JAKs can then bind to the SH2 structural domain of STATs, which are activated by phosphorylation modification and ultimately enter the nucleus as homodimers or heterodimers, thus promoting transcription of specific target genes (12). STAT is then dephosphorylated in the nucleus and returned to the cytoplasm (12). Among the STAT family, STAT3 has been recognized to play a central role in signaling from the plasma membrane to the nucleus (13). STAT3 is activated by phosphorylation of tyrosine (Y705) or serine (S727) residues in the transactivation domains, creating a STAT3 dimer that moves into the nucleus, where it promotes the transcription of target genes. Phosphorylation of STAT3 at the Y705 site occurs predominantly through members of the JAK family, whereas phosphorylation at the S727 site is usually carried out by mitogen-activated protein kinase, cell cycle protein-dependent kinase 5, and protein kinase C.

2.2 Negative regulation of the JAK/STAT pathway

The JAK/STAT pathway is primarily regulated negatively by three types of factors: suppressor of cytokine signaling (SOCS), protein inhibitor of activated STAT (PIAS), and protein tyrosine phosphatase (PTP) (14). (Figure 1). The SOCS family is the main signaling molecule that weakens the JAK/STAT pathway, including CIS, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, and SOCS7. Activated STAT entering the nucleus promotes the transcription of SOCS, which has a negative regulatory effect on JAK/STAT signaling by inhibiting STAT receptor binding, inactivating JAK through N-terminal kinase inhibition, or binding and ubiquitinating JAK or STAT for proteasomal destruction (15). PIAS can interact with STAT to prevent STAT dimerization or prevent STAT dimers from binding to DNA. PTP can dephosphorylate JAK by interacting with receptors as a phosphatase. It can also directly dephosphorylate STAT dimers to block JAK/STAT signaling transmission (16).

2.3 JAK/STAT pathway and melanocortin system

The melanocortin system is a complex signaling system composed of multiple hormones, neuropeptides, and receptors, which exerts a widespread regulatory role in the physiological and pathological processes in the body. α-, β- and γ-melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone are important components of the melanocortin system. Melanocortin receptors (MCR, MC1R-MC5R) are important members of the G protein-coupled receptor superfamily. The latest clinical research data indicate that the expression of MC3R

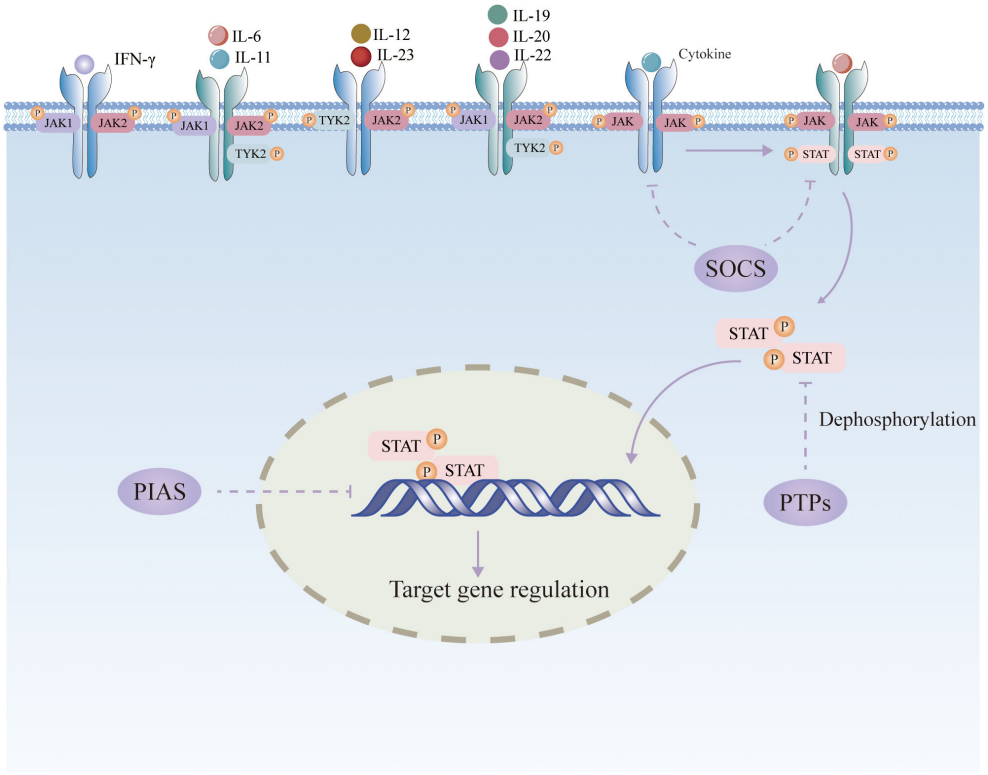

FIGURE 1
Schematic diagram of the JAK/STAT signaling pathway.

and MC5R is significantly increased in inflamed mucosa of inflammatory bowel disease (IBD) patients compared to normal mucosa (17). Importantly, the melanocortin system plays a key role in inflammation and immune regulation (18, 19). The melanocortin system is involved in the development of IBD through multiple pathways (20). Melanocortin peptides, especially α-MSH, have potent anti-inflammatory and immunomodulatory activities (21). It has been suggested that α-MSH may indirectly affect the activity of the JAK/STAT signaling pathway by regulating cytokine production (22, 23). Melanocortin attenuates myocardial ischemia/reperfusion injury by activating JAK/STAT signaling (24). Seemingly paradoxically, α-MSH was shown to activate the JAK2/STAT1 pathway by binding its MC5R receptor (25). The regulatory mechanism of melanocortin system on the JAK/STAT pathway remains to be further investigated in depth.

2.4 Cross-talk between the JAK/STAT pathway and other signaling networks

Diverse components of the JAK/STAT pathway, such as JAK, STAT, receptors, and gene transcription factors, are embedded in a dynamic cross-talk with other signaling networks. For example, the cross-talk between nuclear factor-kappa-B (NF-κB) and STAT3 has been observed in numerous inflammatory disorders and cancers. First, IL-6, a gene production regulated by NF-κB pathway, serves as a critical STAT3 activator (26). Second, STAT3-mediated acetylation of NF-κB p65 enhances its transcriptional activity in the nucleus and promotes the expression of pro-inflammatory factors such as IL-6 and TNF-α (27). Finally, STAT3 stimulates the expression of p52 and CD30, which induces sustained activation of non-canonical NF-κB signaling (28). Furthermore, dimerization of IL-6-type cytokine receptors not only activates the JAK/STAT signaling pathway, but also induces the mitogen-activated protein kinase (MAPK) cascade by recruiting SH2-domain-containing tyrosine phosphatase (SHP2) to tyrosine-phosphorylated gp130 and phosphorylating it in a JAK1-dependent manner. The phosphorylated SHP2 combines with the growth factor receptor-bound protein/Son of Sevenless (Grb2-SOS) complex, resulting in the activation of the Ras-Raf-MAPK pathway (29). Autophagy in epithelial cells is usually considered to exert a protective effect in UC (30). On the one hand, it was found that the activation of JAK2/STAT3 pathway directly suppressed the transcription of autophagy regulator Beclin-1, contributing to the inhibition of autophagy and the initiation of intestinal cell death (31). On the other hand, autophagy promotes IFN-γ-induced Jak2/STAT1 activation by inhibiting the expression of reactive oxygen species and SHP2 (32).

Other inflammatory pathways such as the NF-κB pathway also play important roles in inflammation, but the NF-κB pathway is widely involved in a variety of physiopathologic processes with relatively limited specificity. In contrast, the JAK/STAT pathway is more direct and critical in cytokine-mediated inflammatory signaling. Thus, targeting the JAK/STAT pathway enables more precise intervention in the inflammatory process of UC. Besides, the

JAK/STAT pathway involves diverse cytokines and immune cells. Compared to this, the regulatory scope of NF-κB is relatively limited. Natural products may target the JAK/STAT pathway to comprehensively regulate the inflammatory response through multi-target effects. Importantly, JAK inhibitors (e.g., tofacitinib) have shown significant efficacy in the treatment of UC, further validating the feasibility of the JAK/STAT pathway as a therapeutic target (33). In addition, some inflammatory pathways have complex regulatory mechanisms, making intervention difficult. For example, there are multiple upstream and downstream kinases in the MAPK pathway, with complex interactions between members. Comprehensive intervention may induce more adverse effects. The JAK/STAT pathway is comparatively clear and its intervention by natural products has been more intensively studied. Consequently, JAK/STAT a privileged target for natural products in UC.

3 Role of JAK/STAT pathway in the pathogenesis of UC

3.1 Influence on inflammatory response

The JAK2/STAT3 axis is a major pathway for transcription factors associated with mediating proinflammatory cytokine in intestinal mucosal inflammation. Inflammatory factors such as interferon-γ (IFN-γ) and interleukin (IL) have been found to promote the activation of JAK/STAT pathway, which in turn exerts immunomodulatory functions (34, 35). In recent years, increasing evidence suggested that aberrant activation of the JAK/STAT signaling pathway is related to the pathogenesis of UC. The expression of four JAK genes was upregulated in the intestinal mucosal epithelium of patients with active UC (36). Polymorphisms in JAK2 and STAT3 genes correlate with the severity of UC patients (37). Most cytokines mediate inflammatory responses by activating JAK/STAT pathway in UC (summarized in Figure 2). Previous studies have revealed that IL-6 is involved in the pathogenesis of UC (38). IL-6 binding to its receptor activates JAK1/2 and TYK2 and contributes to the phosphorylation and transcriptional activation of STAT3, which ultimately regulates T cell differentiation and inflammatory response (35, 39). Moreover, IL-12 and IL-23 activate STAT3 and STAT4 through JAK2 and TYK2, respectively (40, 41).

3.2 Impact on intestinal epithelial cells

Intestinal mucosal structures are maintained by a balance between apoptosis and proliferation of intestinal epithelial cells (IECs). However, IECs in UC patients exhibit a higher rate of apoptosis (42). Abnormal apoptosis leads to the loss of intestinal epithelial structures, disrupting the intestinal mucosal barrier and further activating excessive immune responses, eventually leading to uncontrollable inflammatory responses and mucosal damage. The JAK pathway is known to play an influential role in the regulation of cell proliferation and apoptosis (43). Studies showed

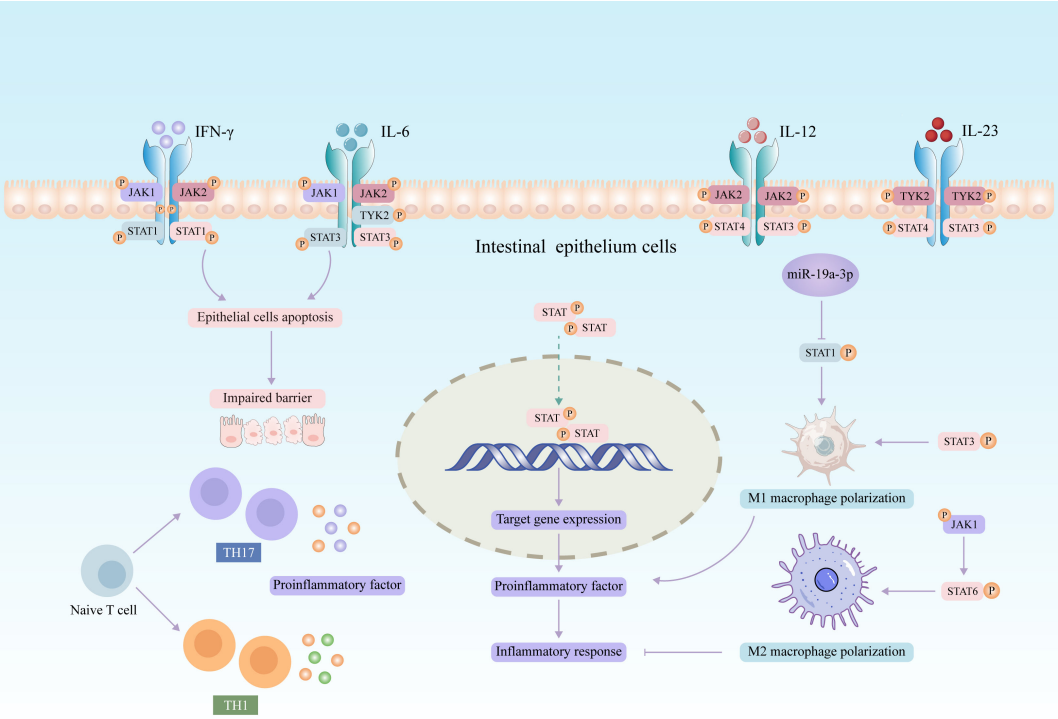

FIGURE 2
Potential role of JAK/STAT signaling pathway in the pathogenesis of UC.

that the activation of STAT1 or STAT3 promotes apoptosis in IECs (44, 45). miR-124-3p can directly target the STAT3 3'-UTR to modulate STAT3 expression (46). A recent study indicated that the overexpression of miR-124-3p attenuates apoptosis and reactive oxygen species production by targeting STAT3 in lipopolysaccharide (LPS)-induced colonocytes (47).

3.3 Modulation of intestinal macrophages

Under homeostatic conditions, circulating lymphocyte antigen 6 complex (Ly6C) monocytes in mice and CD14 monocytes in humans are constantly attracted to the intestinal tract and differentiate into mature F4/80 macrophages with high levels of CX3C chemokine receptor 1 ($CX3CR1^{hi}$). CX3CR1 macrophages directly activate neighboring T cells to amplify the inflammatory response (48). A recent study found that gut-resident $CX3CR1^{hi}$ macrophages trigger tertiary lymphoid structures and IgA response in situ (49). Furthermore, intestinal mucosal $CXCR4^{+}$ IgG plasma cells drive the activation of CD4 macrophage and exacerbate UC (50). Compared to the lamina propria of the normal mucosa, the number of macrophages is greatly increased and activated in the localized colonic tissues of individuals with active UC, indicating that intestinal macrophages are implicated in the occurrence and progression of UC (51). Particularly, different expression of Tim-4 and CD4 can divide intestinal macrophages into three subsets, including locally maintained macrophages ($Tim\text{-}4^{+}CD4^{+}$), circulating monocyte-renewing macrophages ($Tim\text{-}4^{-}CD4^{+}$), and macrophages with the high monocyte-replenishment rate ($Tim\text{-}4^{-}CD4^{-}$) (52). Furthermore, macrophages from colonic lamina propria cells can be divided into subpopulations based on the expression of F4/80 and CD11b. $F4/80^{hi}$ macrophages are considered to be intestinal resident macrophages, whereas $CD11b^{hi}$ macrophages are regarded as infiltrative macrophages supplemented by circulating monocytes (53, 54). Interestingly, JAK/STAT signaling has an important regulatory effect on macrophage (55–58). It was found that inhibition of the JAK2/STAT3 pathway resulted in a significant reduction in apoptosis, collagen deposition, and immunoreactivity of intestinal macrophages (59). Notably, the levels of IFN-γ are markedly elevated in the mucosa of IBD patients. This cytokine promotes the pro-inflammatory characteristics of $CD14^{hi}$ macrophages in humans (60). Consistently, the complete deletion of IFNγR1 or its downstream transcription factor STAT1 suppresses the formation of immature Ly6C MHCII macrophages (61).

Macrophages are highly plastic in different environments, exhibiting different phenotypes and functions depending on microenvironmental stimuli and signals (62). Macrophages are divided into classically activated M1-type macrophages with proinflammatory effects and alternatively activated M2-type macrophages with anti-inflammatory effects (63, 64), both of which are involved in UC pathology (65). Recently, a growing amount of studies indicated that STAT1 plays a critical role in the modulation of M1 macrophage polarization (66–68). It was reported that miR-19a-3p inhibited M1 macrophage polarization as an upstream regulator of STAT1 (69). Moreover, SOCS3-deficient macrophages showed increased STAT3 expression and M1 polarization (70). Interestingly, the JAK1/STAT6 pathway is an important pathway in the induction of M2 macrophage polarization (71). IL-4 binds to its receptor to activate JAK1, which recruits STAT6 phosphorylation and promotes the expression of M2 macrophage markers (72).

Notably, most of the current studies have been conducted mainly using *in vitro* bone marrow-derived macrophages (BMDMs). When cultured *in vitro*, BMDMs rely on specific cytokines to induce differentiation. However, the induction process is significantly different from the complex intestinal microenvironment *in vivo*. Moreover, BMDMs differ from intestinal macrophages in their degree of differentiation and maturation, leading to their distinct performance in the expression and activity of some key functional proteins. Apart from participating in immune defense, intestinal macrophages also play a crucial role in maintaining intestinal homeostasis and regulating intestinal microbiota balance. On the other hand, BMDMs lack gut-related signaling stimulation in the *in vitro* environment to fully exhibit these complex functions of intestinal macrophages.

3.4 Regulation of T cell balance

T cells are another important type of immune cells involved in adaptive immunity. Studies demonstrated that the JAK/STAT signaling pathway is critical in modulating T cell differentiation (35, 73, 74). A recent study showed a significant increase in the number of CD4 T cells in UC patients (75). Naive CD4 T cells are induced to differentiate into different types of T cells in different cytokine microenvironments, including T helper cell 1 (Th1), Th2, Th17, and regulatory T cells (Tregs). Abnormally activated CD4 T cells differentiate into subpopulations of Th1 and/or Th17 cells that subsequently infiltrate the colon to mediate autoimmune responses in UC (76). Cytokines such as Th1-induced IL-2 and IFN-γ, and Th17-induced IL-17 and IL-21 promote inflammatory responses and exacerbate colitis (77). In contrast, Tregs control effector T cell immunosuppression through intercellular contacts or secretion of anti-inflammatory cytokines. Th17/Treg balance facilitates the maintenance of intestinal immune homeostasis, an imbalance of which is the source of immune dysfunction in intestinal mucosa (78). Increasing data suggested that proinflammatory cytokines that stimulate the JAK/STAT signaling pathway govern the differentiation of naive Th1 and Th17 cell subsets and aggravate the development of UC (35, 73). STAT5 and forkhead box P3 (Foxp3) are key transcription factors for Tregs, whereas retinoic acid-related orphan receptor γt (RORγt) and STAT3 are key transcription factors for Th17 cells (79, 80). The overactivation of STAT3 promotes the Th17-like transformation of Treg and exacerbates immune responses (81). IL-12 or IFN-γ binds to their receptors to activate STAT1, STAT4, and the T-box transcription factor, driving the differentiation and function of Th1 cells. Similarly, IL-6 binds to its receptor and drives Th17 differentiation by activating RORγt and STAT3 (82, 83). Interestingly, TAK-242, a specific inhibitor of Toll-like receptor-4

(TLR4), was shown to alleviate UC by regulating macrophage polarization and Th homeostasis through the TLR4/JAK2/STAT3 signaling pathway (84).

4 Natural products involved in the regulation of JAK/STAT signaling in UC

4.1 Glycosides

Arbutin (molecular formula: $C_{12}H_{16}O_7$, molecular weight: 272.25) is a glycoside compound mainly extracted from the leaves of arbutus. The chemical structure of arbutin is shown in Figure 3. Arbutin is a hydroquinone glucoside, with two different configurations: α and β arbutin. Compared to α-arbutin, β-arbutin is more frequently found in nature and typically occurs in higher concentrations in plants. β-arbutin has been widely researched for its whitening, anti-inflammatory, antimicrobial, antioxidant, and anticancer properties (85). Arbutin has been reported to significantly down-regulate the levels of inflammatory cytokines (IL-1β, IL-6, and TNF-α), iNOS, and cyclooxygenase-2 (COX-2) in colitis mice (86). In addition, arbutin remarkably inhibited the phosphorylation of JAK2 and STAT3 and suppressed IECs apoptosis, thereby improving barrier function (Table 1). *In vivo* experiment demonstrated that p-JAK2 expression was significantly inhibited by arbutin and AG490, a JAK2 inhibitor (86). No additional therapeutic efficacy was observed with the combination of arbutin and AG490. *In vitro* experiment showed that the inhibitory effect of arbutin on p-STAT3 and inflammatory factors (TNF-α and IL-6) was significantly reversed by AG490, further suggesting that arbutin may be a potential JAK2 inhibitor. These results indicated that the effect of arbutin on JAK was primary rather than secondary to broader anti-inflammatory effects. Interestingly, a recent study found that arbutin also inhibited the formation of neutrophil extracellular traps and increased the diversity and abundance of gut microbiota (87).

4.2 Polysaccharides

Chrysanthemum morifolium Ramat (Juhua), as a medicinal and edible homeopathic plant with strong heat-removing and detoxifying abilities, has long been widely used for the treatment of various diseases, including influenza, colitis, stomatitis,

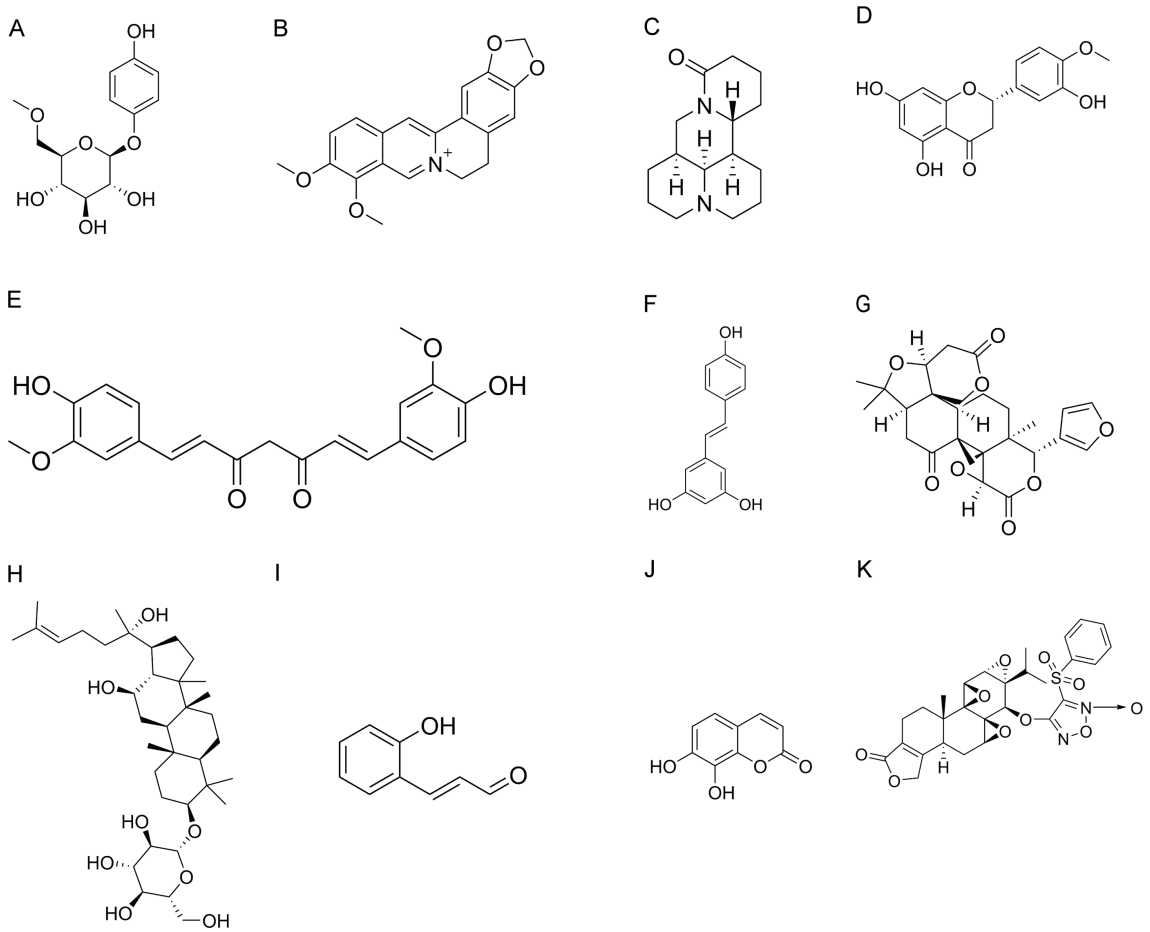

FIGURE 3
Chemical structures of natural products. **(A)** Arbutin. **(B)** Berberine. **(C)** Matrine. **(D)** Hesperetin. **(E)** Curcumin. **(F)** Resveratrol. **(G)** Limonin. **(H)** Ginsenoside Rh2. **(I)** 2′-Hydroxycinnamaldehyde. **(J)** Daphnetin. **(K)** ZT01.

TABLE 1 Summary of natural compounds involved in the regulation of JAK/STAT signaling in UC.

Phytochemicals	Type	Experimental model	Mechanisms	References
Arbutin	Glycoside	DSS-induced UC mice, LPS-stimulated IEC-6 and RAW264.7 cells	↓TNF-α, IL-1β, and IL-6, ↓iNOS and COX2, ↑Bcl2, ↓MLCK, ↓p-JAK2, p-STAT3, and SOCS3	(86)
Chrysanthemum polysaccharide	Polysaccharide	TNBS-induced colitis rats	↓TNF-α, IFN-γ, IL-6, and IL-1β, ↓MDA and MPO, ↑SOD, ↓p-p65, TLR4, p-STAT3, and p-JAK2	(94)
Tetrastigma hemsleyanum polysaccharide	Polysaccharide	DSS-induced UC mice, Caco-2 cells induced by LPS in combination with IL-6	↑Claudin-1, ↓IL-6, TNF-α, MCP-1, and IFN-γ, ↓IL-17A, ↑Foxp3, ↓p-JAK2, JAK2, p-STAT3, and STAT3, ↑SOCS1	(97)
Aloe polysaccharide	Polysaccharide	TNBS-induced colitis rats, HT-29 cells stimulated by TNF-α and LPS	↓IL-6, ↓p-JAK2, JAK2, p-STAT3, and STAT3	(7)
Berberine	Alkaloid	DSS-induced UC mice	↓NLRP3, ASC, and cleaved caspase-1, ↓IL-1β, IL-6, and IL-18, ↑ZO-1, E-cadherin, occludin, claudin-1, and MUC2, ↓OSM and OSMR, ↓p-JAK1, p-JAK2, p-STAT1, and p-STAT3	(112)
Matrine	Alkaloid	DSS-induced NCM460 cells	↓TNF-α, IL-1β, IL-2, and IL-6, ↓MPO and NO, ↓Bax and cleaved caspase-3, ↑Bcl-2, ↓p-JAK2/JAK2 and p-STAT3/STAT3	(116)
Hesperetin	Flavonoid	TNBS-induced colitis rats	↑GSH and SOD, ↓NO content, ↓IL-6, NF-κB, RAGE, and TNF-α, ↓p-JAK2 and p-STAT3, ↑SOCS3	(117)
Curcumin	Polyphenol	DSS-induced UC mice	↓IL-7, IL-15, and IL-21, ↓JAK1, STAT5, and p-STAT5, ↑PIAS1	(122)
			↓p-STAT3, ↓DNA-binding activity of STAT3 dimers, ↓MPO, IL-1β, and TNF-α	(123)
		TNBS-induced colitis mice	↓p-JAK2, p-STAT3, and p-STAT6, ↑SOCS1, SOCS3, and PIAS3, ↓Activation of dendritic cells	(119)
Resveratrol	Polyphenol	DSS-induced UC mice, HCT116 cells	↓IL-6, IL-1β, and TNF-α, ↑ IL-10, ↓O-GlcNAcylation, ↓p-JAK2 and p-STAT3, ↓NOS2 and COX2	(133)
Ginsenoside Rh2	Terpenoid	DSS-induced UC mice, IL-6-stimulated NCM460 cells	↓TNF-α, IL-6 and IL-1β, ↓p-STAT3, ↓miR-214, ↑PTEN	(138)
Limonin	Terpenoid	DSS-induced UC mice, IL-6-stimulated NCM460 cells	↓IL-6 and TNF-α, ↑IL-10, p-STAT3, ↓miR-214, ↑PTEN and PDLIM2	(140)
2′-Hydroxycinnamaldehyde	Other types	DSS-induced UC mice, LPS-treated FHC cells	↓TNF-α, IL-6 and IL-1β, ↑IL-10, ↑ZO-1, occludin, Bcl-2, E-cadherin, and Claudin-3, ↓Bax, ↓p-STAT3 and the translocation of STAT3 from cytoplasm to nucleus	(145)
Daphnetin	Other types	DSS-induced mice, LPS-challenged Caco-2 cells	↓TNF-α, IFN-γ, IL-6, and IL-1β, ↑ZO-1, occludin, and BCL-2, ↓Bax and cleaved caspase 3, ↓MDA and SOD, ↓JAK2 and STAT3	(152)
ZT01	Other types	DSS-induced UC mice, BMDMs stimulated by LPS and IFN-γ	↑ZO-1 and E-candherin, ↓p-JAK1, p-JAK2, p-STAT1, and p-STAT3, ↓the differentiation of Th1 and Th17 cell, ↓the proinflammatory macrophage phenotype polarization	(154)

The symbol "↓" indicates inhibition, while "↑" represents promotion.

cardiovascular diseases, and various chronic diseases (88). Polysaccharide is one of the key active components in *Chrysanthemum morifolium* Ramat. It exhibits anti-inflammatory, antioxidant, immunomodulatory, anti-cancer, hepatoprotective, and gastrointestinal function regulatory effects (89, 90). The monosaccharides of *Chrysanthemum* polysaccharides (CP) mainly include galactose, glucose, arabinose, and mannose (89). Several studies have reported that the biological activity of CP is related to their chemical propertiesm, molecular weights, and monosaccharide composition (91, 92). The high antioxidant activity of snow CP is partly attributed to the low molecular weight and high content of unmethylated galacturonic acid (93). In rats with 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, CP reduced the expression of proinflammatory cytokines and blocked the phosphorylation of STAT3 and JAK2, resulting in significant relief of colitis (94). Furthermore, CP influenced biomarkers and metabolic pathways in plasma and urine. Although this study found that CP decreased the expression of IL-6/JAK2/STAT3 pathway-related proteins and mRNAs, it was not clear whether the effect of CP on JAK/STAT was primary or secondary to broader anti-inflammatory actions. For a deeper understanding of its mechanism of action, further experiments need to be designed to distinguish the primary and secondary effects. For example, after treatment with specific pathway inhibitors or activators, the changes in the effects of CP on the JAK/STAT pathway and inflammatory response should be observed.

Tetrastigma hemsleyanum Diels et Gilg (Sanyeqing) is a traditional herb native to China. Its whole plant is medicinally used and it is of great concern for its remarkable medicinal value. In particular, it shows strong potential in anti-inflammatory, antibacterial, and antiviral properties (95). *Tetrastigma hemsleyanum* Diels et Gilg is rich in active substances such as flavonoids, phenolic acids and polysaccharides. *Tetrastigma hemsleyanum* polysaccharide (THP) consists of various monosaccharides, mainly including galactose, glucuronic acid, mannose, glucose, rhamnose, and arabinose (96). THP has shown great potential in the treatment of UC (97, 98). THP reduces the expression of the proinflammatory cytokines IL-6, TNF-α, and IL-17 and promotes the regulatory factors forkhead box protein P3 and Tregs (97). Importantly, it exerts anti-inflammatory effects by promoting SOCS1 expression and inhibiting JAK2/STAT3 signaling (97). Additionally, THP elevates levels of tight junction proteins in colonic tissues and decreases colonic permeability, improving the intestinal mucosal barrier. Interestingly, it can also modulate the gut microbiota structure and corresponding short-chain fatty acid metabolites in mice with IBD (98). Notably, although most studies used p-JAK2 and p-STAT3 as efficacy biomarkers, they did not distinguish whether natural products inhibited them directly or regulated them indirectly through upstream cytokines or microbiota-derived metabolites. The specific mechanism by which natural products regulate JAK/STAT signaling is currently not precise enough. Follow-up studies could construct STAT knockdown models by CRISPR/Cas9 technology to verify the specific action targets of active ingredients.

Moreover, relevant upstream regulators can be knocked down or overexpressed to further explore the regulation mechanism of STAT3 phosphorylation by THP.

Aloe polysaccharide is an active macromolecule extracted from Aloe vera. It shows excellent protection against ulcers and significantly prevents ulcer formation (99). *In vivo* and *in vitro* experiments have demonstrated that aloe polysaccharide effectively ameliorated apoptosis in colon tissue by inhibiting the JAK2/STAT3 signaling pathway (7). Meanwhile, aloe polysaccharide contributed to the reduction of IL-6 levels and restoration of colon length in TNBS-induced UC rats. Similarly, this study only used methods such as immunohistochemistry, Western blot, and RT-PCR to detect the expression level of the JAK/STAT pathway, with insufficient depth and specificity of the mechanism of action.

4.3 Alkaloids

Berberine (molecular formula: $C_{20}H_{18}NO_4^+$, molecular weight: 336.4), a natural pentacyclic isoquinoline alkaloid, is the most representative and abundant constituent of the TCM *Coptis chinensis* Franch (Huanglian). Berberine contains two benzene rings, an isoquinoline ring, and functional groups such as methoxy groups, which endow it with unique biological activities. In addition to its anti-inflammatory and antioxidant activities, berberine exhibits a variety of pharmacological effects, including anti-apoptotic, antitumor, hepatoprotective, and cardiovascular protective effects (100–102). Interestingly, it is widely known for its anti-inflammatory effects in inflammatory gastrointestinal diseases (103). In a double-blind phase I trial, berberine was demonstrated to improve colonic mucosal histologic scores in Chinese patients with UC (104). Furthermore, the Xijing Hospital of Digestive Diseases is currently undertaking a phase IV clinical trial to determine the impact of berberine on the annual recurrence rate of UC in remission (NCT02962245, ClinicalTrials.gov). Interestingly, a recent *ex vivo* study explored the synergistic effects of berberine, *Hericium erinaceus*, and quercetin, providing a more effective therapeutic option for UC patients. Their combination reduced the expression of proinflammatory cytokines and promoted the expression of the anti-inflammatory cytokine IL-10 in IBD tissues (105). Berberine has been discovered to relieve experimental colitis by altering the inflammatory response of immunological and epithelial cells, improving intestinal barrier function, and modulating intestinal microbiota (106–109). Oncostatin M (OSM) belongs to the IL-6 cytokine family and is primarily produced by activated macrophages, neutrophils, dendritic cells, and T cells (110). Previous studies have confirmed that recombinant OSM induces the activation of the JAK-STAT pathway via a heterodimeric receptor consisting of OSMR and gp130 (111). Importantly, berberine has been found to alleviate intestinal fibrosis by inhibiting the OSM-mediated JAK-STAT pathway and interfering with the interaction between intestinal stromal cells and immune cells (112). Furthermore, berberine was shown to inhibit M1 macrophage polarization and induce M2 macrophage polarization, by activating the IL-4-STAT6 signaling pathway, thereby exerting a therapeutic effect on UC (113).

Radix Sophorae Flavescentis is the dried root of *Sophora flavescens* Aiton (Kushen), belonging to the Leguminosae family. It is a promising traditional herb with the effect of clearing heat and dampness and has long been used to treat UC. Alkaloids and flavonoids are the main components of *Sophora flavescens* Aiton. Matrine is isolated from the roots of *Sophora flavescens* Aiton, *Sophora tonkinensis*, and *Sophora alopecuroides* (Kudouzi). Matrine is a tetracyclic quinolizidine alkaloid with the chemical formula $C_{15}H_{24}N_2O$ and a molecular weight of 248.36. Matrine exhibits a wide range of pharmacological activities, including analgesic, anticancer, anti-inflammatory, antiviral, antifibrotic, and immunomodulatory effects (114). Because of its anti-inflammatory and immunomodulatory properties, matrine has great potential in the treatment of UC (115). Apart from this, matrine improved the composition and function of intestinal microbiota in mice with dextran sulfate sodium (DSS)-induced colitis. It decreased the proportions of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, increasing the relative abundance of *Lactobacillus* and *Akkermansia* (115). A recent study confirmed that matrine inhibited proinflammatory factors, MPO activity, NO production, and apoptosis, thus effectively alleviating UC (116). Furthermore, matrine was found to suppress the phosphorylation levels of JAK2 and STAT3, but did not affect the phosphorylation of STAT5.

4.4 Flavonoids

Hesperetin (molecular formula: $C_{16}H_{14}O_6$, molecular weight: 302.28) is a naturally occurring flavonoid compound in citrus fruits and is widely found in various traditional herbal medicines such as grapefruit peel, orange peel, and tangerine peel. In TNBS-induced UC rats, hesperetin significantly enhanced glutathione levels and superoxide dismutase activity to reduce colonic oxidative stress, while significantly reducing NO levels (117). Hesperetin also mitigated the inflammatory injury by significantly decreasing IL-6 as well as inhibiting the expression of NF-κB, receptor for advanced glycation end products, and TNF-α. In addition, hesperetin significantly inhibited the phosphorylation of JAK2 and STAT3 and promoted the expression aof SOCS3, thereby alleviating colitis. As mentioned above, the present study did not clarify whether natural products directly inhibit JAK2/STAT3 phosphorylation through small-molecule binding or indirectly modulate this pathway through upstream cytokines. Natural products may affect JAK2 and STAT3 phosphorylation through different mechanisms in different studies. If all do not distinguish between direct and indirect regulation and only use them as biomarkers of efficacy, it will cause incomparability between the results of studies.

4.5 Polyphenols

Curcumin (molecular formula: $C_{21}H_{20}O_6$, molecular weight: 368.4) is an active polyphenol obtained from the dry rhizomes of herbs such as turmeric and tulip. It is also considered one of the potential drugs for the treatment of UC (118). Curcumin could alleviate UC by inhibiting dendritic cell-mediated expression of proinflammatory factors (119), modulating Th17/Treg homeostasis (120), and regulating M1/M2 macrophage polarization (121). Interestingly, the regulation of memory T cell homeostasis by curcumin is associated with the inhibition of JAK1/STAT5 signaling activity (122). In addition, curcumin not only suppressed STAT3 phosphorylation and STAT3 dimer binding to DNA, but also significantly inhibited the expression of proinflammatory cytokines, consequently ameliorating UC (123). Another study revealed that curcumin inhibited the phosphorylation of JAK2, STAT3, and STAT6 and upregulated the expression of downstream proteins (SOCS1, SOCS3, and PIAS3) in TNBS-induced UC rats (119). Moreover, curcumin inhibits dendritic cell activation and restores immune homeostasis by modulating the JAK/STAT/SOCS signaling pathway, effectively treating colitis (119). A randomized, double-blind, placebo-controlled trial demonstrated that the herbal combination of curcumin-QingDai significantly reduced the Disease Activity Index (DAI) score in patients with active UC and effectively induced their response and remission (CLINICALTRIALS: gov ID: NCT03720002).

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a polyphenolic stilbenoid isolated from Veratrum grandiflorum and abundantly found in grapes, mulberries, peanuts, rhubarb and several other plants. It is a well-known antioxidant (124). Due to its planar stilbene motif, resveratrol exhibits relatively high hydrophobicity. As a result, it demonstrates a comparatively strong affinity for hydrophobic pockets and binding sites within proteins. Furthermore, the polar hydroxyl (OH) groups serve as both hydrogen-bond donors and acceptors. These groups are capable of establishing numerous interactions with amino acid side chains and backbone amide groups (125). Clinical and preclinical studies have demonstrated that resveratrol exerts protective effects in numerous disease models, including digestive diseases, cardiovascular diseases, diabetes, tumors, and neurodegenerative diseases, which may be related to its multi-targeting properties (126–129). Notably, resveratrol has been demonstrated to restore the homogeneity and diversity of gut microbiota to some extent in colitis mice (130). Moreover, dietary resveratrol attenuated the inflammatory status and down-regulated the expression of proinflammatory cytokines such as IL-2, IFN-γ, IL-1β, IL-6, and TNF-α in colitis mouse model (131). Among the known resveratrol targets, JAK-STAT signaling has received widespread attention (132). It was shown that increased O-linked N-acetylglucosamine modification (O-GlcNAcylation) of STAT3 upregulated the expression of proinflammatory cytokines such as IL-6, IL-1β, and TNF-α, while downregulating the level of the anti-inflammatory cytokine IL-10 and aggravating colitis in mice (133). In addition, the levels of COX-2 and iNOS were elevated. Encouragingly, resveratrol inhibited the O-GlcNAcylation of STAT3, thereby inhibiting its phosphorylation as well as the activity of JAK2/STAT3 pathway, and consequently alleviating IBD (133). Moreover, resveratrol induced Tregs in mice with colitis, which was dependent on the downregulation of miR-31 (134). Meanwhile, it suppressed inflammatory T cells (Th1 and Th17). A randomized, double-blind, placebo-controlled study showed that supplementation with 500 mg resveratrol for 6 weeks improved the quality of life and

reduced colonic inflammation in UC patients (135). Unfortunately, resveratrol's low bioavailability and poor water solubility restrict its therapeutic use. The stability and oral bioavailability of resveratrol should be improved by future research using different delivery methods and changes (136).

4.6 Terpenoids

Ginseng, a traditional herbal medicine, is the dried root of *Panax ginseng* C. A. Meyer., a plant of the family Wujiaceae. As a valuable medicinal herb, it has been used in China for more than 2,000 years. Ginsenoside Rh2 (molecular formula: $C_{36}H_{62}O_8$, molecular weight: 622.9) is one of the active ingredients extracted from ginseng root. It possesses various pharmacological activities and has great potential in the treatment of UC (137). *In vivo* and *in vitro* experiments revealed that ginsenoside Rh2 effectively inhibited STAT3 phosphorylation and miR-214 expression (138). Ginsenoside Rh2 was found to indirectly suppress STAT3 phosphorylation by inhibiting the upstream cytokine IL-6.

Limonin (molecular formula: $C_{26}H_{30}O_8$, molecular weight: 470.5) is a triterpenoid derived from citrus and possesses favorable anti-inflammatory and antiapoptotic effects. Limonin reduced the generation of proinflammatory cytokines TNF-α, IL-1β, and IL-6 as well as the expression of inflammatory proteins COX-2 and iNOS in the colonic tissues of mice with DSS-induced colitis (139). Moreover, limonin was found to ameliorate DSS-induced chronic colitis in mice by inhibiting the endoplasmic reticulum-stressed PERK-ATF4-CHOP pathway and NF-κB signaling (139). In addition to this, limonin also improved the prognosis of UC by downregulating p-STAT3/miR-214 levels (140).

4.7 Other types

2'-Hydroxycinnamaldehyde (HCA) (molecular formula: $C_9H_8O_2$, molecular weight: 148.16) is an active component isolated from the stem bark of *Cinnamomum cassia* (Rougui) (141). HCA was proved to have anticancer, anti-inflammatory, antioxidant, and immunomodulatory effects (141–143). Interesting, HCA was screened as a natural STAT3 inhibitor (141, 144). A recent study showed that HCA directly binds to STAT3 and inhibits its activation (145). The hydroxyl group of HCA may interact with the protein-binding site of STAT3 via hydrogen bonding to enhance binding specificity. Thanks to this property, it inhibits inflammatory cytokine expression, reduces apoptosis of IECs, and attenuates intestinal mucosal barrier damage, thus effectively alleviating UC (145).

Daphnetin (molecular formula: $C_9H_6O_4$, molecular weight: 178.14), a coumarin derivative isolated from the Daphne plant, is a natural compound with multiple therapeutic potential (146–148). Daphnetin possesses oxygen-containing heterocycles with a characteristic benzo-α-pyrone framework (149). The catechol moiety served as the crucial pharmacophore for the antioxidant activity of daphnetin (149). Apart from its antioxidant activity, daphnetin also exhibits diverse therapeutic potentials, including anti-inflammatory, analgesic, antibacterial, neuroprotective, hepatoprotective, nephroprotective, and anticancer activities (150). A previous study has demonstrated that daphnetin ameliorates colitis by regulating microbiota composition and TH17/Treg balance (151). A recent study reported that Daphnetin attenuated intestinal inflammation, oxidative stress, and apoptosis in UC, which was associated with the inhibition of REG3A-dependent JAK2/STAT3 signaling (152).

Triptolide, a natural diterpenetriepoxide which is isolated from *Tripterygium wilfordii* Hook F (Leigongteng), has prominent anti-inflammatory and immunosuppressive properties. ZT01 is a newly obtained tretinoin derivative with strong anti-inflammatory effects and low toxicity (153). ZT01 may be an attractive candidate for future development as an anti-UC drug. Importantly, ZT01 significantly inhibits T cell differentiation into Th1 or Th17 cell subsets and prevents macrophage polarization to an inflammatory phenotype by modulating the JAK/STAT signaling pathway (154).

5 Conclusion and perspective

The increasing incidence of UC has placed a heavy burden on the global health system. The JAK/STAT signaling pathway mediates the pathogenesis of UC to some extent. There are limitations to some of the current studies on the JAK/STAT pathway and UC. The sample sizes of the studies detecting the activation level of the JAK/STAT pathway in colon biopsies from UC patients are small. Although it is possible to obtain information on a specific patient group to some extent, it is difficult to fully reflect the real situation of the entire UC patient population and is prone to bias. Moreover, single-center studies may be affected by factors such as geography and medical level, which makes the generalizability of the findings questionable. Future studies need to expand the sample size, use multicenter studies, and deeply investigate the reasons for the differences in JAK/STAT pathway activation. In terms of findings, higher levels of JAK and STAT expression were detected in the inflamed colonic mucosa of UC patients compared to the uninflamed mucosa. However, the current study did not further investigate the reasons for this difference, whether it is genetic differences in individuals, living environment or other factors. The lack of in-depth analysis would limit a comprehensive understanding of the pathogenesis of UC.

In recent years, natural products have received extensive attention from the medical community. They have the advantages of multiple pathways and multiple targets. It has been demonstrated that herbal active ingredients alleviate UC through various pathways, such as targeting the JAK/STAT pathway to reduce intestinal inflammation, improving the function of IECs, regulating Th17/Treg balance, and modulating macrophage status. This review systematically summarized the recent advances in natural products targeting the JAK/STAT pathway to treat UC, including polysaccharides, alkaloids, polyphenols, terpenoids, flavonoids, glycosides, and other types of compounds. Natural products are potential candidates to treat UC by targeting the JAK/STAT pathway.

Nevertheless, there are many challenges. Firstly, the current studies on the targeting of the JAK/STAT pathway by natural products for the treatment of UC mainly focus on the animal and cellular experimental level, which cannot be fully equated with the immunohistopathology of UC patients. At present, there is insufficient research on the pharmacokinetic properties of many natural products, such as their absorption, distribution, metabolism, and excretion in the body. These uncertainties affect the design of standardized dosages and the formulation of dosing regimens. Furthermore, natural products such as resveratrol have poor water solubility and low bioavailability, making it difficult to make suitable dosage forms for clinical use. There is an urgent need to improve the stability, solubility, and bioavailability of natural products to overcome the transformation challenges. Besides, when herbal active ingredients are combined with other drugs, they may affect pharmacokinetics and pharmacodynamics through multiple pathways, but their specific mechanisms and links are difficult to be clearly defined. These limitations pose a great challenge to mechanism research and efficacy assessment in the translation process of TCM. Preclinical and clinical studies are needed to validate the safety and efficacy of herbal active ingredients for the treatment of UC. Secondly, crosstalk exists between the JAK/STAT pathway and other signaling pathways, which means that targeting only one of JAK or STAT may not be sufficient for significant therapeutic effects. Exactly how natural products interfere with the JAK/STAT pathway and whether they interact with other signaling has not been fully elucidated. Therefore, subsequent scholars still need to conduct profound research on the mechanism of herbal active ingredients in the treatment of UC. Finally, the vast majority of studies have been limited to the effect of natural products on the JAK/STAT pathway and have not analyzed the in-depth laws between their chemical structures and pharmacological activities. The structural features of phytochemicals may influence their specificity for JAK/STAT through factors such as molecular size, shape, functional groups, charge distribution, and conformational flexibility. Although the intrinsic laws have not been fully revealed, structure-activity relationship studies and molecular docking will provide important clues for understanding these interactions.

Author contributions

DL: Conceptualization, Investigation, Supervision, Visualization, Writing – original draft. CM: Conceptualization, Formal analysis, Investigation, Writing – original draft. WZ: Conceptualization, Investigation, Writing – original draft. YZ: Funding acquisition, Supervision, Writing – review & editing. YX: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Baicalin mitigates hyperglycemia-linked intestinal epithelial barrier impairment in part by inhibiting the formation of neutrophil extracellular traps

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Background: Under hyperglycemic conditions, impaired intestinal barrier integrity leads to heightened level of inflammation, playing important roles in driving diabetic complications. Emerging evidence supports the implications of neutrophil extracellular traps (NETs) in the pathogenesis of diabetes. However, whether NETs contribute to hyperglycemia-linked intestinal barrier impairment remains to be investigated. Moreover, baicalin, the major chemical component of Scutellaria baicalensis Georgi, is equipped with twofold intestinal protective and neutrophil suppressive activities. Yet, it is unclear if baicalin is effective at mitigating hyperglycemia-linked NETs-mediated intestinal barrier impairment.

Methods: To directly address the mechanistic implications of NETs in hyperglycemia-linked intestinal epithelial barrier impairment, the impact of DNase I treatment or *Padi4* gene deficiency on intestinal epithelial integrity was first examined in the streptozotocin (STZ)-induced hyperglycemic mice *in vivo*. Next, the pharmacological impact of baicalin on NETs formation and intestinal epithelial barrier impairment was investigated in high glucose- and/or lipopolysaccharides (LPS)-stimulated neutrophils *in vitro* and in STZ-induced hyperglycemic mice *in vivo*, respectively.

Results: The *in vitro* experiments confirmed that high glucose and/or LPS induced NETs formation. NETs directly impaired the viability and tight junction of the intestinal epithelial cells. The histological and immunohistochemical examinations unveiled that along with impaired intestinal epithelial morphology, citrullinated histone H3 (H3Cit), a marker of NETs, and neutrophil specific Ly6G were readily detected in the intestinal epithelium in the hyperglycemic mice. Without affecting the presence of neutrophils, DNase I treatment or *Padi4* gene deficiency markedly mitigated intestinal NETs formation and improved the intestinal morphology in the hyperglycemic mice. Notably, baicalin suppressed NETs formation and inhibited histone H3 citrullination stimulated by high glucose, LPS or both *in vitro*. Furthermore, baicalin blunted

NETs formation and partially preserved the integrity of the intestinal epithelium in the hyperglycemic mice *in vivo*.

Conclusions: The current study sheds new light on the pathophysiological implications of NETs in intestinal epithelial barrier impairment under hyperglycemic conditions. Most importantly, the findings here demonstrate for the first time that baicalin directly inhibits NETs formation stimulated by high glucose and/or LPS, which may in part account for its pharmacological effects at protecting against hyperglycemia-linked intestinal epithelial barrier impairment.

KEYWORDS

hyperglycemia, intestinal epithelial barrier, neutrophils, neutrophil extracellular traps, baicalin

1 Introduction

Chronic hyperglycemia leads to the development of complications in multiple organs, accounting for diabetes-associated morbidities and mortalities. Systemic inflammation runs through the course of diabetes and promotes the development of diabetic complications (1). Hyperglycemia-associated systemic inflammation is in part caused by intestinal barrier impairment, which directly leads to aberrant translocation of the intestinal microbial contents into the bloodstream, triggering innate immune responses that augment the level of inflammation (2, 3). Understanding the mechanisms underlying hyperglycemia-linked intestinal barrier impairment may help to develop treatments to lower systemic inflammation under hyperglycemic conditions.

Neutrophils, the first-line defense of the innate immune system, contribute substantially to inflammation and host defense. Once activated, neutrophils execute the function of entrapping and killing exogenous pathogens by releasing neutrophil extracellular traps (NETs) consisting of nucleic acids, histones and granular proteins (4, 5). In addition to the canonical roles in host defense, the pathophysiological significance of NETs in the pathogenesis of systemic disorders such as diabetes has been gaining increased attention. Elevated circulating levels of NETs have been reported in the diabetic patients, correlating positively with inflammation as well as renal and cardiovascular complications in the patients. Moreover, high glucose directly induces NETs formation and the neutrophils from the diabetic patients and hyperglycemic mice are primed to produce NETs. Available evidence has also demonstrated that NETs play an indispensable role in promoting wound healing impairment and retinopathy under hyperglycemic conditions (6–10). It is also worth noting that altered intestinal homeostasis initiates transmigration of circulating neutrophils into the intestinal mucosa, leading to increased neutrophil infiltration and NETs formation in the intestinal epithelium. NETs can cause direct damage to the intestinal epithelial cells, impairing the integrity of the intestinal epithelial barrier (11). However, whether NETs formation is implicated in hyperglycemia-linked intestinal epithelial barrier impairment remains unknown.

Baicalin is a glycosidic flavone abundantly present in Scutellaria baicalensis Georgi, a herb that has long been used for the treatment of inflammation-related diseases and intestinal disorders in traditional Chinese medicine (12–15). Baicalin alleviates the intestinal barrier impairment under conditions such as hypertension and inflammation (16, 17). Moreover, baicalin suppresses neutrophil activation induced by phorbol-12-myristate-13-acetate (PMA) or N-formyl-methionyl-leucyl-phenylalanine (fMLP) (18). However, the pharmacological implications of baicalin in protecting against hyperglycemia-associated intestinal epithelial barrier impairment are unclear. Moreover, the impact of baicalin on high glucose-induced NETs formation and potential hyperglycemia-associated intestinal NETs formation remains to be addressed.

Therefore, based on addressing the implications of NETs in hyperglycemia-linked intestinal epithelial barrier impairment, the current study investigated the pharmacological potential and mechanisms of baicalin in attenuating hyperglycemia-associated NETs formation and intestinal epithelial barrier impairment.

2 Materials and methods

2.1 Neutrophil isolation and NETs formation assay

Neutrophils were isolated using a Neutrophil Isolation Kit (P8550, Solarbio, China) and maintained at 37°C in a humidified incubator with 5% CO_2. Briefly, after removing the erythrocytes from the bone marrow cells collected from the femurs and tibias, the neutrophil isolation reagent was applied, followed by centrifugation at 900 g for 30 min. The isolated neutrophils were seeded at 2×10^5 cells per well in 48-well plates in low-glucose (1 g/L glucose) or high-glucose (4.5 g/L glucose) DMEM medium (11965,

Gibco, USA) for 2.5 h in the presence or absence of 10 μg/mL LPS (L2630, Sigma-Aldrich, USA). For the experiments involving baicalin treatment, baicalin was added to the high-glucose-stimulated neutrophils at 25, 50 or 100 μM or the neutrophils stimulated by LPS in both low-glucose and high-glucose culture conditions at 100 μM, followed by incubation at 37°C for 2.5 h in a humidified incubator with 5% CO_2. To assess NETs formation, the neutrophils were fixed in 4% paraformaldehyde at room temperature for 10 min, permeabilized using 0.1% Triton X-100 in PBS at room temperature for 10 min and blocked with 10% donkey serum at room temperature for 1 h. The cells were then stained with rabbit anti-histone H3 (citrulline R2 + R8 + R17) antibody (1:500, ab5103, Abcam, USA) at room temperature for 2 h and Alexa Fluor 488 donkey anti-rabbit IgG-H&L secondary antibody (1:1000, ab150073, Abcam, USA) in the dark at room temperature for 1 h, respectively. Nuclei were counterstained using 4-6-diamidino-2-phenylindole (DAPI) (10236276001, Roche, Germany). Images were acquired using a fluorescence microscope (DMI6000, Leica, Germany) and the imaging settings were kept the same for all the images. Citrullinated histone 3 (H3Cit) positive area was quantified using Image J.

2.2 NETs isolation and quantification

The neutrophils were isolated as described above. Isolated neutrophils were seeded in 10-cm cell culture dishes at the number of 2×10^7 per dish and cultured at 37°C in a humidified incubator with 5% CO_2. NETosis was then induced by incubation with 500 nM PMA (P8139, Sigma-Aldrich, USA) for 4 h. Afterward, the culture medium was gently aspirated, leaving the layer of NETs and neutrophils adhered to the cell culture dishes. NETs and neutrophils were then dislodged and collected in cold PBS, followed by centrifugation at 450 g at 4°C for 10 min. The supernatants containing NETs were collected and the amount of DNA in the supernatants was measured using a Nanodrop (ThermoFisher Scientific, USA) or Quant-iT PicoGreen dsDNA Assay Kit (P11496, ThermoFisher Scientific, USA). The isolated NETs were stored at -80°C prior to the indicated experiments.

2.3 Cell culture and treatments

The rat small intestinal epithelial cell line IEC-6 (CRL-1592, ATCC, USA) was maintained in DMEM supplemented with 10% fetal bovine serum (10099-141, Gibco, USA), 100 unit/ml streptomycin (15140122, Gibco, USA) and 0.1 unit/mL bovine insulin at 37°C in a humidified incubator with 5% CO_2. To assess the tight junction, IEC-6 cells were seeded in 48-well plates at the density of 1×10^5 cells/well, followed by treatment of NETs at 25, 50, 100, 200 and 400 ng/mL for 12 h. The cells were then fixed in 4% paraformaldehyde at room temperature for 10 min, permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min and blocked with 10% sheep serum at room temperature for 1 h. Afterwards, the cells were sequentially stained with the rabbit anti-ZO1 tight junction protein monoclonal antibody (1:500, ab221547, Abcam, USA) at room temperature for 2 h and sheep anti-rabbit IgG antibody, Cy3 conjugate (1:1000, AP510C, Sigma-Aldrich) at room temperature for 1 h. Nuclei were counterstained by DAPI. Images were acquired using a fluorescent microscope with the same imaging settings (DMI6000, Leica, Germany). Five or six non-overlapping images captured from each well were subjected to the analysis of the fluorescence intensity of ZO-1 using the Analyze Particles function in ImageJ. Moreover, tight junction organization rate (TiJOR) was assessed following the previously described method (19). For cell viability assessment using thiazolyl blue tetrazolium bromide (MTT) (M8180, Solarbio, China), IEC-6 cells were seeded in 96-well plates at the density of 1×10^4 cells/well and treated by NETs at 25, 50, 100, 200 and 400 ng/mL for 12 h. After the indicated treatments, the cells were incubated in the presence of MTT reagent (50 μg/well) at 37°C for 4 h, followed by the measurement of the absorbance at 490 nm using a plate reader (Epoch, BioTek, USA).

2.4 Western blotting

The neutrophils were snap frozen and homogenized in RIPA buffer (P0013C, Beyotime, China) supplemented with protease inhibitor cocktails (04693116001, Roche, Germany). Electrophoresis was performed in 15% SDS-PAGE gels at room temperature, which was started at a constant voltage of 80 V. When the bromophenol blue indicator formed a straight line and the molecular weight markers were separated, the voltage was adjusted to 100 V and the electrophoresis was carried out at a constant voltage of 100 V for additional 2 h. Afterwards, the protein was transferred to polyvinylidene fluoride membrane (IPVH85R, Millipore, USA) in an ice pack for 90 min under a constant current of 200 mA. The levels of H3Cit and histone H3 were examined using rabbit anti-histone H3 (citrulline R2 + R8 + R17) antibody (1:1000, ab5103, Abcam, USA) and rabbit anti-histone H3 polyclonal antibody (1:1000, ab1791, Abcam, USA) at room temperature for 2 h, respectively. Goat anti-rabbit IgG (H+L)-HRP conjugate secondary antibody (1:3000, W4011, Promega, USA) was applied at room temperature for 1 h following the primary antibody incubation. The blots were developed with enhanced chemiluminescence substrate (34096, ThermoFisher Scientific, USA) and the images were acquired using an UVP imaging system (BioSpectrum 410, USA). The densitometry of the protein bands was performed using ImageJ.

2.5 Animals and treatments

Male C57BL/6 mice at 6-8 weeks of age were purchased from Shanghai Laboratory Animal Co., Ltd., China. *Padi4*-/- mice in the C57BL/6 background (NM-KO-190334) were ordered from Shanghai Model Organisms, China. The animals were maintained

under controlled temperature (20 ± 2°C), humidity (35-55%) and lighting (12 h light/dark cycle) conditions with free access to food and water in the animal resources facility at Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine. Two-week acclimatization was allowed prior to further treatments. All animal procedures were approved by the Institutional Animal Care and Use Committee of Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine (YYLAC-2019-044). All animal experiments were in compliance with the ARRIVE guidelines and conducted following National Research Council's Guide for the Care and Use of Laboratory Animals. To induce hyperglycemia, streptozotocin (STZ) (S8050, Solarbio, China), freshly prepared in sodium citrate buffer (pH 4.4), was intraperitoneally injected at 50 mg/kg/day for 5 consecutive days. Normal controls received the vehicle injection in the same manner. One week after the last injection of STZ, fasting blood glucose was measured using a blood glucose meter (Contour TS, Bayer, Germany). Hyperglycemia was defined by significantly elevated fasting blood glucose compared to the normal controls without STZ administration. Baicalin (B20570, Yuanye Biotechnology Co., Ltd, Shanghai, China) was prepared in 0.5% sodium carboxymethyl cellulose solution (vehicle). Hyperglycemic mice received the 2-week daily treatment of either vehicle or baicalin at 240 mg/kg or 1200 mg/kg via oral gavage. Normal controls were given the vehicle solution in the same manner. Or DNase I (D8071, Solarbio, China), dissolved in saline, was administered to the hyperglycemic mice at the daily dose of 2.5 mg/kg or 10 mg/kg via oral gavage for 2 weeks. Body weight and fasting blood glucose were measured before and after the indicated treatments.

2.6 Histological examination

The ileal specimens were fixed in 4% paraformaldehyde at room temperature for 24 h and processed for paraffin embedding and sectioning. Ileal sections (4 μm thick) were subjected to hematoxylin & eosin (HE) or Alcian Blue staining. Micrographs were acquired using a light microscope (DM2000, Leica, Germany). The number of goblet cells was quantified using ImageJ.

2.7 Immunohistochemistry

The ileal paraffin sections were deparaffinized and antigen-retrieved in 10 mM sodium citrate (pH 6.0) prior to the detection of cingulin, H3Cit and Ly6G using rabbit anti-cingulin polyclonal antibody (1:500, NBP1-89600, Novus, USA), rabbit anti-histone H3 (citrulline R2 + R8 + R17) antibody (1:500, ab5103, Abcam, USA) and rat anti-mouse Ly6G monoclonal antibody (1:250, 551461, BD Pharmingen, USA) at room temperature for 2 h, respectively. The secondary antibodies included Alexa Fluor 488 donkey anti-rabbit IgG-H&L (1:500, ab150073, Abcam, USA), Cy3 sheep anti-rabbit IgG (whole molecule)-F(ab′)2 fragment (1:1000, Sigma-Aldrich, C2306, MO, USA) and Cy3 goat anti-rat IgG-H&L (1:500, ab98416, Abcam, USA). The secondary antibody incubation was performed at room temperature for 2 h. Counterstaining with DAPI was performed to visualize the nuclei. The fluorescent images were acquired using a fluorescence microscope with the imaging parameters (e.g., exposure and gain) kept the same for each marker (DM6000B, Leica, Germany).

2.8 Statistics analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using the Graphpad Prism 9 software. A two-tailed Student's t-test was performed for comparisons between two groups. One-way ANOVA was performed to compare the means of three or more experimental groups. *Post-hoc* test with Tukey correction or Mann-Whitney U test was applied to multiple comparisons. Statistical significance was defined as $p < 0.05$.

3 Results

3.1 NETs impair the tight junction and viability of the intestinal epithelial cells

Prior to validating the impact of NETs on the barrier integrity and survival of the intestinal epithelial cells, the effects of high glucose on the formation of NETs in the presence or absence of LPS were confirmed. H3Cit was adopted to mark the formation of NETs after the indicated treatments. Measurement of the area of NETs revealed increased formation of NETs in the neutrophils exposed to either high glucose or LPS. Meanwhile, a further increase in the NETs formation was found in the neutrophils exposed to both high glucose and LPS (Figures 1A, B). Next, IEC-6 intestinal epithelial cells were incubated with vehicle or NETs at 25, 50, 100, 200 or 400 ng/mL. Diminished ZO-1 immunopositivity was observed in the NETs-exposed IEC-6 cells at all the doses examined (Figures 1C, D). Additionally, significantly decreased TiJOR was associated with the cells incubated with 50, 100, 200 or 400 ng/mL NETs, suggesting that the tight junction network was disorganized as a result of NETs exposure (Figure 1E). Meanwhile, the survival of IEC-6 was remarkedly compromised in the presence of relatively higher doses of NETs (e.g., 100, 200 or 400 ng/mL). The deleterious impact of NETs on the survival of the IEC-6 cells appeared to reach a plateau at around 100 ng/mL since no statistical significance was noted among the cells treated with 100 ng/mL NETs, 200 ng/mL NETs and 400 ng/mL NETs (Figure 1F). These results confirm that high glucose promotes NETs production and NETs directly impair the barrier integrity of the intestinal epithelial cells, suggesting potential contribution of NETs formation in hyperglycemia-linked intestinal epithelial barrier impairment.

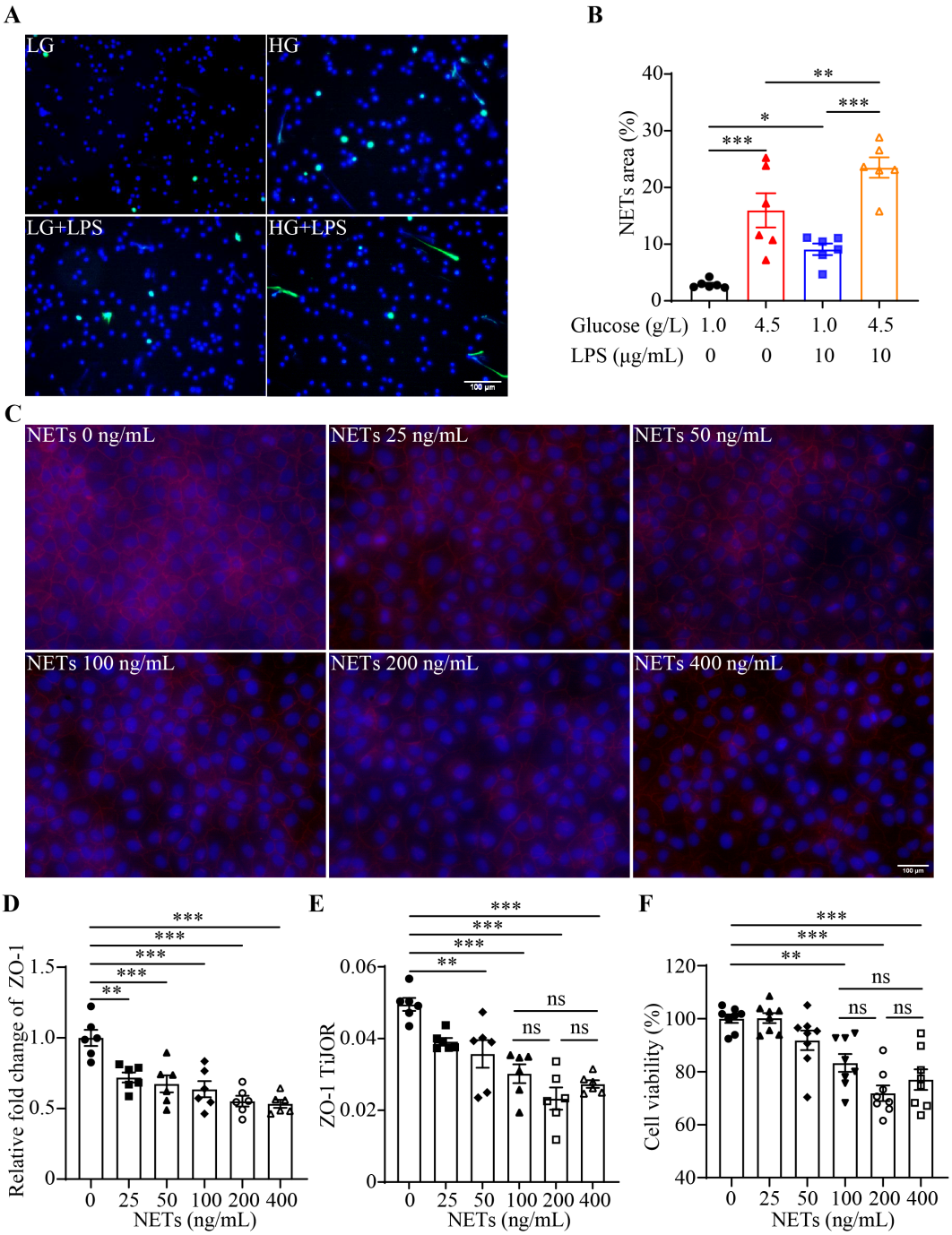

FIGURE 1
High glucose and/or LPS induces NETs formation and NETs impair the tight junction and viability of the intestinal epithelial cells. **(A)** Isolated mouse neutrophils were exposed to 1 g/L glucose (LG), 4.5 g/L glucose (HG), 1 g/L glucose along with 10 ug/mL (LG+LPS) or 4.5 g/L glucose along with 10 ug/mL LPS (HG+LPS) for 2.5 h, followed by the assessment of the H3Cit positivity (in green). Nuclei were visualized by DAPI counterstaining (in blue). Scale bar, 100 μm. **(B)** NETs were quantified as the percentage of H3Cit positive area (n=6 per group). **(C)** IEC-6 cells were stimulated with NETs at 25, 50, 100, 200 or 400 ng/mL for 12 h, followed by the assessment of the ZO-1 positivity (in red). Nuclei were visualized by DAPI counterstaining (in blue). Scale bar, 100 μm. **(D)** Quantification of the ZO-1 positivity (n = 6 per group). **(E)** Quantification of the TiJOR for ZO-1 (n = 6 per group). **(F)** The viability of IEC-6 cells was examined by MTT assay 12 h after NETs stimulation at 25, 50, 100, 200 or 400 ng/mL (n = 8 per group). Data were expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant.

3.2 Hyperglycemia leads to morphological impairment and NETs formation in the intestinal epithelium

Next, the possible implication of NETs formation in hyperglycemia-linked intestinal barrier impairment was explored. HE staining showed that compared to the intact ileal morphology detected in the normal controls, leukocyte infiltration, capillary dilation and congestion in the villi, villus shedding and necrosis, and focal mucosal ulceration were readily detected in the intestinal mucosa and submucosa from the STZ mice (Supplementary Figure 1). Alcian blue staining revealed that the number of the goblet cells, the essential component of the intestinal epithelial barrier, was significantly reduced in the STZ mice compared to the normal controls (Figures 2A, B). IHC demonstrated that the immunopositivity cingulin, which regulates tight junction permeability, was orderly distributed around the villus and crypts in the ilium from the normal control. In contrast, remarkably diminished cingulin immunopositivity was observed in the ileum from the STZ mice (Figures 2C, D). Furthermore, overt immunopositivity of Ly6G, a neutrophil marker, was readily

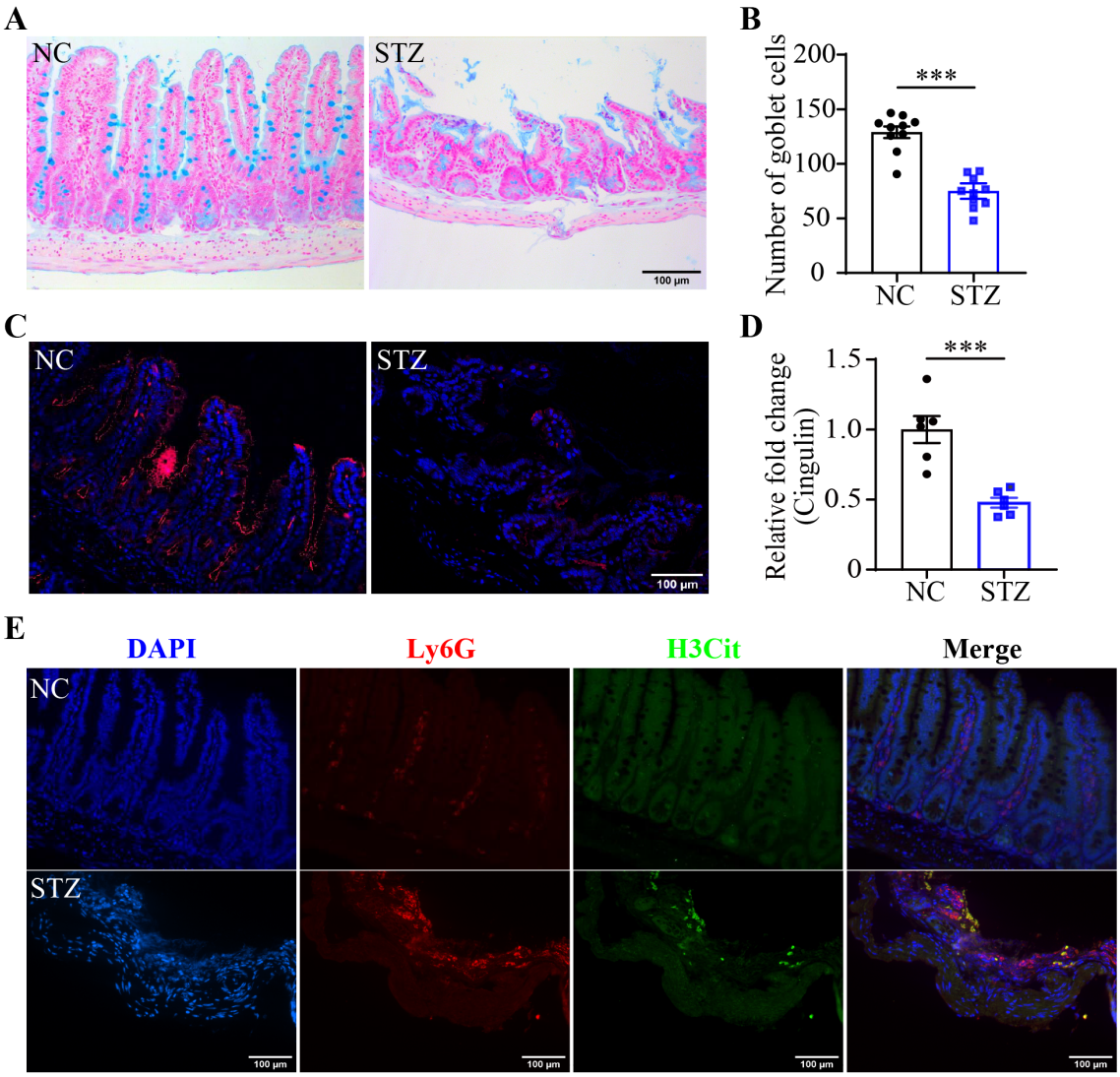

FIGURE 2
Hyperglycemia leads to mucosal barrier impairment and NETs formation in the intestinal epithelium. **(A)** Representative micrographs of Alcian blue-stained goblet cells in the ileum. Scale bar, 100 μm. **(B)** The number of goblet cells per unit area (n = 10 per group). **(C)** Representative micrographs of cingulin immunopositivity (in red). The nuclei were counterstained by DAPI (in blue). Scale bar, 100 μm. **(D)** Cingulin immunopositivity per unit area (n = 6 per group). **(E)** Representative images of Ly6G (in red) and H3Cit (in green) immunopositivity. DAPI was counterstained to visualize the nuclei (in blue). Scale bar, 100 μm. Data were expressed as mean ± SEM. *** $p < 0.001$. NC, normoglycemic controls; STZ, STZ-induced hyperglycemic mice.

detected in the ilium from the STZ mice but not the normal controls. Moreover, H3Cit immunopositivity was found to be colocalized with Ly6G positive neutrophils in the ilium from the STZ mice (Figure 2E). These results indicate that hyperglycemia leads to barrier integrity impairment and NETs formation in the intestinal epithelium.

3.3 NETs promote hyperglycemia-associated intestinal epithelial barrier impairment

To further clarify the causal relationship between NETs and hyperglycemia-linked intestinal barrier impairment, DNase I was administered to the hyperglycemic mice to degrade the DNA backbone of NETs. Compared to the vehicle-treated STZ mice, no significant changes in the fasting blood glucose were observed in the DNase I-treated STZ mice (Supplementary Figure 2A). IHC revealed significantly diminished H3Cit immunopositivity but not Ly6G immunopositivity in the ilium from the STZ mice treated with DNase I at both 2.5 and 10 mg/kg (Figures 3A–C). The ileal morphology was partially preserved in the DNase-treated STZ mice compared to the vehicle-treated STZ mice (Supplementary Figure 2B). Moreover, the number of goblet cells was significantly higher in the DNase-treated STZ mice compared to the vehicle-treated STZ mice (Figures 3D, E). These observations support a causal relationship between NETs and intestinal epithelial barrier impairment under hyperglycemic conditions. To further validate this causality, *Padi4*-/- mice, which are deficient in the gene encoding PAD4, an essential driver of NETs formation, were subjected to STZ-induced hyperglycemia. Decreased immunopositivity of H3Cit but not that of Ly6G was observed in the hyperglycemic *Padi4*-/- mice compared to the hyperglycemic wild type controls (Figures 4A–C). Meanwhile, similar to DNase I treatment, no significant changes in the fasting blood glucose were observed in the *Padi4*-/- mice. However, improved ileal morphology was observed in the hyperglycemic *Padi4*-/- mice compared to the hyperglycemic wild type controls (Supplementary Figures 3A, B). Moreover, compared to the hyperglycemic wide type controls, the number of goblet cells was significantly increased in the hyperglycemic *Padi4*-/- mice (Figures 4D, E). These results collectively indicate that it is the NETs formation but not the mere presence of neutrophils that causes the intestinal epithelial barrier impairment under hyperglycemic conditions.

3.4 Baicalin suppresses NETs formation induced by high glucose, LPS or both high glucose and LPS

The results above suggest the possibility that inhibiting NETs formation may mitigate hyperglycemia-linked intestinal epithelial barrier impairment. Available evidence supports the intestinal barrier protective activities of baicalin under hypertensive or inflammatory conditions (16, 17). Moreover, it has been shown that baicalin directly attenuates neutrophil activation (18). To further evaluate the pharmacological activity of baicalin in protecting against hyperglycemia-linked, NETs-mediated intestinal epithelial barrier impairment, we assessed the effects of baicalin on NETs formation in the neutrophils subjected to high glucose and/or LPS stimulation. The results showed that baicalin dose-dependently reduced high glucose-stimulated NETs formation (Figures 5A, B). Furthermore, high glucose-induced increases in H3Cit were significantly blunted in the baicalin-treated neutrophils (Figures 5C, D). In addition, baicalin attenuated NETs formation and histone citrullination in the neutrophils stimulated by LPS (Figures 6A–D) or in the neutrophils exposed to both high glucose and LPS (Figures 7A–D). These results demonstrate that baicalin is pharmacologically effective at suppressing histone citrullination, thereby attenuating NETs formation in the presence of high glucose, LPS or combination of high glucose and LPS.

3.5 Baicalin attenuates hyperglycemia-associated NETs formation and intestinal barrier impairment *in vivo*

Next, the putative effects of baicalin at alleviating hyperglycemia-associated NETs formation was validated *in vivo*. Although no significant changes in the level of the fasting blood glucose were noted (Supplementary Figure 4A), significantly decreased Ly6G immunopositivity was observed in the STZ mice treated with high-dose baicalin compared to the mice receiving vehicle treatment (Figures 8A, B). Furthermore, both low-dose and high-dose baicalin treatments resulted in remarkably lower H3Cit immunopositivity in the ileum compared to the vehicle-treated STZ mice (Figures 8A, C). Histological examination further revealed improvement in the ileal morphology in the STZ mice that received both low-dose and high-dose baicalin treatment compared to the vehicle-treated STZ mice (Supplementary Figure 4B). Consistently, more goblet cells (Figures 9A, B) and increased cingulin immunopositivity (Figures 9C, D) were detected in the ileum from the baicalin-treated STZ mice compared to the vehicle-treated STZ mice. Taken together, these results support that baicalin is effective at attenuating NETs formation and intestinal epithelial barrier impairment in the hyperglycemic mice.

4 Discussion

Although the associations between hyperglycemia and intestinal epithelial barrier impairment, hyperglycemia and NETs formation

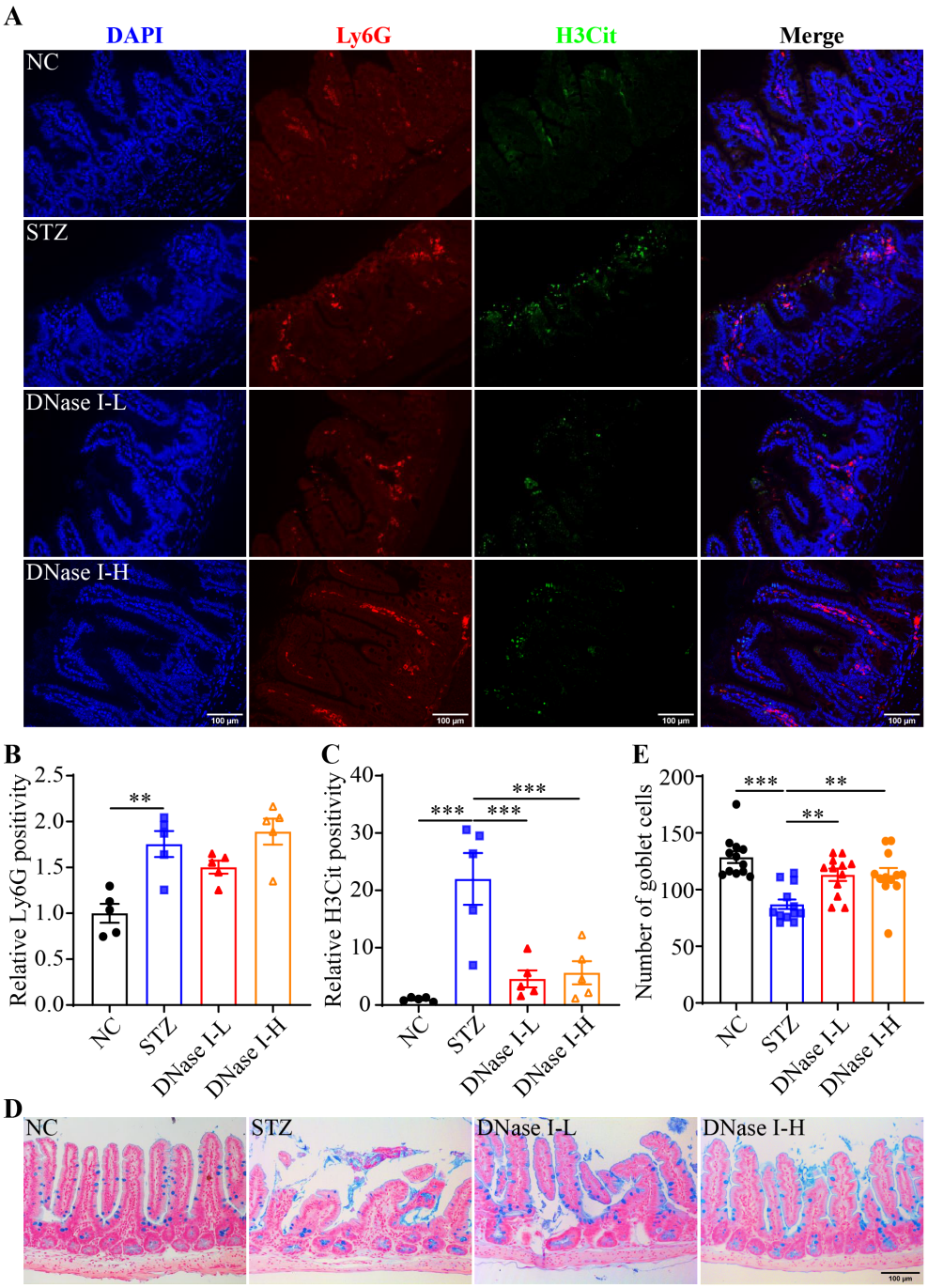

FIGURE 3
DNase I treatment attenuates intestinal NETs formation and mucosal barrier impairment in the hyperglycemic mice. **(A)** Representative micrographs showing Ly6G immunopositivity (in red), H3Cit immunopositivity (in green) and DAPI positivity (in blue) in the ileal sections. Scale bar, 100 μm. **(B)** Relative Ly6G positivity (n = 5 per group). **(C)** Relative H3Cit positivity (n = 5 per group). **(D)** Representative images of Alcian blue-stained goblet cells. Scale bar, 100 μm. **(E)** The number of goblet cells per unit area (n = 12 per group). Data were expressed as mean ± SEM. ** $p < 0.01$, *** $p < 0.001$. NC, the vehicle-treated normoglycemic controls; STZ, the STZ-induced hyperglycemic mice treated with vehicle; DNase I-L, the STZ-induced hyperglycemic mice treated with a daily dose of 2.5 mg/kg DNase I; DNase I-H, the STZ-induced hyperglycemic mice treated with a daily dose of 10 mg/kg DNase I.

as well as NETs and intestinal epithelial injuries have been separately established, the causality of hyperglycemia-induced NETs formation in intestinal epithelial barrier impairment remains to be addressed *in vivo*. Therefore, one of the major aims of the current work was to clarify the mechanistic contribution of NETs to intestinal epithelial barrier impairment under hyperglycemic conditions. The *in vivo* findings here unveil that NETs are present in the intestinal epithelium in the hyperglycemic

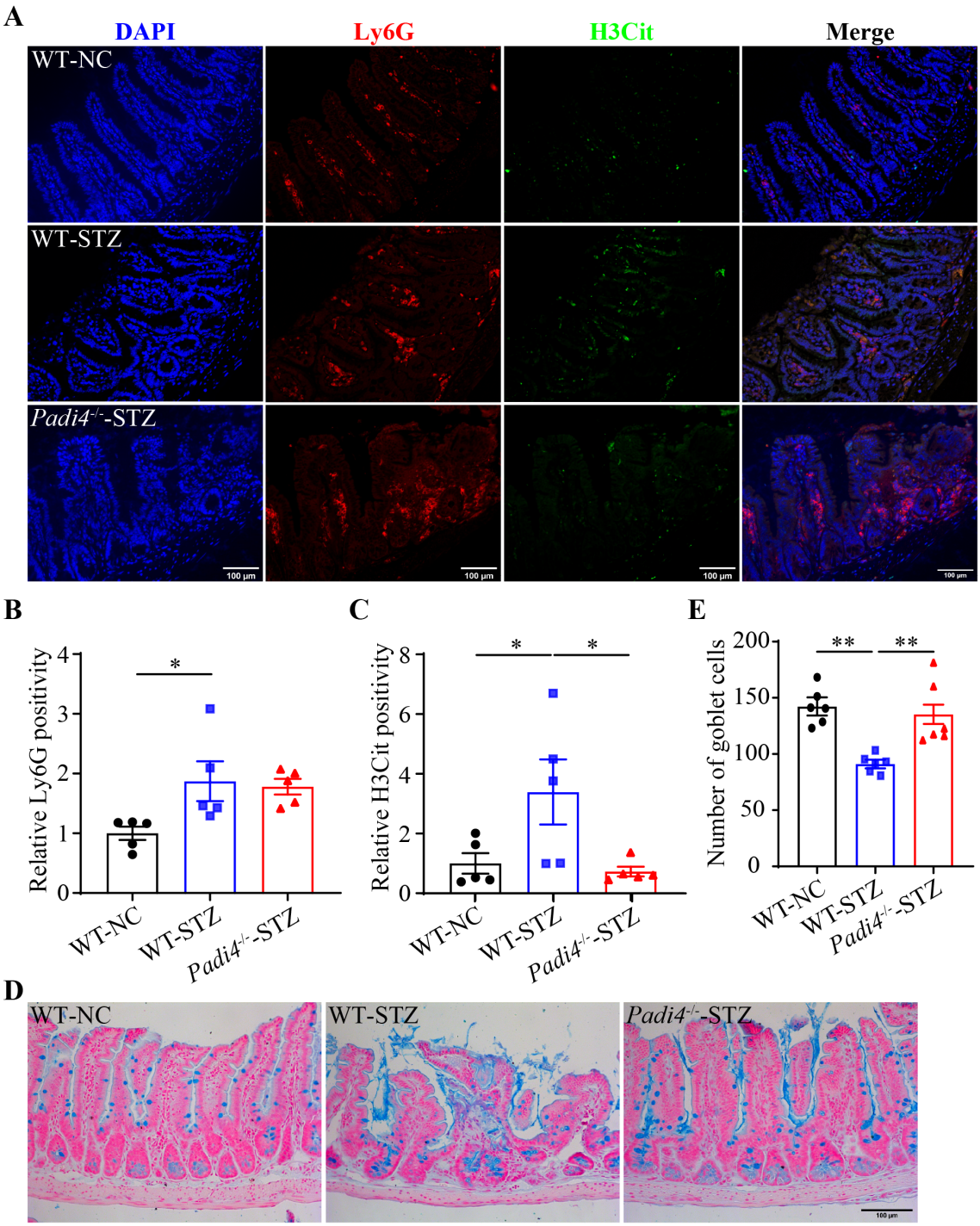

FIGURE 4
Padi4 gene deficiency mitigates intestinal NETs formation and mucosal barrier impairment in the hyperglycemic mice. **(A)** Representative micrographs showing Ly6G immunopositivity (in red), H3Cit immunopositivity (in green) and DAPI positive nuclei (in blue) in the ileal sections. Scale bar, 100 μm. **(B)** Relative Ly6G positivity (n = 5 per group). **(C)** Relative H3Cit positivity (n = 5 per group). **(D)** Representative images of Alcian blue-stained goblet cells. Scale bar, 100 μm. **(E)** The number of goblet cells per unit area (n = 6 per group). Data were expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$. WT-NC, the wild type normoglycemic controls; WT-STZ, the wild type mice subjected to STZ-induced hyperglycemia; *Padi4*-/–STZ, the *Padi4*-/- mice subjected to STZ-induced hyperglycemia.

mice. DNase I treatment or *Padi4* gene deficiency abolishes NETs formation in the intestinal epithelium and mitigates intestinal epithelial barrier impairment in the hyperglycemic mice, supporting the causal relationship between NETs and hyperglycemia-associated intestinal epithelial barrier impairment. Based on these findings, we further demonstrate that baicalin suppresses NETs formation in the neutrophils stimulated by high glucose, LPS, or both. Most importantly, baicalin attenuates NETs

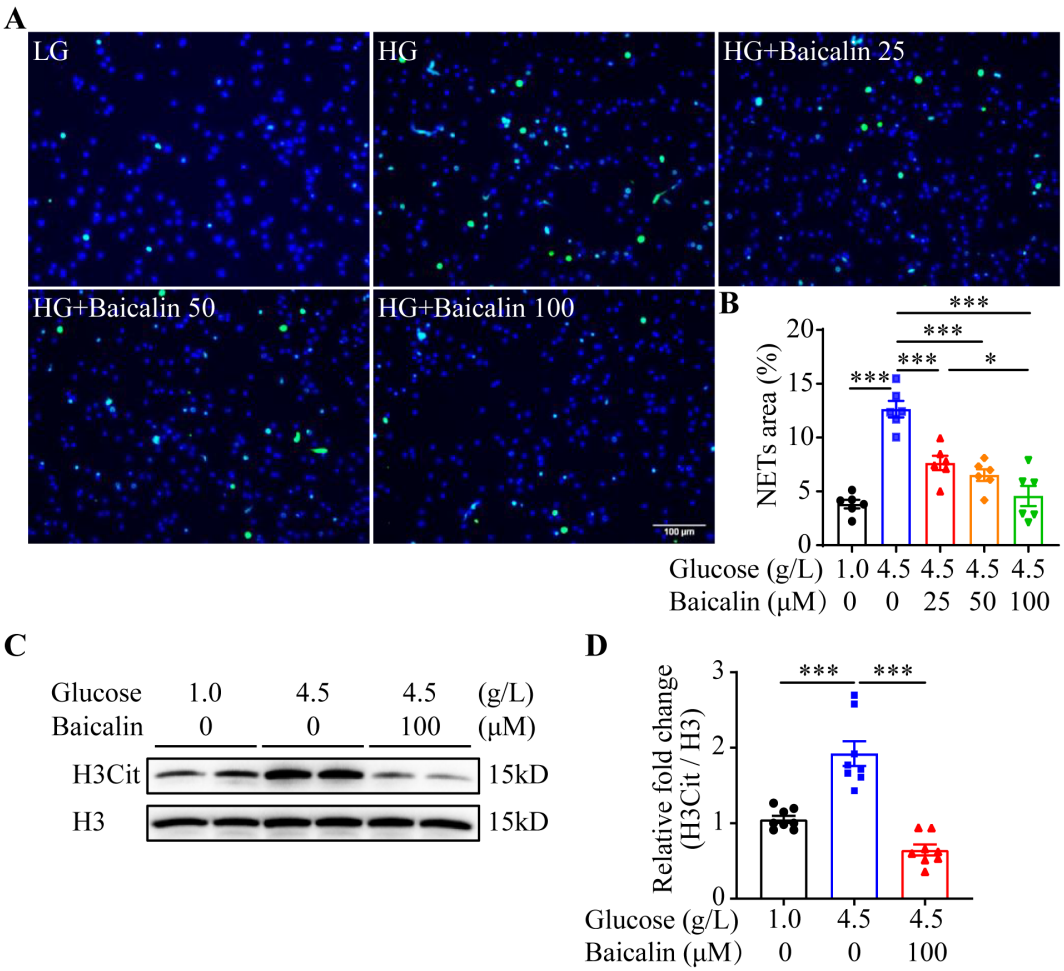

FIGURE 5
Baicalin suppresses high glucose-induced NETs formation. **(A)** The isolated mouse neutrophils were exposed to 1 g/L glucose (LG) or 4.5 g/L glucose (HG). The HG-exposed neutrophils were treated with either vehicle or baicalin at 25, 50, 100 μM for 2.5 h, followed by the assessment of H3Cit immunopositivity. Representative images showing H3Cit immunopositivity. Scale bar, 100 μm. **(B)** NETs formation was quantified as the percentage of the H3Cit positive area (n = 6 per group). **(C)** Isolated neutrophils were exposed to 1 g/L glucose or 4.5 g/L glucose. The cells exposed to 4.5 g/L glucose were treated with vehicle or 100 μM baicalin for 2.5 h, followed by the evaluation of the level of histone H3 citrullination. Histone H3 served as the loading control. **(D)** Relative fold change in the level of H3Cit (n = 8 per group). Data were expressed as mean ± SEM. * $p < 0.05$, *** $p < 0.001$.

formation and preserves the intestinal epithelial barrier integrity in the hyperglycemic mice (Figure 10).

First of all, our major findings here provide *in vivo* evidence supporting the mechanistic implications of NETs formation in the pathogenesis of hyperglycemia-linked intestinal epithelial barrier impairment. Impaired intestinal epithelial barrier plays an important role in driving the development of systemic inflammation under hyperglycemic conditions. However, the mechanisms accountable for hyperglycemia-linked intestinal epithelial barrier impairment remain to be fully elucidated. It has been demonstrated that high glucose directly stimulates NETs formation. Increased NETs formation promotes diabetes-associated impairment of wound healing and diabetic retinopathy (6, 9, 10). Consistently, our results here demonstrate that high glucose alone or in combination with LPS leads to increased NETs formation in the neutrophils. Our *in vitro* findings also confirm that NETs directly impair the viability of the intestinal epithelial cells in a dose-dependent manner. Of note, at the doses that are not detrimental to the survival of the intestinal epithelial cells, NETs sabotage the barrier integrity of the intestinal epithelial cells. Most importantly, *in vivo* evidence here uncovers that along with impaired epithelial integrity, NETs could be readily detected in the intestinal epithelium in the mice 2 weeks into hyperglycemia, suggesting that NETs formation and impairment of the intestinal epithelial barrier

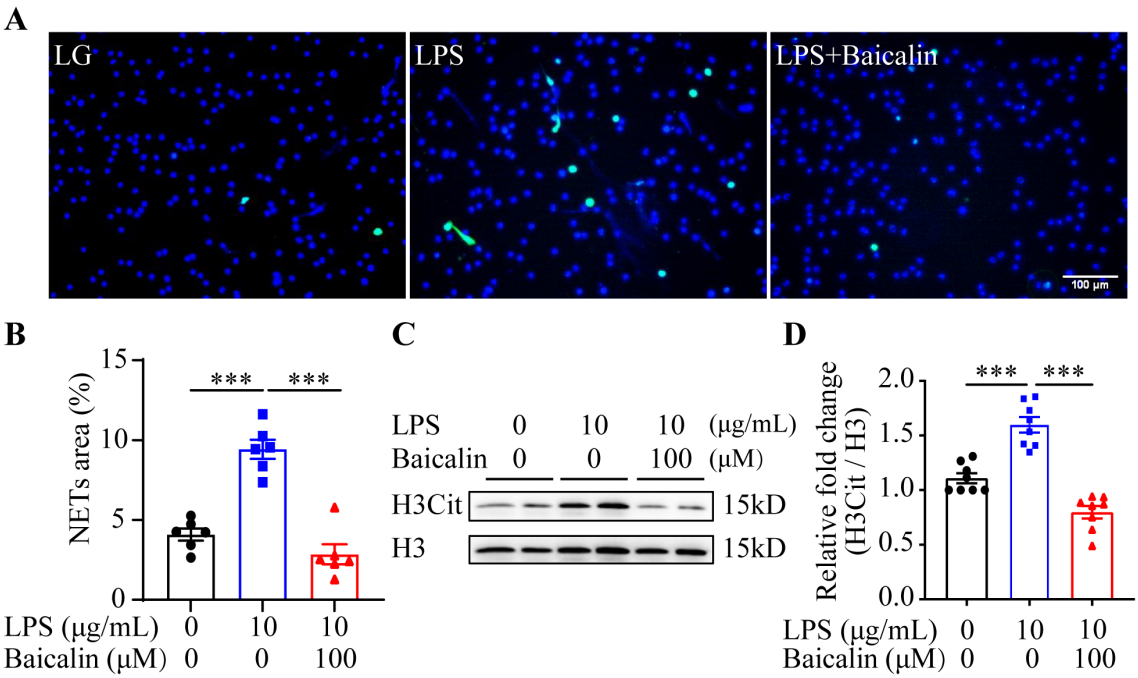

FIGURE 6
Baicalin inhibits LPS-induced NETs formation. **(A)** The isolated mouse neutrophils cultured in 1 g/L glucose were stimulated with 10 μg/mL LPS (LPS) in the presence or absence of 100 μM baicalin for 2.5 h, followed by the examination of the H3Cit immunopositivity. The cells unexposed to LPS and treated by vehicle (VC) served as the baseline for the indicated analysis. Representative immunofluorescent images showing H3Cit immunopositivity. Scale bar, 100 μm. **(B)** NETs formation was quantified as the percentage of the H3Cit positive area (n = 6 per group). **(C)** Histone H3 citrullination was assessed by examining the protein level of H3Cit. Histone H3 was probed as the internal control. **(D)** Relative fold change of H3Cit (n = 8 per group). Data were expressed as mean ± SEM. *** $p < 0.001$.

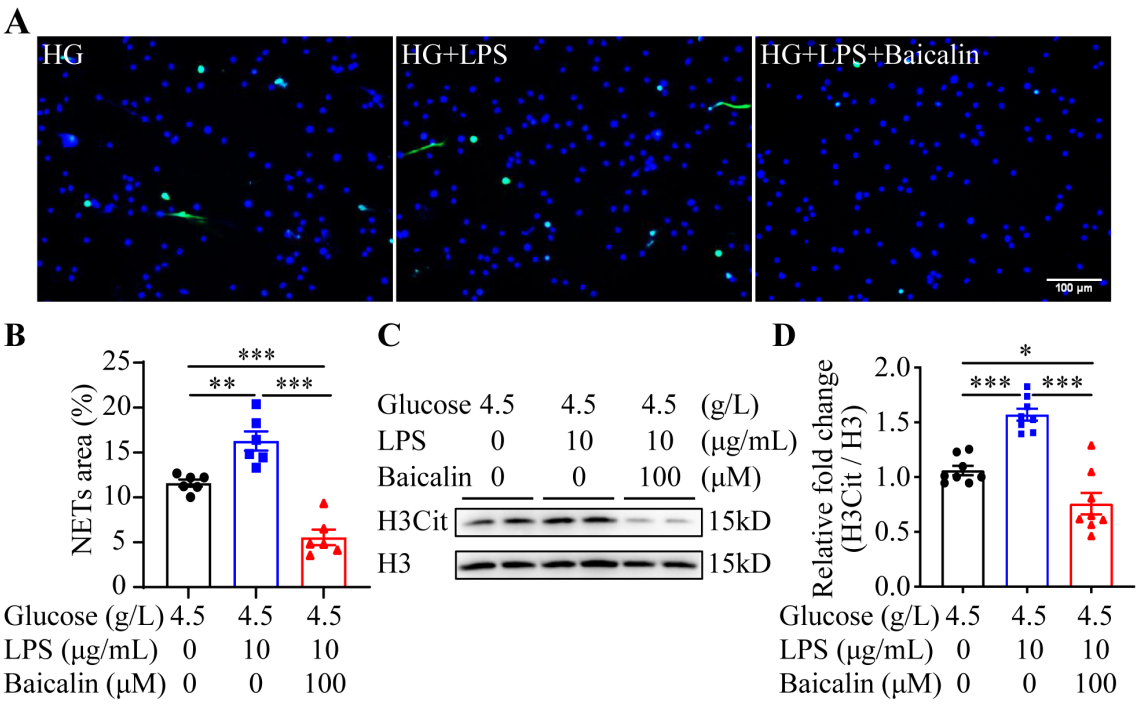

FIGURE 7
Baicalin inhibits NETs formation in the presence of both high glucose and LPS. **(A)** The isolated neutrophils were stimulated by 4.5 g/L glucose (HG) or both 4.5 g/L glucose and 10 μg/mL LPS (HG+LPS) in the presence or absence of 100 μM baicalin for 2.5 h, followed by the assessment of the H3Cit immunopositivity. Representative immunofluorescent images showing the H3Cit immunopositivity. Scale bar, 100 μm. **(B)** NETs formation was quantified as the percentage of the H3Cit positive area (n = 6 per group). **(C)** Histone H3 citrullination was assessed by examining the protein level of H3Cit. Histone H3 served as the internal control. **(D)** Relative fold change of H3Cit (n = 8 per group). Data were expressed as mean ± SEM.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

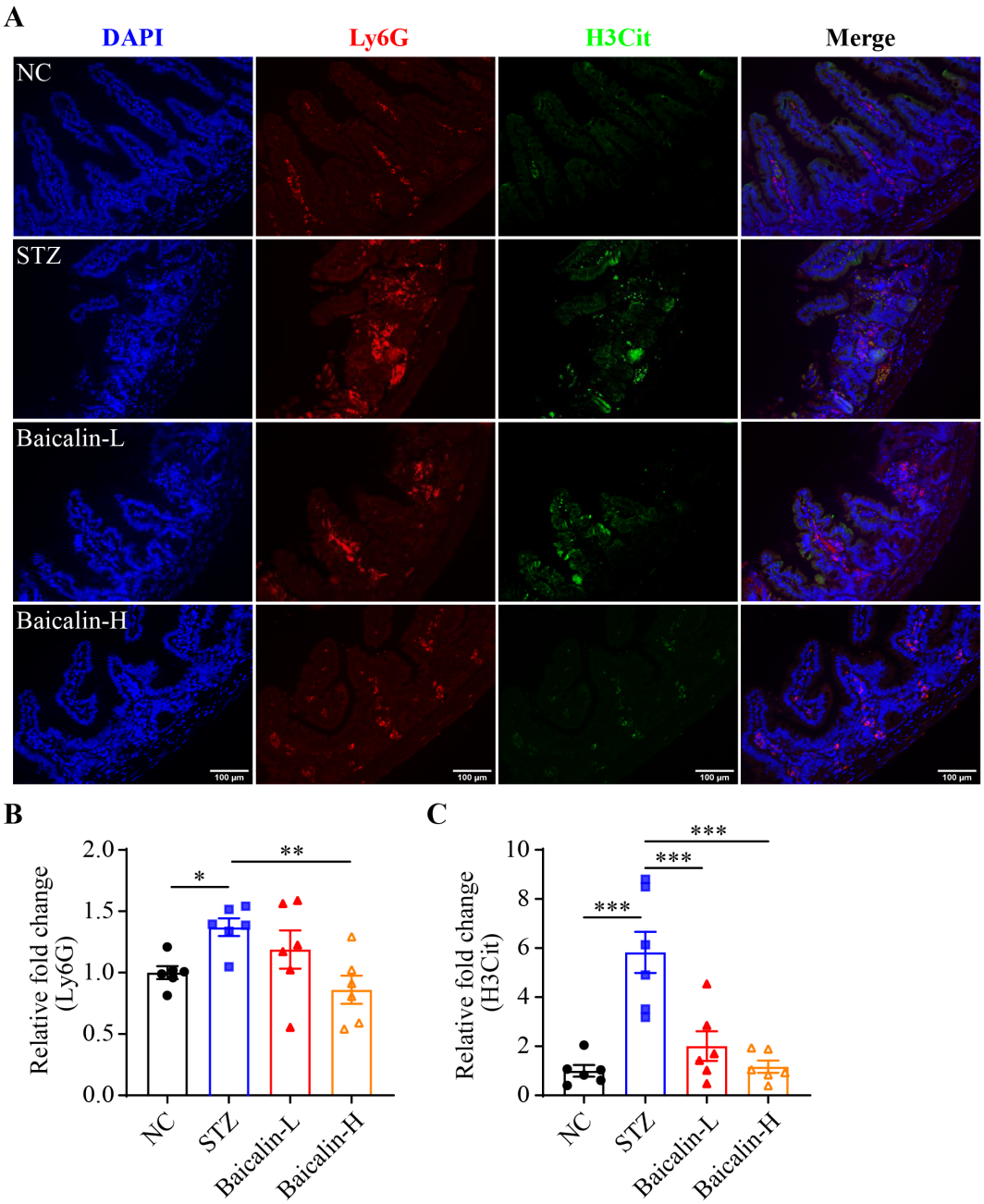

FIGURE 8
Baicalin attenuates hyperglycemia-linked intestinal NETs formation *in vivo*. **(A)** Representative micrographs showing Ly6G (in red) and H3Cit (in green) immunopositivity. DAPI was counterstained to visualize the nuclei (in blue). Scale bar, 100 μm. **(B)** Relative fold change in the Ly6G positivity (n = 6 per group). **(C)** Relative fold change in the H3Cit positivity (n = 6 per group). Data were expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NC, the vehicle-treated normoglycemic controls; STZ, the vehicle-treated STZ-induced hyperglycemic mice; Baicalin-L, the STZ-induced hyperglycemic mice treated with a daily dose of 240 mg/kg baicalin; Baicalin-H, the STZ-induced hyperglycemic mice treated with a daily dose of 1200 mg/kg baicalin.

are early pathological events during the course of hyperglycemia. Eliminating the DNA components in NETs by DNase I treatment or abolishing NETs formation by genetic ablation of *Padi4* improves the intestinal epithelial morphology in the hyperglycemic mice. These findings collectively support the causal relationship between hyperglycemia-induced NETs formation and intestinal epithelial barrier impairment. Meanwhile, the *in vitro* and *in vivo* findings suggest the possibility that DNA is involved in driving the compromised survival and tight junctional impairment of the intestinal epithelial cells under hyperglycemic conditions. Impaired

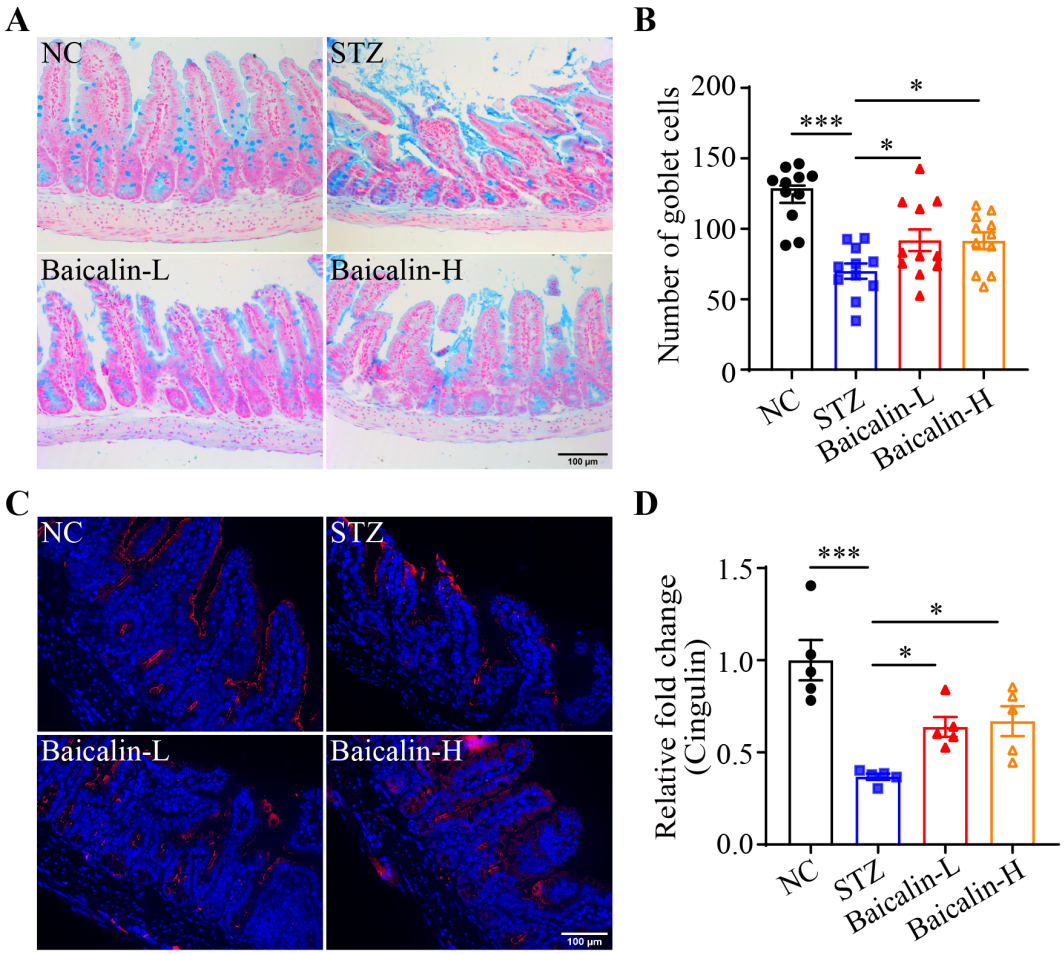

FIGURE 9
Baicalin protects against the intestinal epithelial barrier impairment in the hyperglycemic mice. **(A)** Representative micrographs showing Alcian blue-stained ileal goblet cells. Scale bar, 100 μm. **(B)** The number of the goblet cells per unit area (n = 11 per group). **(C)** Representative micrographs revealing cingulin immunopositivity in the ileal sections. Scale bar, 100 μm. **(D)** Relative fold change in the cingulin immunopositivity in the ilium (n = 5 per group). Data were expressed as mean ± SEM. * $p < 0.05$, *** $p < 0.001$. NC, the vehicle-treated normal controls; STZ, the vehicle-treated STZ-induced hyperglycemic mice; Baicalin-L, the STZ-induced hyperglycemic mice treated with a daily dose of 240 mg/kg baicalin; Baicalin-H, the STZ-induced hyperglycemic mice treated with a daily dose of 1200 mg/kg baicalin.

integrity of the intestinal epithelial barrier is of paramount significance to the development of diabetes-associated systemic inflammation (2). The findings here further support that NETs contribute to hyperglycemia-linked intestinal barrier disruption, supporting the possibility that aberrant activation of neutrophils could be therapeutically targeted to control hyperglycemia-associated inflammation.

Secondly, our work here underpins the pharmacological value of baicalin in attenuating hyperglycemia-initiated NETs-mediated intestinal epithelial barrier impairment. Baicalin directly suppresses histone citrullination and NETs formation in the neutrophils stimulated by high glucose, LPS or both high glucose and LPS. These *in vitro* findings support the possibility that the beneficial impact of baicalin on preserving the integrity of the intestinal epithelium under hyperglycemic conditions could in part be ascribed to its direct action at inhibiting NETs formation promoted by high glucose and/or bacterial products such as LPS. Although the mechanisms underlying high glucose-triggered formation of NETs remain to be fully elucidated, it has been shown that NADPH oxidase-mediated overproduction of reactive oxygen species (ROS) is involved in high glucose-induced NETs formation (6). Under platelet-free conditions, which is more relevant to our *in vitro* experimental setting, LPS induce NETosis via mechanisms implicating autophagy and ROS production (20). Therefore, overproduction of ROS appears to be a common mechanism underlying NETs formation stimulated by high glucose, LPS or both high glucose and LPS. It has been demonstrated that without affecting the assembly of NADPH

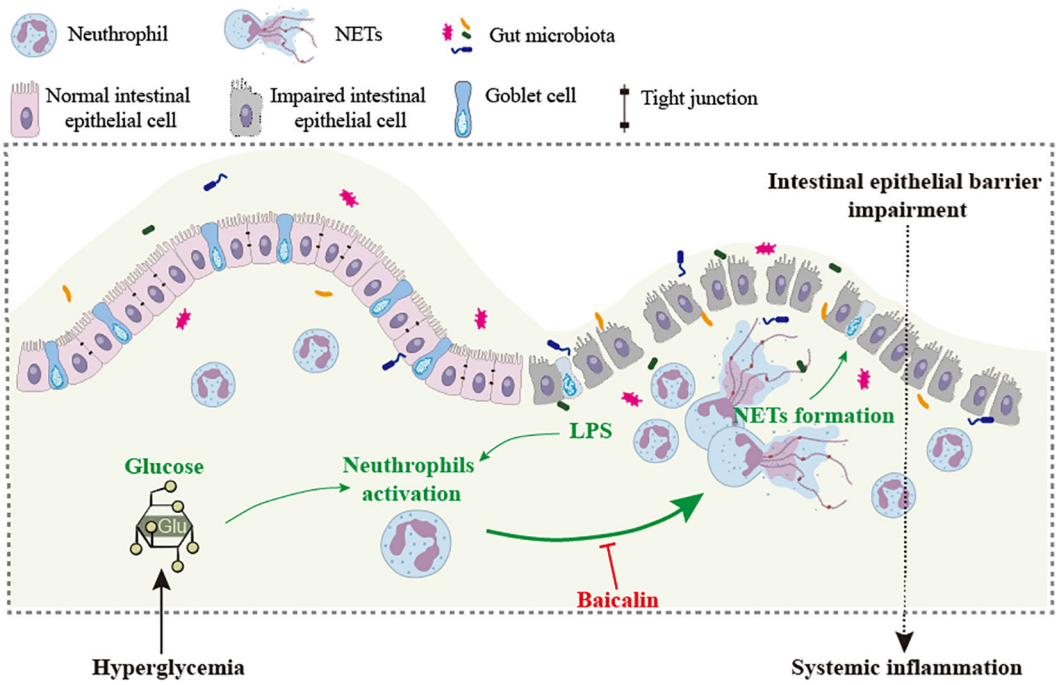

FIGURE 10
Baicalin mitigates hyperglycemia-linked intestinal epithelial barrier impairment in part via suppressing NETs formation. Intestinal barrier impairment contributes to hyperglycemia-associated systemic inflammation. NETs induced by high level of glucose and/or bacterial products such as LPS are directly involved in the pathogenesis of intestinal epithelial barrier impairment. Baicalin directly suppresses NETs formation triggered by high level of glucose, LPS or both, which may in part account for baicalin-conferred protection against intestinal epithelial barrier impairment under hyperglycemic conditions.

oxidase, an essential step required for the activation of NADPH oxidase, baicalin inhibits fMLP or PMA-induced ROS production in human neutrophils, supporting its pharmacological activity in scavenging reactive oxygen intermediates (18). Moreover, baicalin lowers the activity of myeloperoxidase in the presence of fMLP (18). Myeloperoxidase is required for NETs formation as neutrophils from the donors with myeloperoxidase deficiency fail to form NETs in the presence of PMA (21). Both hyperglycemia and LPS exposure are associated with heightened level of myeloperoxidase activity (22, 23). Therefore, it is possible that the antioxidant property of baicalin may contribute to its effects at attenuating NETs formation in the presence of high glucose and/or LPS. Meanwhile, myeloperoxidase is likely a molecular target of baicalin in this process. Future studies are necessary to delineate the molecular mechanisms underlying the effects of baicalin at inhibiting NETs formation under hyperglycemic conditions. In terms of the potential mechanistic implications of myeloperoxidase, it is worth delineating if baicalin directly interacts with myeloperoxidase and/or how baicalin suppresses the activation of myeloperoxidase in neutrophils.

Additionally, there are several limitations associated with the current study. First, the histopathological features associated with NETs formation were not evaluated in the other segments of the small intestine in the hyperglycemic mice, thus it remains unknown if hyperglycemia has a broad impact on the morphological integrity of the small intestine and if NETs formation is involved. Second, the intestinal permeability assays, for instance, FITC-dextran leakage assay, remained to be conducted in the hyperglycemic mice to directly assess the pharmacological impact of baicalin treatment on the intestinal barrier integrity *in vivo*. Moreover, the impact of baicalin on intestinal barrier impairment-linked metabolic inflammation under hyperglycemic conditions remained unknown. Future studies are warranted to address these remaining questions.

Taken together, the work here demonstrates for the first time that NETs formation promotes intestinal epithelial barrier impairment under hyperglycemic conditions. Most importantly, our findings here shed new light on the pharmacological mechanisms of baicalin in protecting against hyperglycemia-linked intestinal epithelial barrier impairment, which in part implicates direct effects of baicalin at suppressing NETs formation. The novel understanding of the mechanisms of actions of baicalin justifies its potential as an adjunct agent in controlling hyperglycemia-associated inflammation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YQC: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. QY: Data curation, Investigation, Methodology, Validation, Writing – original draft. XT: Investigation, Methodology, Writing – original draft. PW: Investigation, Methodology, Writing – original draft. JC: Investigation, Methodology, Writing – original draft. XD: Investigation, Methodology, Writing – original draft. TZ: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. YC: Conceptualization, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1551256/full#supplementary-material

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An updated review on immune checkpoint inhibitor-induced colitis: epidemiology, pathogenesis, treatment strategies, and the role of traditional Chinese medicine

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Immune checkpoint inhibitor-induced colitis (irColitis) is a common and severe adverse reaction to immune checkpoint inhibitors (ICIs), significantly impacting the treatment outcomes and quality of life of cancer patients. Epidemiological studies indicate that the incidence of irColitis is associated with factors such as the type of ICIs, the patient's gender, age, and medical history. Although the exact pathophysiology remains unclear, irColitis is thought to be related to immune system activation and dysregulation, gut microbiota imbalance, and impaired epithelial barrier function. This review summarized the epidemiology, clinical presentation, diagnostic criteria, and pathogenesis of irColitis. Additionally, the standard and novel therapeutic strategies of irColitis, including corticosteroids, biologics, and gut microbiota interventions, more importantly the potential and application of Traditional Chinese Medicine (TCM). Future researches call for deeper mechanistic investigations, the development of biomarkers, and reveal the integration of TCM therapies within individual immunotherapy frameworks.

KEYWORDS

immune checkpoint inhibitors (ICIs), immune checkpoint inhibitor induced colitis (irColitis), traditional Chinese medicine (TCM), gut microbiota, review

1 Introduction

Immune checkpoint inhibitors (ICIs) have made significant advancements in cancer treatment, being widely used for non-small cell lung cancer (NSCLC), melanoma, gastric cancer, and other malignancies. This has transformed the treatment landscape for both solid tumors and hematological malignancies. The ICIs are associated with a range of immune-related adverse events (irAEs) that can affect multiple organs, including

dermatological reactions, hepatitis, myocarditis, colitis, and others (1). Among these, immune checkpoint inhibitor-induced colitis (irColitis) is one of the most common and severe adverse effects. Patients with irColitis typically experience diarrhea, abdominal pain, and hematochezia, and even bowel perforation in severe cases. The irColitis significantly impacts patients' quality of life, more seriously, causing drug dose reduction, discontinuation, or even death. The pathogenesis of irColitis remains unclear, but it is likely associated with immune system activation and dysregulation, gut microbiota imbalance, and impaired epithelial barrier function (2–4). Current treatments include corticosteroids and immunosuppressive agents; however, some patients exhibit inadequate responses or develop resistance, highlighting the urgent need for new therapeutic options for irColitis.

Under the principle of treatment based on syndrome differentiation, Traditional Chinese Medicine (TCM) has accumulated extensive experience in treating irAEs. For irColitis, TCM aims to restore the body's balance and improve immune and intestinal functions (5). This holistic approach, along with syndrome differentiation and individualized treatment, have gradually demonstrated unique advantages.

This review aims to summarize the recent advances in the pathogenesis, clinical features, and TCM-based treatments of irColitis, while also exploring potential future research directions in this field. The goal is to provide new insights into clinical practice and offer reference points for future research endeavors.

2 Incidence and risk factors of irColitis

2.1 Incidence and risk factors of irColitis in previous literature

Studies have demonstrated that the incidence of irColitis varies significantly among different patient populations. A systematic review and meta-analysis (6) revealed that the incidence of colitis in patients treated with anti-programmed cell death protein 1 (PD-1) antibodies alone was 2%. In contrast, the use of Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) inhibitors, such as ipilimumab, resulted in a notable increase in the incidence of colitis to 7%. In CheckMate 920 (7), the combination of nivolumab and ipilimumab led to an 18% incidence rate of all-grade colitis, with a 7% incidence rate of grade 3 or higher. As a consequence, one participant discontinued treatment. These findings suggested that the treatment modality of ICIs significantly influences the incidence of colitis. Other potential risk factors for irColitis have been identified in a cohort study of data from the Explorys, a US population database, indicating that irColitis occurred in 3.6% of patients. The study identified several risk factors associated with an increased likelihood of developing irColitis, including being female, white, older than 65 years, obese, and having a history of alcohol abuse (8).

Furthermore, a retrospective, comparative cohort study focused on the comorbid diseases associated with irColitis. This study showed patients with rheumatoid arthritis (RA) demonstrated a lower incidence of irColitis than those without RA, but the difference was not statistically significant(6 cases [7%] vs. 28 cases [14%]; p = 0.094) (9). This observed difference may be due to the distinct underlying pathological and immune mechanisms between irColitis and RA.

2.2 Incidence and risk factors of irColitis in clinical trials from the past five years

To characterize the epidemiology of irColitis, we reviewed clinical studies published on PubMed between 2020 and 2024 (10–49). The literature search used the keywords "Immune checkpoint inhibitor" and "colitis." Clinical trials published in the past five years were selected based on the following inclusion criteria: (1) studies included more than 10 participants to ensure statistical robustness, minimized random variation, and reduced biases associated with small sample sizes or case reports; and (2) studies explicitly reported the incidence of irColitis to directly compare, minimizing consistency across different studies. These criteria were established to enhance the reliability of our findings and minimize data heterogeneity. The search results are presented in Table 1, including the year, country, sample size, drug type, and dosage (with treatment cycles and study endpoints detailed in the Supplementary Table), the incidence of irColitis (overall and grades 3–4), and the number of patients who discontinued treatment or died due to irColitis.

As shown in Table 1, these studies focused on the incidence of irColitis across various malignancies. Among the 41 included studies, 39 were prospective, including 28 phase I/II trials and 7 phase III/IV trials. These trials investigated various types of cancer, including melanoma, NSCLC, Renal Cell Carcinoma (RCC), Colorectal Cancer (CRC), Head and Neck Squamous Cell Carcinoma (HNSCC), gastroesophageal adenocarcinoma, classical Hodgkin lymphoma, cervical cancer, bladder cancer, soft tissue sarcoma, and anal cancer. The incidence of colitis varied significantly, ranging from 0 to 32%, with the rate of grade 3 or higher events varying between 0 and 21%.

These differences were primarily influenced by tumor type, the immunotherapy drugs and doses used, treatment frequency, and patient characteristics. Regarding the relationship between immunotherapy drug types and the incidence of irColitis, our findings aligned with previous literature. Compared to treatment with a single drug, the incidence of irColitis was significantly higher in combination therapies. For example, the incidence of irColitis reached 18% in patients treated with nivolumab and ipilimumab (3 mg/kg + 1 mg/kg) (25). In contrast, monotherapy with nivolumab at 3 mg/kg showed a lower incidence of 0.9% compared to ICIs combination therapy (49). Additionally, high-dose and high-frequency application of ICIs appeared to be associated with an increased incidence of irColitis. For instance, pembrolizumab at 10 mg/kg every 2 weeks resulted in an overall irColitis incidence of 11.9% (35), whereas fixed-dose pembrolizumab at 200 mg every 3 weeks led to an overall irColitis incidence of 2%-9.3% (19, 40). Furthermore, patients with different tumor types had varying

TABLE 1 Incidence of irColitis in recent five years.

Author (Year)	Country	Tumor Type	Drug and Dosage	Number of patients	Male, N (%)	Age, median (range)	ECOG PS:n (%)	Any grade, N (%)	grade 3-4, N (%)	Discontinuation, N (%)	Death, N (%)
Diaz (10) (2022)	Global	CRC[1]	pembrolizumab 200 mg q3w	153	71 (46.4)	63.0 (24–93)	0:75 (49.0)	10 (7.0)	5 (3)	2 (1)	–
Shitara (11) (2024)	Global	Gastric or Gastroesophagealadeno Carcinoma,	pembrolizumab 200 mg q3w	402	288 (72)	64 (56–70)	0:302 (75.0); 1:100 (25.0)	19 (5.0)	10 (3)	–	–
Lynch (12) (2023)	United States	Classic Hodgkin Lymphoma	pembrolizumab 200 mg q3w	30	12 (40)	33 (18-69)		1 (3.0)	0	–	–
Ready (13) (2023)	Global	NSCLC[2]	nivolumab (240 mg q2w) +ipilimumab (1 mg/kg q6w)	391	236 (60.4)	65.0 (26–89)	0-1	11 (2.8)	8 (2.0)	–	-
				139	90 (64.7)	67.0 (39–90)	2	3 (2.2)	1 (0.7)	–	–
Tykodi (14) (2022)	United States	RCC[3]	Nivolumab (3 mg/kg)+ ipilimumab (1 mg/kg)	52	36 (69.2)	64 (23–86)		7 (13.5)	4 (7.7)	–	–
Frentzas (15) (2024)	Australia	Solid Tumors	Ivonescimab (d1 and d15 of q28d) 0.3、1、3、10、20 and 30 mg/kg	52	18 (35.3)	63 (30, 76)	0:33 (64.7); 1:18 (35.3)	3 (5.9)	2 (3.9)	–	–
Schoenfeld (16) (2022)	United States	NSCLC	durvalumab 1,500 mg q4w/ tremelimumab 75 mg q4w+ HFRT[5]	78	50 (64)	66 (59-72)	0:20 (26); 1:57 (73); 2:1 (1)	1 (3.8)	1 (3.8)	–	–
Oaknin (17) (2024)	Global	Cervical Cancer	nivolumab 240 mg q2w	19	0	51 (43–57)	0:10 (53); 1:8 (42)	0		–	–
			nivolumab 3 mg/kg q2w + ipilimumab 1 mg/kg q6w	45	0	48 (41–55)	0:23 (51) 1:22 (49)	1 (2)	1 (2)	–	–
			nivolumab 1 mg/kg q3w+ ipilimumab 3mg/kg q3w for 4cycles, followed by nivolumab 240 mg q2w	112	0	46 (38.5–54)	0:52 (46); 1:52 (46)	13 (12)	6 (5)	–	1 (0.9)
Monge (18) (2023)	United States	CRC	PexaVec+durvalumab 1500 mg q28d	16	6 (37)	53.5 (28-69)	0:11 (69); 1:5 (31)	0		–	–
			PexaVec+a single dose of tremelimumab (day1) +durvalumab 1500 mg q28d	18	8 (44)	56.5 (28-76)	0:16 (89); 1:2 (11)	2 (11)	1 (6)	1 (6)	–
Necchi (19) (2024)	Global	Bladder Cancer	pembrolizumab 200 mg q3w	132	104 (79)	72 (64.5–77.5)	0:101 (77); 1:28 (21); 2:3 (2)	3 (2)	3 (2)	1 (1)	–

(Continued)

10.3389/fimmu.2025.1551445

TABLE 1 Continued

Author (Year)	Country	Tumor Type	Drug and Dosage	Number of patients	Male, N (%)	Age, median (range)	ECOG PS:n (%)	Any grade, N (%)	grade 3-4, N (%)	Discontinuation, N (%)	Death, N (%)
Grimm (20) (2023)	Europe	RCC	nivolumab 240 mg q2w;nivolumab 3mg/kg+ipilimumab 1 mg/kg q3w	207	147 (71)	65 (57–71)		16 (8)	13 (6)	–	–
Lakhani (21) (2024)	Global	Solid Tumors	retifanlimab 3 mg/kg q2w	134	42 (31)	60 (18-86)	0:42 (31); 1:92 (69)	5 (4)	3 (2)	4 (3)	–
Schöffski (22) (2023)	Belgium, France, Denmark, United States	Soft-Tissue Sarcoma	Olaratumab (15or20 mg/kg, d1d8) + pembrolizumab (200 mg,d1)	41	15 (36.6)	56.83 (43.76-69.9)	0:19 (46.3); 1:22 (53.7)	–	2 (4.9)	–	–
Zhao (23) (2024)	Global	NSCLC	SBRT (24 Gy in three fractions) with sequential tislelizumab (2 cycles of 200 mg) and chemotherapy	46	42 (91)	62 (58–65)	0:36 (78); 1:10 (22)	2 (4)	1 (2)	1 (2)	–
Saba (24) (2024)	United States	HNSCC[4]	nivolumab 240mg q2w and IMRT[6] reirradiation	51	42 (82)	62 (56-67)	0:13 (25); 1:36 (71); 2:2 (3.9)	2 (3.9)	1 (2)	1 (2)	–
Emamekhoo (25) (2022)	United States	RCC	nivolumab 3mg/kg +ipilimumab 1mg/kg q3w; followed by nivolumab 480mg/4weeks	28	24 (86)	60 (38-87)		5 (18)	2 (7)	1 (4)	–
Morano (26) (2022)	Italy	CRC	temozolomide+nivolumab 480 q4w+ipilimumab 1 mg/kg q8w	33	17 (52)	58 (53-65)	0:22 (67); 1:11 (33)	6 (18)	1 (3)	–	–
Kim (27) (2024)	France	Squamous Cell Carcinoma of the anus	atezolizumab 800 mg, q2w+Chemotherapy	64	18 (28)	63.2 (56.0–71.9)	0:37 (58); 1:27 (42)	1 (2)		–	–
George (7) (2022)	United States	RCC	nivolumab 6 mg/kg+ipilimumab 1 mg/kg q8w; alternating with nivolumab 480 mg q8w, staggered q4w	106	86 (81.1)	64.5 (40–84)		15 (14.2)	8 (7.5)	4 (3.8)	–
Ferris (28) (2022)	United States	HNSCC	Cetuximab, Radiotherapy, and Ipilimumab (1,3,10mg/kg)	18	18 (95)	57 (43–74)	0:15 (79);1:4 (21)	1 (6)	1 (6)	1 (6)	–
Xiao (29) (2022)	United States	Solid Tumors	Pembrolizumab 200 mg q3w and SBRT[7]	73					2 (3)	–	–
Marabelle (30) (2020)	Global	Solid Tumors	pembrolizumab200mg q3w	233	96 (41.2)	60.0 (20-87)	0:113 (48.5) 1:120 (51.5)	9 (3.9)	2 (0.9)	–	–

(Continued)

10.3389/fimmu.2025.1551445

TABLE 1 Continued

Author (Year)	Country	Tumor Type	Drug and Dosage	Number of patients	Male, N (%)	Age, median (range)	ECOG PS:n (%)	Any grade, N (%)	grade 3-4, N (%)	Discontinuation, N (%)	Death, N (%)
Stratigos (31) (2021)	Canada, Europe, and the USA.	advanced Basal Cell Carcinoma	cemiplimab 350 mg q3w	84			0 or 1		4 (5)	–	–
Qian (32) (2021)	United States	Melanoma	ipilimumab, nivolumab, or pembrolizumab, or a combination of these	299	197 (66)			54 (18)		–	–
Tawbi (33) (2021)	United States	Melanoma brain metastases	nivolumab 1 mg/kg +ipilimumab 3 mg/kg q3w; nivolumab 3 mg/kg	Asymptomatic patients101	68 (67)	59 (51–66)	0 or 1	7 (7)	7 (7)	–	–
Ascierto (34) (2020)	Global	Melanoma	ipilimumab 10 mg/kg q3w	453					2 (0.4)		1 (0.2)
Goldberg (35) (2020)	United States	NSCLC with brain metastasis	pembrolizumab 10mg/kg q2w	42	14 (33)	60 (56-71)	0:4 (10);1:38 (90)	5 (11.9)	1 (2)	–	–
Campbell (36) (2021)	United States	RCC	tremelimumab 10mg/kg q4w	29	24 (83)	59 (23-82)	0:18 (62);1: 9 (31);2: 2 (7)	6 (21)	6 (21)		
Cacciotti (37) (2020)	United States	Central Nervous System Tumors	ipilimumab,nivolumab and/ or pembrolizumab	11	6 (55)	13.9 (4.1-20.7)	–	3 (27.3)	1 (9)	2 (18.2)	–
Gao (38) (2020)	United States	high-risk Urothelial Carcinoma	durvalumab (1500mg) + tremelimumab (75 mg)	28	20 (71)	71 (24–83)	–	–	2 (7)	–	–
Brastianos (39) (2021)	United States	Leptomeningeal Carcinomatosis	nivolumab+ipilimumab, dosage vary depending on the type of cancer.	18	7 (38.9)	54 (36–70)	0:4 (22.2);1: 10 (55.6);2:4 (22.2)	1 (5.6)	–	1 (5.6)	–
Kawazoe (40) (2020)	Japan	Gastric/ Gastroesophageal Junction Cancer	Pembrolizumab 200 mg q3w	54	43 (79.6)	66 (32–75)	0:46 (85.2);1:8 (14.8)	5 (9.3)	3 (5.6)	–	–
Tolaney (41) (2020)	United States	Breast Cancer	pembrolizumab, 200 mg, q3w	44	0	58 (30-76)	0:35 (80);1:9 (21)	2 (5)	2 (5)	–	2 (5)
Sanborn (42) (2021)	Global	Solid Tumors	pacmilimab (0.3, 1, 3, or 10 mg/ kg) +ipilimumab (3 or 6 mg/kg) q3w for 4 doses, followed by pacmilimab monotherapyq2w.	27	11 (41)	56 (28–70)	0:11 (41);1:16 (59)	2 (7)	2 (7)	1 (3.7)	–

(Continued)

10.3389/fimmu.2025.1551445

TABLE 1 Continued

Author (Year)	Country	Tumor Type	Drug and Dosage	Number of patients	Male, N (%)	Age, median (range)	ECOG PS:n (%)	Any grade, N (%)	grade 3-4, N (%)	Discontinuation, N (%)	Death, N (%)
Desai (43) (2020)	Australia, Korea, New Zealand and Taiwan	Solid Tumors	tislelizumab	451	246 (54.5)	61.0 (18.0- 81.0)	0:169 (37.5);1:282 (62.5)	6 (1.3)	3 (0.7)	–	–
Diefenbach (44) (2020)	United States	Hodgkin Lymphoma	brentuximab1-8mg/kg, nivolumab3mg/kg, and ipilimumab1mg/kg	22	11 (50)	35 (19–60)	0:12 (55);1-2:10 (45)	1 (2)	1 (2)	–	–
Apolo (45) (2020)	United States	Genitourinary Malignances	cabozantinib 40 mg/d, nivolumab 3 mg/kg, and ipilimumab 1 mg/kg	24				2 (8)	2 (8)		
Yap (46) (2021)	Global	Mesothelioma	pembrolizumab 200 mg q3w	118					3 (2.5)		
McDermott (47) (2021)	Global	CRC	pembrolizumab 200 mg q3w	110	86 (78.2)	64 (29-87)	–	6 (5.5)	6 (5.5)	–	–
Boutros (48) (2020)	France	Melanoma	ipilimumab10 mg/kg q3w and radiotherapy	19	10 (53)	58 (35–85)	0 or 1	6 (32)	2 (10.5)	–	–
Felip (49) (2020)	Global	NSCLC	nivolumab 3mg/kg q2w	811	640 (78.9)	66 (31–86)	0:173 (21.3);1:534 (65.8);2:103 (12.7);3:1 (0.1)	7 (0.9)	5 (0.6)	2 (0.2)	–

[1] CRC, colorectal cancer; [2] NSCLC, non-small cell lung cancer; [3] RCC, renal cell carcinom; [4] HNSCC, head and neck squamous cell carcinoma; [5] HFRT, Hypofractionated Radiotherapy; [6] IMRT, Intensity-Modulated Radiation Therapy; [7] SBRT, Stereotactic Body Radiation Therapy.

10.3389/fimmu.2025.1551445

prevalences of irColitis. For example, in CRC, the incidence of irColitis with pembrolizumab was 5.5%-7% (10, 47), whereas a much lower incidence of 2% in bladder cancer (19). Age was also found to be related to the occurrence of irColitis. In patients with a median age of 13.9 years old, the incidence of irColitis was 27.3% (37), a higher incidence than that in elderly patients (aged over 55 years), ranging from 0.9% to 32% (48, 49). In contrast, the incidence was lower in middle-aged population (30-55 years), ranging from 0% to 12% (12, 17, 18, 44). However, Necchi found age may not be the primary factor compared to other factors (19); in this study, the median age of patients was 72 years old, but the incidence of irColitis reported 2%.

As shown in Table 1, 13 of 41 studies reported discontinuation of immunotherapy due to irColitis (7, 10, 18, 19, 21, 23–25, 28, 37, 39, 42, 49). The ICIs used in these studies included single-agent pembrolizumab, tremelimumab, durvalumab, retifanlimab, tislelizumab, nivolumab, and ipilimumab, as well as combinations of nivolumab with ipilimumab, and pacmilimab with ipilimumab. Cacciotti's study observed the highest discontinuation rate at 18.2% (37); this study focused on children and young adults with recurrent pediatric high-grade central nervous system (CNS) tumors, which were treated with ipilimumab, nivolumab, and/or pembrolizumab. The high discontinuation rate may be attributed to the combined effects of tumor malignancy, patient age, and the use of combination immunotherapy. Additionally, two studies reported a 6% discontinuation rate due to irColitis (18, 28). Three studies reported irColitis-related deaths. One case involved a female patient with metastatic cervical cancer, who received nivolumab (1 mg/kg) and ipilimumab (3 mg/kg) every 3 weeks for 4 cycles, followed by nivolumab (240 mg) every 2 weeks (17). Another case involved a patient with advanced melanoma treated with ipilimumab (34). Additionally, two cases involved females with receptor-positive, ERBB2-negative metastatic breast cancer, who died of eribulin and pembrolizumab-induced irColitis due to sepsis (41).

However, we acknowledge the potential for selection bias in our review. Focusing on published clinical trials, we have inadvertently excluded smaller retrospective studies and case reports, potentially underestimating rare presentations of irColitis. Additionally, publication bias may have influenced the reported incidence rates. To mitigate these limitations, we conducted a comprehensive keyword search and systematically reviewed the reference lists of relevant articles.

3 Clinical features, diagnosis, and grading of irColitis

3.1 Clinical features

IrColiti typically manifests as diarrhea, the primary clinical symptom. Diarrhea has been found to occur early during immunotherapy. The median onset of diarrhea/colitis typically occurs around 3 to 6 months after treatment with anti-PD-1/Programmed cell death-ligand 1 (PD-L1) agents, while earlier for anti-CTLA-4 agents at around 6 to 8 weeks (50, 51). However, there are also cases of relapse occurring 1-2 years after treatment discontinuation (52). Patients often experience frequent bowel movements, with stools containing mucus or blood. Symptom severity ranges from mild to severe and may significantly impact the patient's quality of life. Besides diarrhea, patients with irColitis frequently report other gastrointestinal symptoms, including abdominal pain, fever, and, in some cases, upper gastrointestinal symptoms such as dyspepsia, reflux, or heartburn (53, 54).

In addition to the typical symptoms of diarrhea and abdominal pain, irColitis may present with atypical manifestations, including anorexia, significant weight loss or dehydration due to prolonged diarrhea, and fatigue. These systemic symptoms further complicate the clinical management of irColitis (55).

These clinical manifestations may resemble those of other gastrointestinal disorders, making it essential to differentiate irColitis from infections, inflammatory bowel disease (IBD), or ischemic colitis. A careful differential diagnosis is required for proper treatment strategies.

3.2 Endoscopic and pathological findings

The pathological changes associated with irColitis are diverse and complex. Although the condition is typically characterized by pancolitis, irColitis may occasionally affect only the descending colon (56, 57). These changes not only influence clinical diagnosis but also have significant implications for treatment strategies and patient prognosis. Common endoscopic findings included erythema, loss of vascular pattern, granular appearance, and ulcerations; however, normal mucosa might also be observed in some cases (57, 58). Therefore, a biopsy has been recommended for suspect irColitis patients.

According to existing literature, the pathological changes associated with irColitis could be classified into several types: (1) Active colitis is the most common histopathological pattern of irColitis. This form is characterized by neutrophil infiltration in the lamina propria, cryptitis, and crypt abscesses. Crypt atrophy or loss was uncommon but could be found Increased epithelial apoptosis and intraepithelial lymphocytes could also be observed (57); (2) Chronic active colitis is commonly observed after long-term ICI treatment. Histopathological findings include persistent lymphocytic infiltration and disruption of intestinal architecture, such as crypt distortion and pseudopyloric or Paneth cell metaplasia (57). Crypt abscesses and ulceration of the intestinal epithelium may also be present (59). (3) Microscopic colitis often appears normal on endoscopy, making it difficult to detect. Histopathological changes do not show significant acute or active inflammation. However, histological examination may reveal increased intraepithelial lymphocytes and lymphoplasmacytic infiltration in the lamina propria (57, 60). Microscopic colitis is potentially required for additional treatment with oral or intravenous corticosteroids and/or nonsteroidal immunosuppressive agents. In addition to these three common types, other patterns, such as increased apoptosis (61), ischemic colitis (58), and nonspecific inflammatory reactive changes (57), have also been observed.

3.3 Diagnosis and grading of irColitis

The diagnosis of irColitis primarily depends on clinical manifestations, laboratory tests, imaging studies, and endoscopic evaluation, making it a complex process that integrates various standards and methodologies. Currently, several grading systems are employed to assess clinical symptoms, including the Common Terminology Criteria for Adverse Events (CTCAE) (62), the treatment guidelines for irAEs from the European Society for Medical Oncology (ESMO) (63), the American Society of Clinical Oncology (ASCO) (64), and Chinese Society of Clinical Oncology (CSCO) Guidelines for the management of toxicities related to ICIs (65) (Table 2). However, the ESMO/ASCO/CSCO guidelines do not address irColitis separately; instead, they group irColitis and diarrhea together for grading and management.

Endoscopic examination plays a crucial role in diagnosing irColitis as the golden standard (66, 67). However, due to the similar symptoms between irColitis and other gastrointestinal disorders, additional diagnostic tests are essential for differentiating diagnosis. For example, laboratory blood tests and stool analyses can help differentiate irColitis from infections and IBD. The imaging modalities of CT and MRI are more valuable in evaluating the extent and severity of intestinal inflammation, as well as in managing complex cases of irColitis that may pose risks for endoscopic procedures (66).

Beyond conventional diagnostic methods, recent advancements in irColitis biomarkers have shown promise for predicting and diagnosing its occurrence. Anti-integrin αvβ6, a heterodimeric cell adhesion receptor, has been identified as a potential biomarker. A study revealed that Anti-integrin αvβ6 autoantibodies were significantly more prevalent in irColitis patients than in control groups, including patients with other organ irAEs, cancer patients without irAEs, and healthy volunteers (30.8% vs. 1.9%) (68). Furthermore, the presence of Anti-integrin αvβ6 autoantibodies was associated with disease activity, supported by characteristic endoscopic findings, high-grade irColitis, and steroid resistance (68). A single-cell sequencing study of 13 irColitis patients revealed that increased mucosal Regulatory T cells (Tregs), $ITGAE^{Hi}$ CD8 tissue-resident memory T cells (TRMs) expressing C-X-C motif chemokine ligand 13 (CXCL13) and T helper 17 (Th17) gene programs, and recirculating $ITGB2^{Hi}$ CD8 T cells may serve as potential biomarkers for irColitis (69). Another study involving 15 irColitis patients showed that activated CD8+ TRMs cells express high levels of transcripts for checkpoint inhibitors and interferon-gamma and identified Interferon-Gamma-Producing CD8+ TRMs as potential targets (3).

4 The possible mechanism of irColitis

Recent advances in research have illuminated key pathological processes, such as T cell overactivation, pro-inflammatory cytokine production, gut microbiota dysbiosis, and epithelial barrier dysfunction, providing novel insights into irColitis pathogenesis (Table 3, Figure 1).

TABLE 2 Grading systems for irColitis.

Guideline	Grade			
	I	II	III	IV
CTCAE5.0[a] (62)	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Abdominal pain; mucus or blood in stool	Severe abdominal pain; peritoneal signs	Life-threatening consequences; urgent intervention indicated
ESMO (63)	increase of <4 stools/day over baseline	increase of 4-6 stools/day over baseline	increase of≥7 stools/day	Life-threatening consequences or any grade of diarrhea and one of the following: hematochezia, abdominal pain, mucus in stool, dehydration, fever
ASCO (64)	Increase of < 4 stools/day over baseline; mild increase in ostomy output compared with baseline	Increase of 4-6 stools/day over baseline; moderate increase in ostomy output compared with baseline	Increase of ≥7 stools/day over baseline; incontinence; hospitalization indicated; severe increase in ostomy output compared with baseline, and limiting self-care ADL	Life-threatening consequences; urgent intervention indicated
CSCO (65)	Asymptomatic; requires only clinical or diagnostic observation (diarrhea ≤ 4 times/day).	Abdominal pain; fecal mucus or blood (diarrhea frequency 4-6 times/day).	Severe abdominal pain; changes in bowel habits; requires pharmacological intervention; signs of peritoneal irritation (diarrhea frequency ≥ 7 times/day).	Life-threatening symptoms; requires urgent intervention.

[a]:A disorder characterized by inflammation of the colon.

TABLE 3 The possible mechanism of irColitis.

Author	Year	Experimental subject	Mechanism of Effect/ effect cells	Effect
Tomm (4)	2024	Biopsies of patients with irColitis	–	Pathology features: intraepithelial lymphocytes↑, epithelial cell apoptosis↑, and active inflammation
Malik (70)	2023	mouse model of acute colitis	upregulation of IFNγR-STAT-IRF1 pathway	IFN-γ↑, IRF1↑, STAT activation; GM-CSF+CD4+ T cells↑, IL-17↑
Lo (71)	2024	irColitis mouse model with dissimilar gut microbiota composition	–	CD4+ T cells↑, IFN-γ↑, Th1 response↑, Treg↑; WildR microbiota (+)
Zeng (72)	2024	colitis mouse model	activation of JAK2/STAT3/ SOCS3 pathway	TIPE2↑, tight junction proteins (Occludin, Claudin-1, ZO-1)↓, SOCS3↑ Epithelial barrier dysfunction
Reschke (73)	2022	patients with irColitis	–	IFN-γ↑, CXCL9↑, CXCL10↑, CXCL11↑, IL-17↑; Th1 response↑; CD8 + TRM↑, CD4 + TRM↑
Halsey (74)	2023	patients with irColitis	–	Collinsella↓, Bifidobacterium↓, Tyzerella↑
Thomas (69)	2024	colon mucosa cells from patients with irColitis	–	IL17A↑, IL26↑, CXCL13↑, Th17↑; CD8 + Tcell↑, CD8 + TRM↑, CD4 + TRM↑; intravascular effector memory populations↑; Epithelial defects: stem cells↓, transit amplifying cells↑, and top crypt epithelial cells (with ISGs, CASP1, ZBP1, ICAM1, CD274/PD-L1, CXCL10/11)↑, aquaporin (AQP) water channel genes↓
He (75)	2024	colon biopsies and blood from patients with irColitis	–	CD8 + T↑, CD8 + TRM↑, CD4 + TRM↑; perturbed stromal metabolism
Ghosh (76)	2024	patients with irColitis	–	CD8 + TRM↑, CD4 + TRM↑,IL-17↑; CXCL8↑, GM-CSF↑, CXCL1↑, IL-6↑, TNF-α↑
Ye (77)	2024	irColitis mouse model	DICB (Double immune checkpoint blockade)/ TNF-α/gut microbiota loop	TNF-α↑, IL-6 ↑, IL-1β↑; Bacteroides↑

"↑" means an increase, while "↓" represents a decrease in expression.

4.1 The activation and disorder of immune system

4.1.1 Excessive T cell activation and pro-inflammatory cytokine production

By blocking inhibitory PD-1/CTLA-4 pathways, ICIs activate inhibited T cells thereby enhancing the immune system's ability to attack tumor cells; however, this activation can cause T cell overactivation, resulting in the loss of immune tolerance, particularly in mucosal tissues, which is a key driver of irColitis (75).

Substantial evidence supports that CD8+ T cells are the key effector cells in irColitis (2). A cross-sectional study of clinical and pathological data analyses from patients with anti-CTLA-4/PD-1 colitis demonstrated that CD8+TRMs constituted the majority of activated TRMs in irColitis, with the degree of activation correlating with clinical and endoscopic severity. Notably, these CD8+ TRMs exhibited significantly higher expression of Interferon-gamma (IFN-γ), which further promoted TRMs activation. This finding was complemented by evidence of upregulation of classic Janus kinase/ Signal transducer and activator of transcription (JAK/STAT) components in the IFN-γ signaling pathway in irColitis (3). Collectively, the upregulation of IFN-γ signaling represents a key pathological feature of irColitis, with the JAK/STAT signaling pathway playing a central role in this process, thereby providing a theoretical basis for potential therapeutic strategies, such as JAK inhibitors (74).

Thomas et al. applied single-cell multi-omics to analyze approximately 300,000 cells from the colonic mucosa and blood samples of patients with irColitis. Their findings revealed that the expansion of mucosal Tregs, CD8+ TRMs, and recirculating CD8+ T cells are hallmark features of irColitis, particularly T cells expressing pro-inflammatory gene programs, such as CXCL13 and Th17. In patients with irColitis, 1.9% of CD8+ T cells expressed Interleukin-17 (IL-17) A, while IFN-γ and CXCL13 were significantly upregulated in intestinal tissue samples (69). These cytokines synergistically impair epithelial integrity and drive chronic inflammation, further elucidating the mechanisms underlying irColitis. CD4+ T cells can differentiate into various phenotypes, including T helper 1 (Th1), Th17, and Treg cells. Th1 cells primarily secrete IFN-γ, while Th17 cells produce cytokines such as IL-17, which is closely associated with the pathogenesis of intestinal inflammation. Numerous studies have indicated that the overactivation of CD4+ T cells contributes to the development and

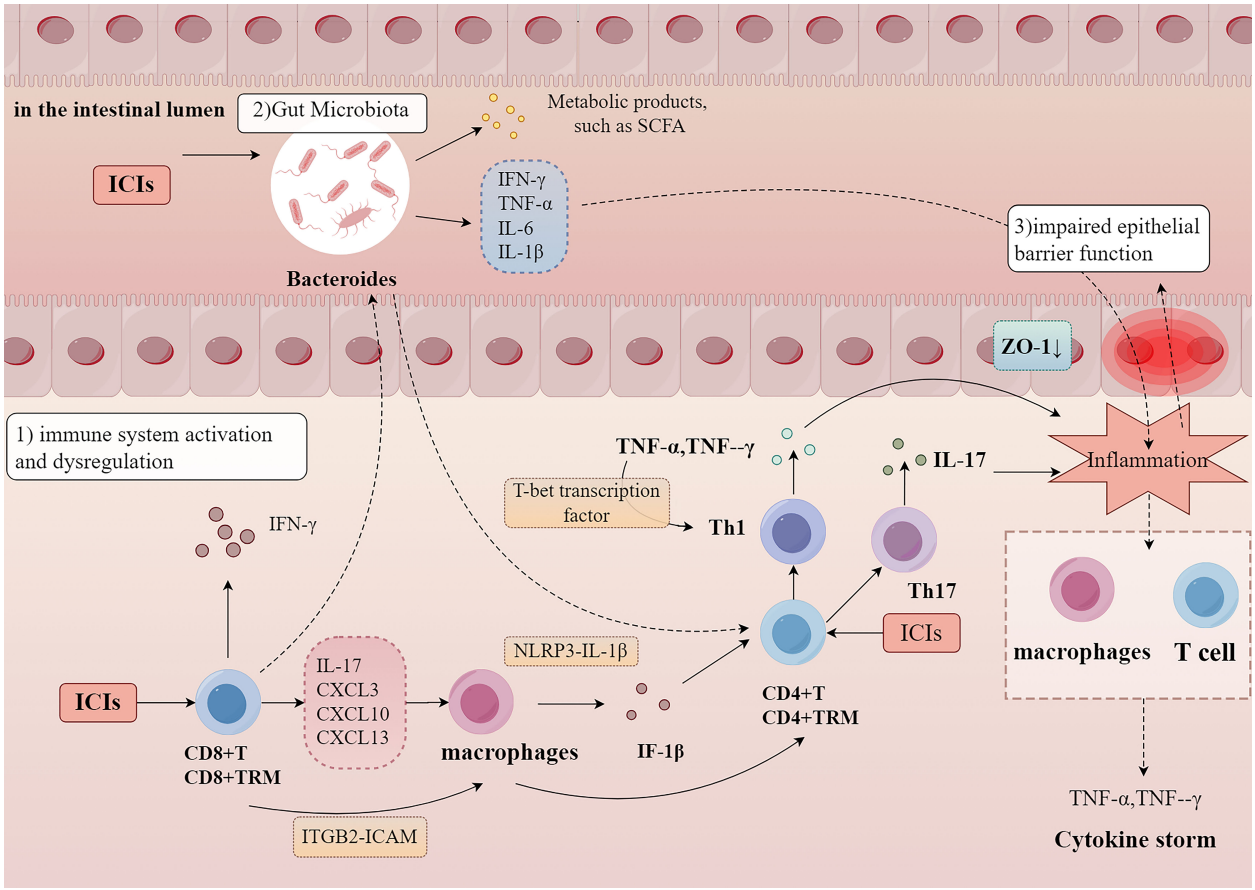

FIGURE 1
The possible mechanism of irColitis. ICIs induce immune system activation and dysregulation, resulting in excessive T cell and macrophage activation, along with increased pro-inflammatory cytokine release. Gut microbiota, particularly *Bacteroides*, contribute to inflammation by producing metabolic byproducts and modulating cytokine expression. Additionally, epithelial barrier dysfunction, characterized by reduced ZO-1 expression, further amplifies inflammation, ultimately leading to immune dysregulation and a cytokine storm. (TRM, tissue-resident memory T cells; SCFA, Short-Chain Fatty Acids; ZO-1, Zonula Occludens-1; IFN, Interferon; TH, T helper cells; IL, Interleukin; TNF, Tumor Necrosis Factor. Detailed abbreviations are presented in Supplementary Table 2. Image created with Figdraw.com).

progression of irColitis (78). To further elucidate the roles of CD4+ T cells in irColitis, Lo et al. established an irColitis mouse model using anti-CTLA-4 treatment. Their findings demonstrated that CTLA-4 blockade significantly promotes the accumulation of IFN-γ+ CD4+ T cells, resulting in intestinal tissue damage, which is considered the initial trigger of irColitis (71). Additionally, they observed that IFN-γ expression was closely linked to the activation of the T-bet transcription factor, which amplified the Th1 response and underscores the Th1-biased inflammatory response in irColitis. Furthermore, a recent study analyzing colon biopsies and blood samples from irColitis patients revealed that CD4+ TRMs were progenitors of cytotoxic effectors such as CD8+ T cells, thereby identifying CD4+ TRMs as novel therapeutic targets (75).

4.1.2 Immune cell interactions

Interactions among immune cells play a pivotal role in disease pathogenesis when irColitis occur. ICIs relieve immune suppression, thereby altering the crosstalk among various immune cell populations within the intestines. These interactions notably include those between T cells and macrophages, dendritic cells, and epithelial cells.

Recent research has shown that activated CD8+ T cells interact with macrophages through C-X-C Chemokine Receptor Type 3 (CXCR3) and macrophage-derived C-X-C Motif Chemokine Ligand 10 (CXCL10), as well as Integrin beta 2-Intercellular Adhesion Molecule (ITGB2-ICAM) pathways. These interactions facilitated macrophage recruitment and activation, initiating localized inflammatory responses in the intestine (55, 69). To further investigate this mechanism, Ankit et al. created an immunodeficient mouse model. Their results demonstrated that conditional deletion of Major Histocompatibility Complex class II (MHC-II) in macrophages reduced the proportion of Granulocyte-macrophage colony-stimulating factor (GM-CSF) + CD4+ T cells by 70%, from 18.3% to 5.5%. This suggests that macrophages present antigens via MHC-II and release Interleukin-1 beta (IL-1β), which promotes the differentiation of CD4+ T cells into GM-CSF+ subsets, contributing to irColitis. Moreover, the study revealed that inhibiting the Nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome significantly reduced IL-1β production in macrophages, which in turn decreased the population of GM-CSF+ CD4+ T cells (70). These findings underscored the role of

macrophages in regulating T cell activation through the NLRP3-IL-1β axis, offering potential therapeutic targets for irColitis.

The interaction between T cells and epithelial cells plays a crucial role in the pathogenesis of irColitis. Epithelial cells enhance the pro-inflammatory activity of CD4+ T cells via MHC-II-mediated antigen presentation. Moreover, the absence of IFN-γ signaling in epithelial cells exacerbates the pathological activation of CD4+ T cells (70). Elevated expression of PD-L1 in intestinal epithelial cells has also been observed; however, this pathway becomes dysregulated in the presence of ICIs, leading to uncontrolled T cell cytotoxic responses. This dysregulation indicates a loss of immune tolerance in patients with irColitis (79).

4.2 The dysbiosis of gut microbiome

Recent metabolomics evidence has shown that dysbiosis-induced alterations in lipid and amino acid metabolism contribute to gut inflammation in irColitis.

Ye et al. suggested that certain microbial species, particularly within the Bacteroides genus, exacerbated irColitis through pro-inflammatory effects. Positive correlations between bacteroides and inflammatory factors, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-1β, highlighted the interactions between microbiota, metabolites, and inflammation. Additionally, gut microbiota dysbiosis intensified TNF-α signaling, further disrupting microbial composition and metabolite production, thereby aggravating irColitis (77).

Activated CD8+ TRMs may contribute to the upregulation of IFN-γ signaling and the development of irColitis by mediating immune responses to both commensal or pathogenic microbes. This suggests that alterations in the microbiome play a critical role in the pathogenesis of irColitis. Therapeutic strategies targeting the gut microbiome, such as fecal microbiota transplantation (FMT), may provide a promising approach to mitigate this condition (3).

The microbiome plays a critical role in the activation of CD4+ T cells and the onset of colitis. Severe intestinal inflammation induced by anti-CTLA-4 treatment occurs only in mice with a fully functional, free-living microbiome. This suggests that the composition of the gut microbiota not only influences CD4+ T cell activation but may also exacerbate inflammatory responses through interactions with the immune system (71).

In conclusion, we find that gut microbiota dysbiosis both drives and results from irColitis. Dysbiosis amplifies local inflammation through pro-inflammatory species and disrupted metabolic pathways, establishing a vicious cycle.

4.3 The disruption intestinal barrier

4.3.1 Epithelial barrier dysfunction

The intestinal epithelial barrier is essential for preventing antigen transfer, and its damage is considered one of the key pathological features of irColitis.

As discussed earlier, the enrichment of pro-inflammatory cytokines, such as IFN-γ and TNF-α, is observed in irColitis. Previous evidence supported that these cytokines could increase epithelial cell apoptosis, thereby impairing the intestinal epithelial barrier and contributing to bowel inflammation, a key factor in the development of irColitis (72). Epithelial defects, including upregulation of apoptosis-related genes (Caspase-1 [CASP1], Caspase-8 [CASP8]), interferon-stimulated genes (STAT1, PD-L1), and pro-inflammatory marker genes (CXCL10), as well as downregulation of aquaporin water channel genes, suggest impaired water and solute transport in the intestines (69). Disruption of tight junction proteins, such as myosin light chain kinase (MLCK) and zonula occludens-1 (ZO-1), increased intestinal permeability and epithelial barrier dysfunction, allowing microbial products to activate immune cells, including T cells and macrophages. This triggered a cytokine storm involving TNF-α and IFN-γ, and further amplified inflammation in irColitis (80).

Interventions targeting the epithelial barrier, such as MLCK inhibitors, show promise in reducing inflammation while preserving the efficacy of tumor immunotherapy. This strategy may effectively manage irColitis by restoring barrier integrity.

4.3.2 Alterations of intestinal structure

Epithelial remodeling, a key feature of irColitis, also represents the precursor to intestinal reconstruction. A clinical study highlighted significant structural alterations in the intestine during irColitis, including epithelial barrier disruption, crypt architectural distortion, and increased immune cell infiltration (4). These changes compromise intestinal integrity, allowing microbial antigens to reach immune cells, which perpetuates inflammation. Chronic inflammation is linked to abnormal epithelial regeneration and the loss of goblet cells, further impairing mucosal defense.

At the cellular and molecular level, evidence suggested that interferon signaling induces changes in epithelial differentiation, leading to a decrease in $LGR5^{Hi}$ stem cells and an increase in transit-amplifying cells, along with the loss of mature epithelial cells and enrichment of interferon-induced epithelial subsets. Additionally, some cases of irColitis feature vascular and fibroblast remodeling, potentially driven by the expansion of angiogenesis-related genes (Hypoxia-inducible factor 1 alpha [HIF1A], Vascular Endothelial Growth Factor A [VEGFA]), highlighting the potential role of neovascularization in the pathological process of irColitis (69, 81).

5 Treatment of irColitis

The recommended treatment of irColitis primarily included corticosteroids, biologic agents, and certain non-pharmacological interventions. Moreover, TCM, as a natural therapeutic approach, has shown a promising potential in the treatment of this condition.

5.1 Corticosteroids

Corticosteroids are the first-line treatment for irColitis, primarily used to suppress T cell activation, thereby inhibiting the production of pro-inflammatory cytokines and regulating overactive innate and adaptive immune responses (82). The NCCN guidelines recommend

that patients experiencing grade 2 or higher irColitis should discontinue immunotherapy and receive prednisone or methylprednisolone (1–2 mg/kg/day) until symptoms improve to grade 1 or lower. Corticosteroid tapering should then be performed over 4 to 6 weeks (83). However, some patients may develop resistance to corticosteroids or exhibit poor responses, with relapses occurring during dose reduction, complicating further management (82, 84). A retrospective study reported a clinical response rate of 70% (14/20) to corticosteroid treatment. Among these 14 patients, 2 (14%) experienced relapse during corticosteroid tapering and became steroid-dependent (85). Another retrospective analysis of 49 irColitis patients found that all received glucocorticoids as first-line treatment; however, immunotherapy was discontinued in 21 cases (86). In such cases, it is essential to promptly assess the patient's clinical condition, differentiate irColitis from other gastrointestinal disorders, and identify potential complications. Alternative therapeutic options should then be considered based on this evaluation.

5.2 Biologic agents

For patients with corticosteroid resistance or inadequate response, biologic agents are an important therapeutic option. Recent studies have shown that anti-TNF-α monoclonal antibody infliximab and α4β7 integrin immunoglobulin G1 (IgG1) monoclonal antibody vedolizumab exhibited promising efficacy in the treatment of irColitis (82, 83). If no improvement is observed within 2 to 3 days after initiating corticosteroid therapy, infliximab or vedolizumab may be considered within 2 weeks of diarrhea onset while continuing corticosteroid treatment (83). Additionally, patients who relapse during corticosteroid tapering or after completing the corticosteroid regimen may also require additional immunosuppressive therapy (67). Infliximab, a second-line treatment, is widely used in corticosteroid-resistant patients. Clinical data demonstrate its effectiveness in severe acute colitis cases, with a retrospective study reporting a 71.4% remission rate for corticosteroid-resistant irColitis (87). Furthermore, infliximab has been shown to help maintain overall survival (OS) in these patients (88). A study comparing infliximab with corticosteroids demonstrated that infliximab resulted in a shorter median time to diarrhea resolution and corticosteroid tapering compared to the corticosteroid group without negatively affecting OS or time to treatment failure (TTF) (89). Another biologic agent, vedolizumab, is commonly administered intravenously at a dose of 300 mg (90). Although vedolizumab had not been as extensively studied in clinical applications as infliximab; a multicenter retrospective study demonstrated that vedolizumab treatment for corticosteroid-resistant irColitis resulted in sustained clinical remission in 84% of patients (90).

5.3 Gut microbiota

The gut microbiota plays a crucial role in regulating intestinal immune function. One study found that *Faecalibacterium prausnitzii* helped reduce intestinal toxicity and boost tumor immunity, enhancing the effectiveness of dual CTLA-4 and PD-1 checkpoint blockade (91). This suggests that supplementary probiotics could potentially reduce the risk of colitis while improving the response to immunotherapy. FMT is an emerging therapeutic approach that has been applied to patients with refractory irColitis. A study involving 12 patients with severe irColitis showed that FMT effectively improved clinical symptoms, with 92% of patients achieving clinical remission after treatment (74). This effect may be mediated through FMT's influence on gut microbiota diversity and composition. Additionally, a significant reduction in total lymphocytes and CD8+ T cells was observed in complete responders, indicating that FMT mediates inflammation reduction in these patients (74).

5.4 Traditional Chinese medicine and irColitis

TCM has a long and rich history in treating digestive disease, with extensive experience in treating irAEs. It also offers valuable insights into the treatment of irColitis. TCM views the human body as an integrated whole. When the balance of Yin and Yang and the normal function of the internal organs are maintained, as described in the *Huangdi Neijing* (Yellow Emperor's Inner Canon), "When Yin is in balance and Yang is hidden, the spirit is healthy," and "When the vital Qi is preserved within, pathogens cannot invade." Immunotherapy with ICIs can disrupt this balance while targeting tumors, leading to irAEs. In TCM, irColitis is often classified under categories such as "diarrhea (Xie Xie)" and "intestinal dysentery (Chang Pi)." The occurrence of irColitis is believed to be closely related to pathogenic heat, damp-heat, damage to the intestinal collateral, and spleen-stomach weakness. As the disease progresses, patients may also develop symptoms of Spleen and Kidney Yang deficiency (5). The treatment principles in TCM involve clearing heat and detoxifying, strengthening the spleen and resolving dampness, and warming and tonifying the spleen and kidneys to regulate immune function, balance the internal organs, alleviate symptoms, and promote intestinal recovery. Additionally, TCM offers various methods for treating "diarrhea," including oral administration, moxibustion, acupoint application, and enemas (5). The rich experience in treating "diarrhea" in TCM can provide valuable insights for future experimental studies and clinical trials on natural product therapies for irColitis.

Since no specific studies have investigated the use of TCM for irColitis, we could retrieved related studies based TCM theory of "treating different diseases with same method". In TCM, ulcerative colitis, Crohn's disease, and irColitis share similar symptoms, including diarrhea, abdominal pain, and rectal bleeding. These conditions are classified under the categories of "diarrhea" or "intestinal dysentery". Therefore, this study conducted a comprehensive review of clinical articles on TCM treatments for diarrhea-related diseases from databases of PubMed and CNKI. This review provides valuable insights for future research (Table 4). The PubMed search utilized the following keywords and MeSH terms: ("Traditional Chinese Medicine" OR "Chinese herbal medicine" OR "Chinese medicine") AND ("ulcerative colitis" OR "Crohn's disease" OR " colitis "), focusing on clinical studies

published within the past five years. A similar search strategy was applied to Chinese databases. The inclusion criteria were as follows: (1) studies reported clinical outcomes of TCM in the treatment of irColitis or IBD; (2) studies had a standard research design. Studies were excluded if they lacked specific outcome measures, had an unclear study design, or involved insufficient sample sizes that could compromise statistical validity. To minimize selection bias, representative studies from both English and Chinese databases were included, with a preference for those demonstrating high methodological rigor. However, potential selection bias remains due to publication bias and variations in study design, which is recognized as a limitation.

Fifty articles focused on treatments such as oral Chinese medicine, bamboo scraping therapy, acupoint application, acupuncture, and moxibustion. Due to the limited number of irColitis cases, conducting large-scale clinical trials remains challenging. Therefore, as indicated in Table 4, most of the reviewed studies explore herbal formulas for the treatment of ulcerative colitis and Crohn's disease. However, a study has explored the use of Chinese patent medicine (105). Given its convenience and cost-effectiveness, further research in this area is warranted.

In TCM, acute inflammatory symptoms are typically associated with Damp-Heat Syndrome. Treatment focuses on clearing heat, detoxifying, and drying dampness, using classic formulas such as Gegen Qinlian decoction, Baitouweng decoction, and Shao Yao decoction. Frequently used herbs include *Phellodendri Cortex* (Huang Bai), *Coptidis Rhizoma* (Huang Lian), *Scutellariae Radix* (Huang Qin), and *Rhei Radix et Rhizoma* (Da Huang) for their heat-clearing, damp-drying, and detoxifying properties. In TCM theory, it is commonly believed that the patient enters a stage of "deficiency" (Zheng Xu), then treatment focused on tonifying the spleen and stomach for improving patient's condition. Additionally, TCM emphasizes supporting the body's vital energy (Zheng Qi) with herbs such as *Atractylodis Macrocephalae Rhizoma* (Bai Zhu), *Glycyrrhizae Radix et Rhizoma* (Gan Cao), and *Dioscoreae Rhizoma* (Shan Yao).

6 Future research directions and challenges

The widespread use of ICIs in oncology has led to increasing clinical and research interest in irColitis. Although increasing findings on irColitis published (8), studies on epidemiological characteristics and underlying mechanisms remain insufficient, needing future researches.

6.1 In-depth mechanistic studies

The exploration of mechanisms on the effect Chinese medicine and integrative therapies for irColitis remains limited. Some studies demonstrated the potential interactions between TCM and ICIs, as well as the underlying mechanisms of integrated TCM and Western medicine in the treatment of irColitis. A meta-analysis evaluating the efficacy, safety, and potential mechanisms of TCM as an adjuvant therapy in cancer immunotherapy indicated that TCM influenced PD-1/PD-L1 inhibitors through tumor microenvironment modulation, gut microbiota regulation, inhibition of PD-1 or PD-L1 expression, and cytokine signaling regulation (107). Previous studies on inflammatory bowel diseases IBD with similar symptoms, such as Crohn's disease and ulcerative colitis, suggested that Chinese medicine might alleviate irColitis through mechanisms including immune regulation, reduction of intestinal inflammation, modulation of gut microbiota, and protection of the intestinal mucosal barrier (2). This highlights the great potential of integrative traditional Chinese and Western medicine in treatment.

For example, the JAK/STAT and NF-κB signaling pathways may serve as important potential targets. PD-1/PD-L1 expression is regulated by JAK/STAT and NF-κB signaling pathways, both of which are targeted by TCM like Baicalin and Gegen Qinlian decoction (108–110). Experimental studies have demonstrated that Baicalin is an effective treatment for IBD. By restoring the Th17/Treg balance through the JAK/STAT signaling pathway and reducing ZO-1 secretion, Baicalin alleviates intestinal inflammation and preserves the integrity of the intestinal epithelial barrier, thereby improving clinical symptoms (108). This signaling pathway is particularly important during the acute phase of irColitis. On the other hand, research suggested that the inhibition of Toll-like receptor 4/Nuclear factor kappa B (TLR4/NF-κB) signaling and the enhancement of gut microbiota abundance might be key mechanisms the therapeutic efficacy of Gegen Qinlian decoction as well as other TCM decoctions in the treatment of IBD (109, 110). Additionally, traditional herbal medicines, such as Sophora flavescens Aiton, P. grandiflorus, and Kuijieling decoction, have also shown significant effects in restoring gut immune function. These herbs achieve this by suppressing excessive T cell activation and rebalancing Th17/Treg (108, 111, 112). Research on P. grandiflorus in the treatment of IBD has revealed that this herbal remedy modulates the homeostasis of colonic immune cells through the mesenteric lymphatic circulation, offering valuable insights for future research directions (113).

Meanwhile, TCM may exert its effects through different targets depending on the progression of irColitis. In the acute phase of irColitis, characterized by severe inflammation and epithelial barrier disruption, TCM interventions targeting rapid immune modulation and inflammation control may be particularly beneficial. For example, Baicalin and Gegen Qinlian decoction have been shown to effectively reduce severe acute bowel inflammation through JAK/STAT and TLR4/NF-κB signaling passageway, regulating the secretion of pro-inflammatory cytokine such as IL-17, IL-6, and IL-1β (108–110). When combined with corticosteroids, they may help achieve faster symptom control. Additionally, the multi-targeted mechanisms of TCM can alleviate the side effects associated with corticosteroid use, thereby improving patient tolerance and adherence to treatment.

The gut microbiome plays a critical role in the pathogenesis of irColitis and represents a promising area for further research. In

TABLE 4 The potential clinical experience of TCM in the treatment of irColitis.

Author (Year)	Number of patients	Treatment Method	Composition of treatment	Therapeutic Principles	Frequency	Therapeutic Effects	Type	TCM Diagnosis	Disease	Potential Mechanisms of Action
Yang (2020) (92)	70	Oral	*Gegen Qinlian decoction*: *Radix Puerariae* (Ge Gen), *Scutellariae Radix* (Huang Qin), *Coptidis Rhizoma* (Huang Lian), *Glycyrrhizae Radix et Rhizoma* (Gan Cao).	heat-clearing and detoxifying	7d (250 mL, twice daily)	diarrhea↓, tenesmus↓,5-HT↓, ZO-1 ↑	RCT[1], single-blind	Damp-Heat Syndrome	colon or rectal cancer	regulating the composition of gut microbiota, enhancing intestinal barrier function
Shen (2021) (93)	119	Oral	*Qing-Chang-Hua-Shi granules: Coptidis Rhizoma* (Huang Lian), *Scutellariae Radix* (Huang Qin), *Rhizoma Sinensis* (Bai Jiang Cao), *Radix Angelicae Sinensis* (Dang Gui), *Radix Paeoniae Albae* (Bai Shao), *Radix Sanguisorbae (Di Yu), Radix Arnebiae* (Zi Cao), *Radix et Rhizoma Rubiae* (Qian Cao), *Radix Angelicae Dahuricae* (Bai Zhi), *Radix Aucklandiae (Mu Xiang), Radix et Rhizoma Glycyrrhizae* (Gan Cao).	heat-clearing and detoxifying	125g, twice daily	mucous and bloody stools↓, Mayo score↓, mucosal response rate, and mucosal healing rate↑	RCT, Multic-enter, double-blind	–	UC[2]	preventing inflammatory responses, inhibiting apoptosis through the MEK/ERK signaling pathway and protecting intestinal epithelial cells
Xu (2019) (94)	124	Enema	*Baitouweng Decoction*: *Pulsatillae Radix* (Bai Tou Weng), *Phellodendri Cortex* (Huang Bai), *Coptidis Rhizoma* (Huang Lian), and *Fraxini Cortex* (Qin Pi).	heat-clearing and detoxifying, Eliminate Dampness	once daily, for 30days	Improving symptoms and shortening hospitalization time.	RCT	Damp-Heat Syndrome	UC	improve intestinal function, and promote the restoration of cellular immune function.
Wu (2024) (95)	86	Oral	*Qingre Zaoshi Jiedu Huyin Decoction*: *Notoginseng Radix et Rhizoma* (San Qi, fried), *Atractylodis Macrocephalae Rhizoma* (Bai Zhu, fried), *Poria* (Fu Ling), *Dioscoreae Rhizoma* (Shan Yao, fried), *Lablab Semen Album* (Bai Bian Dou), *Coicis Semen* (Yi Yi Ren), *Platycodonis Radix* (Jie Geng), *Amomi Fructus* (Sha Ren, taken at the end), *Daemonoropis Resina* (Xue Jie), *Coptidis Rhizoma* (Huang Lian), *Magnoliae Officinalis Cortex* (Hou Po), *Pulsatillae Radix* (Bai Tou Weng), *Atractylodis Rhizoma* (Cang Zhu), *Citri Reticulatae Pericarpium*	Clear heat, dry dampness, detoxify, and protect yin.	120 to 240 mL, once daily, for 2 months.	Improvement in symptoms such as urgency with a feeling of incomplete bowel evacuation, diarrhea, abdominal pain, bloating, blood in stools, loss of appetite, and fatigue.	RCT	Damp-heat syndrome, spleen yin deficiency.	UC	–

(Continued)

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TABLE 4 Continued

Author (Year)	Number of patients	Treatment Method	Composition of treatment	Therapeutic Principles	Frequency	Therapeutic Effects	Type	TCM Diagnosis	Disease	Potential Mechanisms of Action
			(Chen Pi), *Paeoniae Radix Alba* (Bai Shao), and *Glycyrrhizae Radix et Rhizoma* (Gan Cao).							
Peng (2024) (96)	80	bamboo scraping therapy combined with acupoint application	*Shaoyao Decoction* for acupuncture point application: *Paeoniae Radix Alba* (Bai Shao), *Arecae Semen* (Bing Lang), *Angelicae Sinensis Radix* (Dang Gui), *Rhei Radix et Rhizoma* (Da Huang), *Coptidis Rhizoma* (Huang Lian), *Scutellariae Radix* (Huang Qin), *Aucklandiae Radix* (Mu Xiang), *Cinnamomi Cortex* (Rou Gui), and *Glycyrrhizae Radix et Rhizoma* (Gan Cao).	"TongYinTongYong"(Treating the root cause with universal applicability)	bamboo scraping twice a week in a cycle, acupoint application once daily for 2 weeks.	Improvement in symptoms such as diarrhea, abdominal bloating, abdominal pain, purulent blood stools, and tenesmus.	RCT	Damp-Heat Syndrome	UC	Protecting the patient's intestinal mucosa, reducing inflammation, and regulating immune function.
Peng (2022) (97)	1	Oral	*Jianpi Qingre Huashi Granular Decoction: Codonopsis Radix (Dang Shen), Atractylodis Macrocephalae Rhizoma (Bai Zhu), Poria (Fu Ling), Aucklandiae Radix (Mu Xiang), Agastachis Herba (Huo Xiang), Puerariae Lobatae Radix (Ge Gen), Coptidis Rhizoma (Huang Lian), Dioscoreae Rhizoma (Shan Yao), Hordei Fructus Germinatus (Mai Ya), Notoginseng Radix et Rhizoma (San Qi), Atractylodis Rhizoma (Cang Zhu), Coicis Semen (Yi Yi Ren), and Sanguisorbae Radix Carbonisata (Di Yu Tan).*	Strengthen the spleen, clear heat and transform dampness, regulate qi and relieve pain, activate blood circulation, and stop bleeding	150ml, once daily, for 30days	Improvement in the scores of diarrhea, bloating, physical fatigue, and the total TCMSS score.	N-of-1 Trial	Spleen deficiency with damp-heat	UC	–
He (2024) (98)	60	Oral	*Xuchangqing* (Xuchangqing), *Coptidis Rhizoma* (Huang Lian), *Scutellariae Radix* (Huang Qin), *Aucklandiae Radix* (Mu Xiang, processed), *Paeoniae Lactiflorae Radix* (Shao Yao, fried), and *Glycyrrhizae Radix et Rhizoma* (Gan Cao), among others.	Clear Heat, Eliminate Dampness, Promote Qi Circulation, and Relieve Pain	twice daily for 8weeks	Improvement in symptoms such as diarrhea, bloody mucous stools, abdominal pain, bloating, anal burning, and tenesmus.	RCT	Internal Damp-Heat Syndrome	UC	–

(Continued)

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TABLE 4 Continued

Author (Year)	Number of patients	Treatment Method	Composition of treatment	Therapeutic Principles	Frequency	Therapeutic Effects	Type	TCM Diagnosis	Disease	Potential Mechanisms of Action
Ben-Horin (2024) (99)	42	Oral	Curcumin-QingDai Combination	–	3 capsules daily for 4 weeks	Increase in clinical remission rate and improvement in endoscopy, CYP1A1 ↑	double-blind, randomized, placebo-controlled	–	ulcerative colitis	Induction of the transcription factor AhR translocation from the cytoplasm to the nucleus, thereby inducing the expression of the CYP1A1 and CYP1A2 genes.
Erol Doğan (2024) (100)	48	Oral	Curcumin, and Resveratrol	–	two capsules daily,8 weeks	Disease activity↓, inflammation↓, quality of life↑	prospective multicenter three-arm RCT	–	ulcerative colitis	–
Guo (2022) (101)	63	Acupuncture and Moxibustion	–	mechanical or thermal stimulation	12-week	CDAI↓,TGF-β 1↓, TβR2↓, Smad3↓, Snail↓	RCT, single-blind	–	Crohn's Disease	transforming growth factor β 1 (TGF- β 1)/ Smad3/ Snail pathway.
Qi (2021) (102)	69	moxibustion	Qihai (CV6),bilateral Tianshu (ST25), Shangjuxu (ST37)	stimulate channel-qi	randomized, single-blind	SDS↓, SAS↓, IBDQ↑	RCT, single-blind	–	ulcerative colitis	toll-like receptors 4 signaling pathways
Guo (2024) (103)	60	Oral	Huanglian Wendan Tang (Coptis and Warm Gallbladder Decoction): *Pinelliae Rhizoma Praeparatum* (Fa Ban Xia), *Aurantii Fructus Immaturus* (Zhi Shi), *Bambusae Caulis in Taeniam* (Zhu Ru), *Citri Reticulatae Pericarpium* (Chen Pi), *Poria* (Fu Ling), *Coptidis Rhizoma* (Huang Lian), *Glycyrrhizae Radix et Rhizoma* (Gan Cao), *Zingiberis Rhizoma Recens* (Sheng Jiang).	Clear the intestines and resolve dampness, purge heat and relieve pain, tonify the spleen and kidney.	twice daily for 4weeks	The time to clinical symptom relief↓, Mayo score↓, UCEIS score.↓	RCT	Internal Damp-Heat Syndrome	ulcerative colitis	TNF-α↓, NF-κB↓, TLR4↓, IL-10↑
Fan (2020) (104)	60	Oral	Anchang Yuyang Decoction combined with mesalazine:*Astragali Radix* (Sheng Huang Qi), *Atractylodis Macrocephalae Rhizoma Praeparatum* (Chao Bai Zhu), *Coicis Semen* (Yi Yi Ren), *Patriniae Herba* (Bai Jiang Cao), *Coptidis Rhizoma*(Huang Lian), *Scutellariae Radix* (Huang Qin),	Treat both the root cause and symptoms, support the righteous qi and expel pathogens, and combine attacking and tonifying strategies.	once daily,for 12-week	Lesion reduction and symptom relief.	RCT	spleen deficiency and dampness retention	ulcerative colitis	TNF-α↓, IL-10↑

(Continued)

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TABLE 4 Continued

Author (Year)	Number of patients	Treatment Method	Composition of treatment	Therapeutic Principles	Frequency	Therapeutic Effects	Type	TCM Diagnosis	Disease	Potential Mechanisms of Action
			Aucklandiae Radix (Mu Xiang), *Arecae Semen* (Bing Lang), *Sanguisorbae* Radix Carbonized (Di Yu Tan), *Bletillae Rhizoma* (Bai Ji), *Angelicae Sinensis Radix* (Dang Gui), *Paeoniae Radix Alba Praeparata* (Chao Bai Shao), *Saposhnikoviae Radix* (Fang Feng), *Glycyrrhizae Radix et Rhizoma* (Sheng Gan Cao).							
Dai (2024) (105)	140	anus	Qingchang suppositry:Indigo Naturalis (Qing Dai), Portulacae Herba (Ma Chi Xian), Notoginseng Radix et Rhizoma (San Qi), Galla Chinensis (Wu Bei Zi), Borneolum Syntheticum (Bing Pian)	clear heatness and eliminate dampness, improve blood circulation and disperse stasis, stop bleeding and promote ulcer healing.	twice daily for 12weeks	relieve the symptoms, improve mucosa healing and ameliorate histological inflammation	RCT	Damp-Heat Syndrome	ulcerative colitis	–
Zhou (2021) (106)	14	Enema	Baitouweng Tang.(Pulsatilla Decoction): *Pulsatillae Radix* (Bai Tou Weng), *Phellodendri Chinensis Cortex* (Huang Bo), *Fraxini Cortex* (Qin Pi), *Coptidis Rhizoma* (Huang Lian), *Bletillae Rhizoma* (Bai Ji), *Sanguisorbae Radix* (Di Yu), *Notoginseng Radix et Rhizoma* (San Qi), *Aucklandiae Radix* (Mu Xiang), *Citri Reticulatae Pericarpium* (Chen Pi).	Clear heat and detoxify, cool the blood and relieve dysentery.	once daily, for 6weeks	Improve intestinal mucosal injury and enhance endoscopic scores.	RCT	Damp-Heat Syndrome	ulcerative colitis	–

[1] RCT, Randomized controlled trial.
[2] UC, ulcerative colitis.
"↑" means an increase, while "↓" represents a decrease in expression.

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irColitis, where immune dysregulation and microbiota imbalance persist, TCM's long-term regulatory effects on gut microbiota and immune homeostasis may be advantageous. Existing studies have demonstrated that various herbal medicines possess the ability to improve gut microbiota abundance (2). While current studies suggested that gut microbiota significantly influences the development of irColitis (3, 71, 77), future investigations should focus on elucidating the complex interactions between ICIs, gut immune function, and the microbiome. A deeper understanding of these interactions is essential for advancing prevention and treatment strategies and clarifying the mechanisms by which Chinese medicine affects chronic irColitis.

6.2 Treatment strategy optimization—the promising potential of TCM

Currently, the treatment of irColitis primarily relies on corticosteroids and immunosuppressants such as Infliximab and Vedolizumab. However, some patients show inadequate responses to conventional treatments, and there is even the emergence of drug resistance. Therefore, future research should focus on exploring new therapeutic strategies.

In the treatment of irColitis, TCM emphasizes restoring balance within the body, particularly by normalizing the immune system and gut function. TCM theories, including those related to the gut microbiome, spleen and stomach function, and damp-heat, offer valuable insights for treating irColitis. This approach holds significant potential for future therapeutic strategies. Moreover, TCM emphasizes individualized treatment, tailoring therapeutic strategy according to each patient's unique symptoms and physical constitution. In the future, combining individualized TCM approaches could enhance the therapeutic efficacy of irColitis.

The integration of TCM and Western medicine holds significant potential for the treatment of irColitis. During the acute phase, irColitis is often characterized by symptoms such as diarrhea, mucus or bloody stools, and abdominal pain. In severe cases, patients may experience serious complications such as intestinal perforation and sepsis, necessitating rapid inflammation control. Western medicine primarily focuses on immediate immunosuppression through glucocorticoids and biologics. Concurrently, anti-inflammatory Chinese herbal medicines, such as *Scutellariae Radix* (Huang Qin), *Coptidis Rhizoma* (Huang Lian), and *Rhizoma Sinensis* (Bai Jiang Cao), may be used in combination (93). Additionally, spleen-tonifying herbs like *Codonopsis Radix (Dang Shen)*and *Atractylodis Macrocephalae Rhizoma* (Bai Zhu) may help protect the intestinal barrier (97). Future research should further explore the role of integrative therapy in reducing glucocorticoid dosage, shortening treatment duration, and mitigating drug resistance during the acute phase. In the chronic phase or in cases of prolonged disease due to repeated ICI treatment, Western medicine offers limited strategies. At this stage, TCM presents a promising approach, interpreting the condition as an underlying deficiency with excess manifestations (Ben Xu Biao Shi). TCM emphasizes strengthening and protecting the spleen and stomach, often employing formulas that tonify the spleen and dry dampness, with individualized modifications. Studies have shown that Shenling Baizhu San improves colitis by enhancing intestinal epithelial barrier integrity and reducing inflammation (114). Additionally, a meta-analysis suggested that it alleviates diarrhea symptoms by enhancing immune function (115). Therefore, integrating Chinese medicine into standard Western treatment, tailored to the patient's specific symptoms at different stages of irColitis represents a highly promising therapeutic approach. Previous studies on ulcerative colitis have demonstrated that integrative treatment combining TCM and Western medicine yields superior efficacy compared to either treatment alone (104, 106).

However, clinical evidence supporting the use of TCM or integrative approaches for irColitis remains limited. Current clinical practices mainly follow the "treating different diseases with similar symptoms (Yi Bing Tong Zhi)" principle, often based on treatment strategies for diseases with analogous symptoms. Therefore, further high-quality clinical trials are required. Future research should focus on evaluating the clinical efficacy of TCM in treating irColitis, assessing its safety, and exploring its synergistic effects with corticosteroids and immunosuppressants. Multicenter, large-scale, randomized controlled, double-blind trials will be essential to verify the therapeutic effects of TCM in irColitis and provide scientific evidence for its broader clinical application.

Author contributions

HD: Conceptualization, Data curation, Writing – original draft. YP: Conceptualization, Writing – original draft, Writing – review & editing. XW: Writing – original draft. HC: Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that Generative AI was used in the creation of this manuscript. Generative AI was used for language polishing to enhance the clarity and readability of the text.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1551445/full#supplementary-material

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Kurarinone ameliorates intestinal mucosal inflammation via regulating T cell immunity

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Background: Inflammatory bowel disease (IBD) has become an increasingly significant global health concern, imposing substantial economic and psychological burdens on society and public health systems. Herbal medicines, which have shown promise in alleviating IBD symptoms and promoting remission through mechanisms such as immune regulation and anti-inflammatory effects, are gaining increasing attention. Kurarinone (KAR) is a major component of the dried roots of *Sophora flavescens*, which exhibits a range of pharmacological activities, including antioxidant and anti-inflammatory effects. However, research on the therapeutic potential of KAR in IBD, particularly its effect on intestinal mucosal inflammation, remains limited.

Methods: Colitis was induced by trinitrobenzene sulfonic acid (TNBS) in mice and KAR was intraperitoneally given. Hematoxylin and eosin staining, flow cytometry, and immunofluorescence were used for mucosal inflammation evaluation. Changes in gut microbiota were assessed using 16S rRNA sequencing. RNA sequencing was performed to screen for KAR's therapeutic targets, which was verified by *in vitro* T cell culture.

Results: We demonstrated that administration of KAR resulted in a mitigated colonic tissue damage in mice with TNBS-induced colitis and decreased the infiltration of inflammatory cells, including monocytes/macrophages, neutrophils, and T lymphocytes. Moreover, KAR protected TNBS-insulted mice from colonic goblet cell loss and tight junction destruction. Furthermore, KAR treatment led to the restoration of the gut microbiota to a more normal composition. Mechanistically, KAR suppressed T helper (Th) 17 cell response but facilitated interleukin (IL)-10 production via Blimp-1.

Conclusion: Our study investigated the impact of KAR on mice with TNBS-induced colitis and elucidated its underlying mechanisms, thereby uncovering novel possibilities for clinical interventions in IBD.

KEYWORDS

kurarinone, experimental colitis, inflammatory bowel disease, intestinal epithelial barrier, Th17 cells

Introduction

Inflammatory bowel disease (IBD) is a chronic and relapsing non-specific inflammatory disorder of the gastrointestinal tract, encompassing two main types: ulcerative colitis (UC) and Crohn's disease (CD). IBD has become an increasingly significant global health concern, with a rising prevalence observed in both developed and developing countries (1, 2). The exact cause of IBD remains unknown, but it is believed to result from a complex interplay of genetic, environmental, and immunological factors (3). The disease significantly impacts the quality of life of affected individuals, imposing substantial economic and psychological burdens on society and public health systems (4).

Traditional therapies for IBD primarily include sulfasalazine, 5-aminosalicylic acid (5-ASA) agents, corticosteroids, and immunosuppressive agents, which mainly target intestinal inflammation and immune responses (5). However, these conventional treatments often fall short in achieving satisfactory therapeutic outcomes, with many patients experiencing incomplete remission or relapse. Given these limitations, the quest for more effective therapeutic agents continues to be a paramount goal for researchers. Modern pharmacological research has confirmed that traditional Chinese medicine (TCM) is a vast treasure trove of medical knowledge. Numerous effective components have been extracted from Chinese herbal medicine and widely used in clinical treatments (6). The use of herbal medicines for the treatment of IBD has gained increasing attention in recent years (7). Traditional herbal remedies have been used for centuries in various cultures and have shown promising effects in alleviating IBD symptoms and promoting remission (6).

Sophora flavescens Aiton, commonly known as "Kushen" in traditional Chinese medicine, has a history of several thousand years in treating intestinal symptoms such as abdominal pain and diarrhea (8). Kurarinone (KAR) is a major component of the dried roots of *Sophora flavescens* and is found in various other plants. It exhibits a range of pharmacological activities, including anticancer, antifungal, antibacterial, antiviral, neuroprotective, antioxidant, and anti-inflammatory effects (9–14). Previous studies have shown that KAR can activate the KEAP1/Nrf2 pathway to induce heme oxygenase-1 (HO-1) expression, inhibit interleukin (IL)-1β inflammation in RAW264.7 macrophages stimulated by lipopolysaccharides, and induce the expression of inducible nitric oxide synthase (iNOS), thereby exerting immunosuppressive effects (15). Jia et al. discovered that KAR upregulates the expression of insulin-like growth factor 1 (IGF1) in heme-treated HMC3 cells and activates the PI3K/Akt signaling pathway, promoting M2 polarization of microglia and inhibiting heme-induced neuroinflammation and neurotoxicity mediated by M1 polarization of microglia (10). These findings demonstrate the potent anti-inflammatory and immunomodulatory effects of KAR. Furthermore, KAR has been shown to mitigate oxidative stress and apoptosis in human ovarian granulosa cells induced by H_2O_2 through upregulating the expression of IGF1 and activating the PI3K/Akt signaling pathway (11). These antioxidative and anti-apoptotic properties of KAR suggest its potential protective role in maintaining epithelial barrier function.

Despite the growing interest in the therapeutic potential of various compounds for IBD, research on the effects of KAR remains limited. This study aims to fill this gap by investigating the therapeutic potential of KAR in its overall efficacy in IBD and modulating intestinal mucosal inflammation, potentially offering new insights and treatment options for this challenging condition.

Materials and methods

Mice and trinitrobenzene sulfonic acid-induced colitis model

The Animal Care and Use Committee at Sichuan Provincial People's Hospital approved all experimental procedures (No. 2020204), which adhered to the National Institutes of Health guidelines for animal care and use. Male C57BL/6 mice, aged 10–12 weeks, were obtained from Shanghai Model Organisms (Shanghai, China). Blimp-1 knockout (KO) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). The mice were housed in a specific pathogen-free environment in our facility. Experimental colitis was induced as reported previously (16). Briefly, fasted mice received a rectal administration of TNBS dissolved in 50% ethanol, with a dosage of 2.0-2.5 mg. As described previously (17), the severity of colitis was assessed daily during the modeling period by monitoring weight and disease activity index (DAI) as follows: normal stools = 0, soft stools = 1, soft stools and slight bleeding = 2, loose stools and slight bleeding = 3, watery diarrhea or loose stools and gross bleeding = 4. Colonic pathological scoring was assessed as described previously (17). Briefly, a 0–4 grading scale was utilized, considering factors such as the percentage of colon affected by inflammation, the extent of crypt loss, the presence of lymphoid follicles, edema, erosions, and the density of inflammatory cell infiltration. The overall severity score was determined by summing the individual parameter scores. For the KAR+ TNBS group, TNBS-insulted mice received intraperitoneal administration of KAR (dissolved in dimethyl sulfoxide) from day 0 to day 7. The optimal dose of KAR (125 mg/kg/day) for the TNBS model has been established in our previous study (12). On day 7, all mice were euthanized by cervical dislocation, and colon samples were collected for further analysis. The control group consists of mice that only underwent the same procedural steps without the induction of colitis or administration of any therapeutic agents.

Lamina propria leukocyte isolation

Lamina propria were obtained and LPL were purified as reported previously (16, 18, 19). Briefly, intestinal epithelial cells (IEC) were removed in ethylenediaminetetraacetic acid (EDTA) solution (5 mM EDTA, 10% fetal bovine serum (FBS) in Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS)), and the residual tissues were subjected to enzymatic digestion (type IV collagenase, 1mg/ml, Sigma Aldrich, Burlington, MA, USA). After digestion, LPL in the supernatants were purified by gradient separation (40%/80% Percoll gradient).

Intestinal permeability assay

To assess intestinal permeability, a previously described method was employed (17). Briefly, following a 6–8 hour period of food and water deprivation, mice were orally administered FD-4 (FITC-conjugated Dextran, FITC-Dextran, Sigma-Aldrich) at a dosage of 0.5 mg/kg body weight. After 4 hours, blood samples were obtained and the fluorescence intensity in the sera was measured.

Flow cytometry

Flow cytometry analysis was conducted as reported previously (16–19). In brief, cells were stained for viability dye (LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, Invitrogen, Thermo Fisher Scientific, USA) and fluorochrome-conjugated antibodies (purchased from BioLegend) specific to the target markers for 30 minutes at 4 °C. Flow cytometric data were acquired using a BD FACSCanto II and analyzed with FlowJo version 10 for Windows (Tree Star, Ashland, OR, USA).

Immunofluorescence

As reported previously (17, 19), 6 µm-thick frozen sections of colon tissue were prepared, which were then blocked with a solution containing 5% bovine serum albumin (BSA) and 0.5% Triton × 100 in PBS for 2 hours at room temperature. Next, the sections were incubated overnight at 4°C with the specified primary antibodies. Secondary antibodies were added and incubated with the colonic sections at room temperature for 2 hours. Finally, the slides were sealed with neutral resin and photographed for further analysis.

Hematoxylin and eosin and periodic acid-Schiff staining

As reported previously (17), paraffin sections of the colon (4 µm) were stained following the instructions provided in the H&E staining kit (Servicebio, Wuhan, China) after dewaxing. To label the colonic goblet cells, PAS staining was performed on paraffin colon sections. After dewaxing and hydration, the sections were treated with periodic acid solution and then washed and incubated in Schiff's reagent.

16s rRNA sequencing

DNA extraction from fecal samples involved thawing the samples at room temperature and collecting approximately 200 mg on a cotton swab, which was then placed in a centrifuge tube containing 1.2 ml of lysis buffer. After vortexing and centrifugation, the supernatants were transferred to a new tube and treated with proteinase K and AL buffer. Ethanol was added, and the mixture was loaded onto an adsorption column to collect microbial DNA. The DNA extraction quality was assessed using agarose gel electrophoresis. For polymerase chain reaction (PCR) amplification of the target fragment, primers specific to V3-V4 region were designed, along with sample-specific barcode sequences. This allowed for PCR amplification of the variable regions of the rRNA gene or specific gene fragments. Amplification products were purified and recovered using magnetic beads. After mixing the PCR products with the beads and subsequent adsorption, the supernatants were collected. Ethanol was added, and the supernatants were collected again. Finally, the PCR tubes were placed on a magnetic stand, and the supernatants were aspirated into a centrifuge tube. The amplification products were quantified using the Quant-iT PicoGreen dsDNA Assay Kit and a Microplate reader. Based on the fluorescence quantification results, the samples were mixed in the appropriate proportions for sequencing. Sequencing libraries were prepared using the Illumina TruSeq Nano DNA LT Library Prep Kit, and quality checks were performed using the Agilent Bioanalyzer. Once deemed satisfactory, the libraries were subjected to Illumina MiSeq sequencing.

In vitro T cell differentiation and T cell suppression assay

As reported previously (20, 21), mouse naïve $CD4^+$ T cells were isolated from the spleen and then were cultured with transforming growth factor (TGF)-β (10 ng/ml), IL-6 (30 ng/ml), anti-interferon (IFN)-γ (10 µg/ml), and anti-IL-4 (10 µg/ml) (referred to as Th17 polarizing condition) in the presence of plate-bound anti-CD3 plus anti-CD28. For T cell suppression assay, CD45.1 $CD4^+$ T cells were labeled using a CellTrace Cell Proliferation Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. Carboxyfluorescein succinimidyl ester (CFSE)-labeled CD45.1 $CD4^+$ T cells were activated and cultured with the indicated CD45.2 Th17 cells for 5 days. After harvesting, the CFSE intensity was analyzed via flow cytometry by gating on CD45.1.

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 8.4 for Windows (GraphPad Software, San Diego, CA, USA). Unpaired Student's t test (two-tailed) was applied for comparison between two groups were analyzed, and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was applied to analyze differences among three or more groups. Statistical significance was set at $^*p < 0.05$.

Results

KAR ameliorates TNBS-induced colitis

We employed a TNBS-induced colitis mouse model to investigate the therapeutic effects of KAR on colonic inflammation and potential mechanisms. The group treated with KAR (KAR+TNBS) exhibited

significantly less weight loss compared to the non-treated TNBS group (TNBS group) (Figure 1A). DAI scores, used to assess the severity of colitis in mice, were noticeably lower in the KAR+TNBS group than in the TNBS group (Figure 1B). It was observed that the colons of the KAR+TNBS group were longer than those of the TNBS group (Figure 1C). The KAR+TNBS group also exhibited thinner colonic walls and less congestion, swelling, and necrosis compared to the TNBS group (Figure 1C). The KAR+TNBS group showed less pronounced splenomegaly than the TNBS group (Figure 1D). To further evaluate the severity of intestinal mucosal lesions, histological examination of colon sections stained with H&E revealed that the KAR+TNBS group exhibited better mucosal epithelial integrity, less reduction in goblet cell numbers, relatively preserved crypt structures, and only a minimal amount of lymphocyte infiltration compared to the TNBS group (Figure 1E). Pathological scoring of the inflammatory cell infiltration and tissue damage in the H&E -stained sections using a scoring scale showed significantly lower scores in the KAR+TNBS group than in the TNBS group (Figures 1F, G), indicating that colonic inflammation was milder in the KAR +TNBS group. These results demonstrate that KAR effectively alleviates TNBS-induced mucosal pathological damage in the colon of mice.

KAR suppresses mucosal inflammatory cell infiltration in TNBS-induced colitis

There is a significant increase in various inflammatory cell infiltrates within the colonic lamina propria during colitis, which is a typical hallmark. TNBS-induced colitis is characterized by mucosal pro-inflammatory immune cell infiltration (16). To this end, we performed immunofluorescent staining using F4/80, myeloperoxidase (MPO), and CD3 markers to label macrophages, neutrophils, and T cells in the colon, respectively (Figure 2A). In the KAR+TNBS group, the infiltration of these three types of inflammatory cells in the colonic tissue was significantly lower than in the TNBS group (Figure 2B). Next, we further analyzed the immune cell profile in the lamina propria using flow cytometry (Figure 2C). The number of $CD45^+$ $CD11b^+$ cells (total myeloid cells) was significantly reduced after KAR treatment in colitis (Figure 2D). We further marked dendritic cells ($Ly6G^-$ $CD11c^+$), monocytes/macrophages ($Ly6G^-$ $CD11c^-$), and neutrophils ($Ly6G^+$ $CD11c^-$) from the total myeloid cells by labeling CD11c and Ly6G. The total number of dendritic cells also decreased after KAR treatment, but the difference was not statistically significant (Figure 2E), while the infiltration of neutrophils in TNBS mice was significantly reduced after KAR treatment (Figure 2F). Additionally, the infiltration of monocytes/macrophages ($Ly6G^-$ $CD11c^-$) in the lamina propria was significantly lower in the KAR +TNBS group compared to the TNBS group (Figure 2G). Dendritic cells and monocytes/macrophages can be further divided into pro-inflammatory and anti-inflammatory subtypes, which can be identified by specific surface markers (17, 22, 23). Compared to the TNBS group, the number of pro-inflammatory dendritic cell subsets ($Ly6c^+$ $MHCII^-$) in the KAR+TNBS group showed a significant decrease (Figure 2E). The anti-inflammatory subset ($Ly6c^-$) displayed an upregulation trend, but without statistical significance (Figure 2E). The number of pro-inflammatory monocytes/macrophages ($Ly6c^+$ $MHCII^-$) in the KAR+TNBS group was significantly reduced compared to the TNBS group, while the anti-inflammatory subset ($Ly6c^-$) also showed a downward trend in TNBS mice after KAR treatment, but without statistical significance (Figure 2G). These results confirm that KAR reduces mucosal inflammation in the colon of colitic mice.

KAR maintains intestinal epithelial barrier function in TNBS-induced colitis

The intestinal epithelial barrier is the first line of defense against harmful substances entering the internal environment. Impaired IEC barrier function in IBD is a hallmark of inflammation occurrence and development (18). Restoring the damaged intestinal epithelial barrier function can effectively alleviate intestinal inflammation (17). Therefore, we investigated whether KAR could improve the impaired IEC barrier function during colitis. Firstly, we started with the detection of IEC barrier permeability, showing that the serum FD-4 fluorescence intensity in the KAR+ TNBS group was significantly lower than that in the TNBS group (Figure 3A), which suggested that KAR could protect the integrity of the IEC barrier function. Secondly, the mucus barrier is an important component of the IEC barrier, and we examined the expression of Mucin 2 in each group of mice. As shown in Figure 3B, KAR significantly rescued the loss of Mucin 2 caused by TNBS modeling. Goblet cells, which secreted mucins, were also quantified used PAS staining and the number of goblet cells in the colonic mucosa of TNBS group mice was significantly lower than that in the control group, while it was significantly improved in the KAR+ TNBS group mice compared to the TNBS group mice (Figure 3C). These results suggest that KAR can protect goblet cells from damage, increase mucin secretion. The tight junctions adjacent to IEC constitute the physical barrier of the intestinal barrier. We found that the KAR treatment remarkably improved the damage of tight junctions ZO-1, Occludin, and E-cadherin in TNBS-induced colitis (Figures 3D, E). These data suggest that KAR protects the IEC barrier function during intestinal inflammation.

The effect of KAR on gut microbiota during colitis

The occurrence of IBD often accompanies dysbiosis of the gut microbiota, and studies suggest that the gut microbiota may be an initiating factor in the development of IBD (24). As presented above, KAR could alleviate colitis, but its effects on the gut microbiota are still unknown. To this end, we employed 16S rRNA gene sequencing to investigate the changes in gut microbiota homeostasis in colitic mice treated with KAR. We analyzed the community structure of the gut microbiota. Using β diversity analysis (Figure 4A), we calculated the

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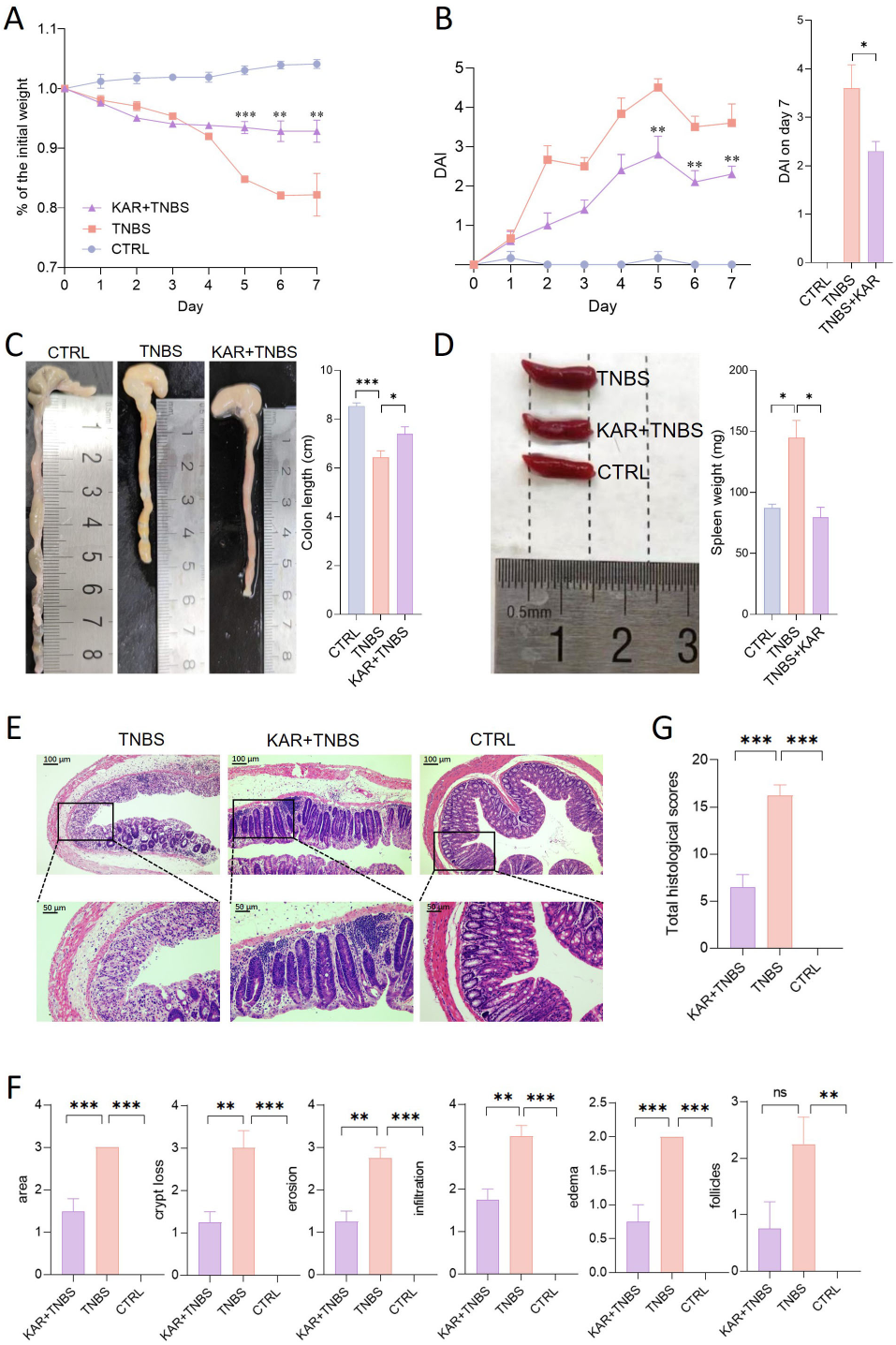

FIGURE 1
Kurarinone (KAR) ameliorates trinitrobenzene sulfonic acid (TNBS)-induced colitis. Colitis was induced by TNBS in mice. **(A)** Changes in body weight of TNBS-induced colitis mice. **(B)** Disease activity index (DAI) scores. **(A, B)** $^{**}p < 0.01$; $^{***}p < 0.001$ versus the TNBS group. **(C)** Comparison of colon lengths and representative images. **(D)** Spleen weights and representative images. **(E)** Colon tissue histology stained with hematoxylin and eosin (H&E). **(F)** Colonic sections were scored on a scale of 0-4 based on the percentage of colon involvement by inflammation, percentage of crypt loss, presence of lymphoid follicles, edema, erosions, and density of infiltrating inflammatory cells. **(G)** Total histological scores were the sum of all sub-scores shown in **(F)**. Data are presented as mean ± SD. Representative data of three independent experiments are shown. N = 5 mice each group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, no statistical difference. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. TNBS+KAR group: TNBS-insulted mice treated with KAR; TNBS group: TNBS-insulted mice without treatment of KAR. CTRL: control mice without TNBS insults.

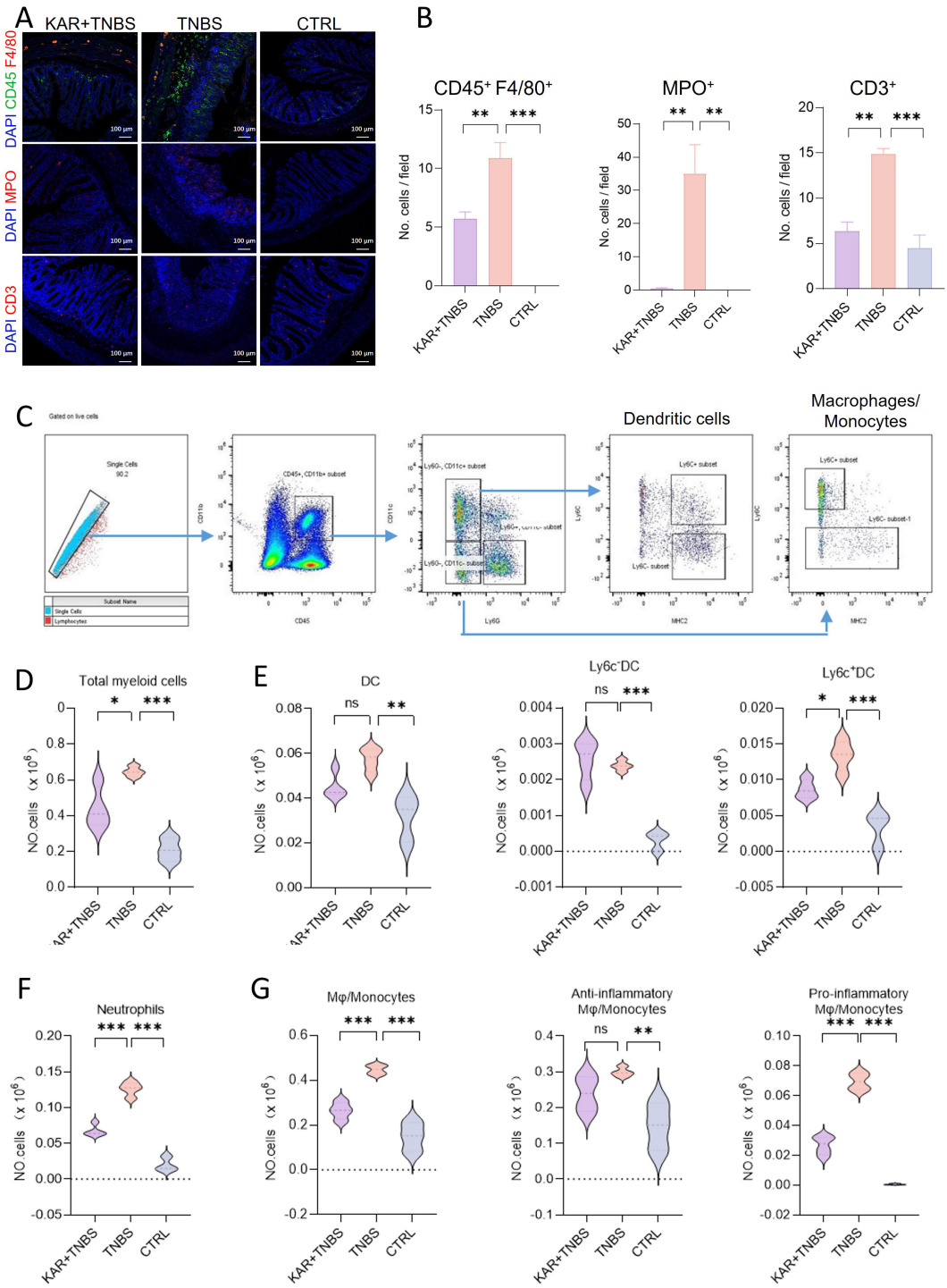

FIGURE 2
KAR suppresses inflammatory cell infiltration in the colon of mice with TNBS-induced colitis. Colon tissues were collected from indicated groups of mice (as described in Figure 1) and prepared for **(A, B)** immunofluorescence staining and **(C–G)** flow cytometry, respectively. **(A)** Immunofluorescence staining of monocytes/macrophages ($CD45^+$ $F4/80^+$, *upper*), neutrophils (MPO^+, *middle*), and T cells ($CD3^+$, *lower*). **(B)** Quantification of indicated cells. **(C)** Immune cell gating strategy in the colonic lamina propria. **(D–G)** Quantification of **(D)** total myeloid cells ($CD45^+$ $CD11b^+$), (**E**, *left*) total dendritic cells (DC, $CD45^+$ $CD11b^+$ $Ly6G^-$ $CD11c^+$), (**E**, *middle*) $Ly6C^-$ DC ($CD45^+$ $CD11b^+$ $Ly6G^-$ $CD11c^+$ $Ly6C^-$), (**E**, *right*) $Ly6C^+$ DC ($CD45^+$ $CD11b^+$ $Ly6G^-$ $CD11c^+$ $Ly6C^+$), **(F)** neutrophils ($CD45^+$ $CD11b^+$ $Ly6G^+$), (**G**, *left*) total monocytes/macrophages ($CD45^+$ $CD11b^+$ $CD11c^-$ $Ly6G^-$), (**G**, *middle*) anti-inflammatory monocytes/macrophages ($CD45^+$ $CD11b^+$ $CD11c^-$ $Ly6G^-$ $Ly6C^-$), and (**G**, *right*) pro-inflammatory monocytes/macrophages ($CD45^+$ $CD11b^+$ $CD11c^-$ $Ly6G^-$ $Ly6C^+$ MHC II^-). Data are presented as mean ± SD. Representative data of three independent experiments are shown. N = 5 mice each group; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, no statistical difference. One-way ANOVA followed by Tukey's multiple comparisons test.

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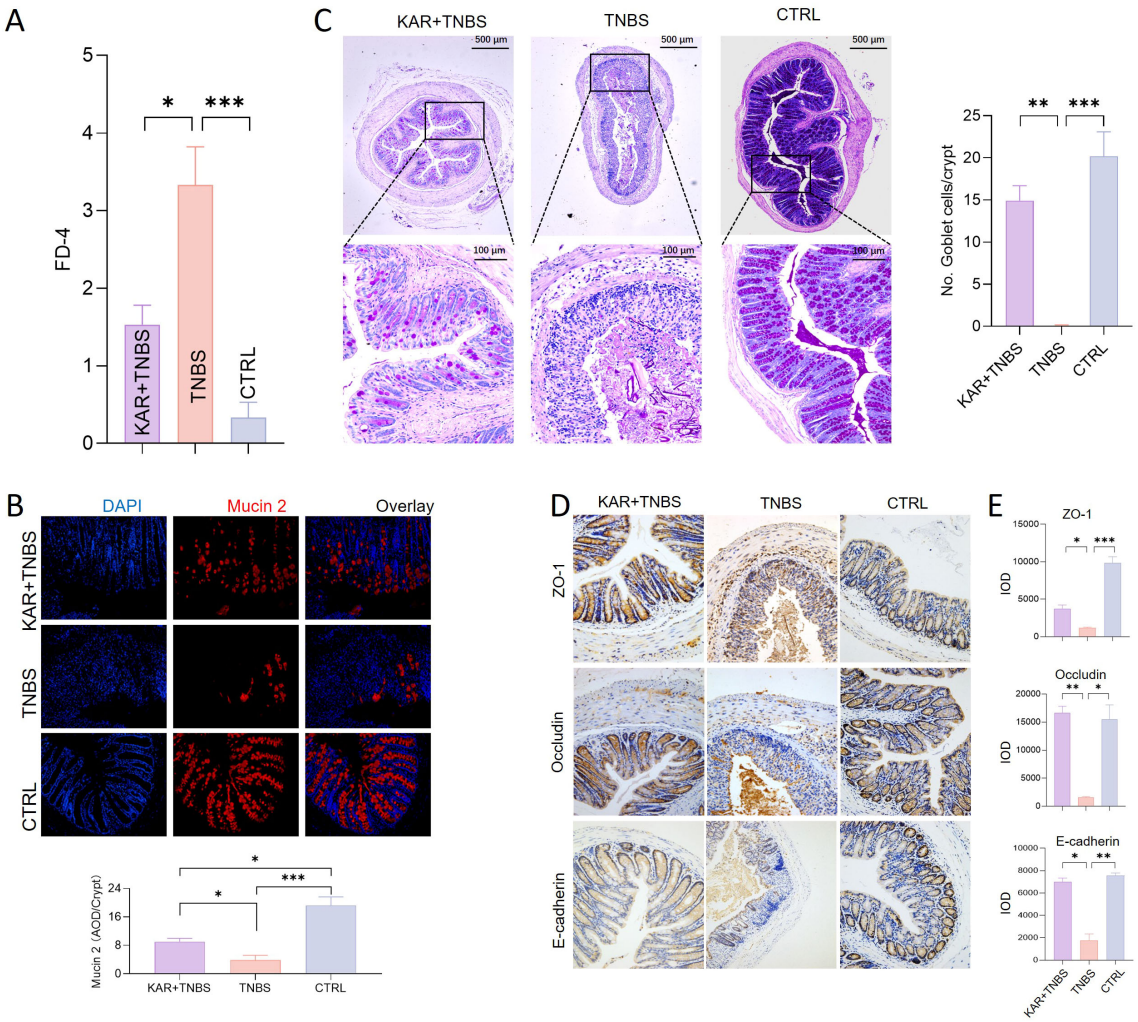

FIGURE 3
KAR maintains intestinal epithelial barrier function. Colitis was induced in mice by TNBS. **(A)** Intestinal permeability in all three groups was assessed on day 7. Fluorescence intensity of FD-4 in serum after 4 hours of FD-4 gavage. **(B)** Colonic sections were stained for Mucin 2 and DAPI. (**B**, *left*) Representative immunofluorescence images are shown. (**B**, *right*) Quantification of Mucin 2 expression was assessed by calculating the average optical density (AOD) of immunofluorescence signal. **(C)** Goblet cells were identified by periodic acid-schiff (PAS) staining. (**C**, *left*) Representative images are shown. (**C**, *right*) The number of goblet cells per crypt was assessed. **(D)** Immunohistochemical staining of tight junction proteins, including (*upper*) ZO-1, (*middle*) Occludin, and (*lower*) E-cadherin. Representative images are shown. **(E)** Quantitative analysis of colonic epithelial (*upper*) ZO-1, (*middle*) Occludin, and (*lower*) E-cadherin. IOD: integrated optical density, representing the cumulative light density. Data are presented as mean ± SD. Representative data of three independent experiments are shown. N = 5 mice each group; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. One-way ANOVA followed by Tukey's multiple comparisons test.

distances between samples in the TNBS and KAR+TNBS group using Unweighted Unifrac, Bray-Curtis, and Jaccard metrics. The samples within the KAR+TNBS group were closer to each other than to those within the TNBS group, and there were no overlapping samples between the two groups, indicating good repeatability within each group. The confidence ellipses of the KAR+TNBS and TNBS group showed clear deviation, suggesting differences in the gut microbiota community between the two groups. Subsequently, we performed permutational multivariate analysis of variance (PerMANOVA) to statistically analyze the distances between the KAR group and the TNBS group. The p-values based on Unweighted Unifrac, Bray-Curtis, and Jaccard were 0.019, 0.018, and 0.016, respectively, indicating significant differences between the two groups. Next, we investigated changes in the abundance of microbial taxa at the genus level, as well as performed LefSe and random forest analyses. We analyzed the changes in the abundance of microbial taxa at the genus level between the two groups of mice. After KAR treatment, the relative abundances of *Lactobacullus*, *Bacteroides*, *Pelomonas*, and *Turicibacter* significantly increased, while the relative abundances of Ruminococcaceae UCG-014, Candid (Figure 4B). Moreover, we further explored the microbial species that exhibited significant differences after KAR treatment using LefSe and random forest analyses. After KAR treatment, the abundance of *Lactobacillus* genus, Ruminococcaceae_UCG_003 genus, Lactobacillales order, Lactobacillaceae family, and Bacilli class significantly increased, while the abundance of Mollicutes class, Mollicutes_RF39 order, and others significantly decreased (Figure 4C). KAR intervention significantly

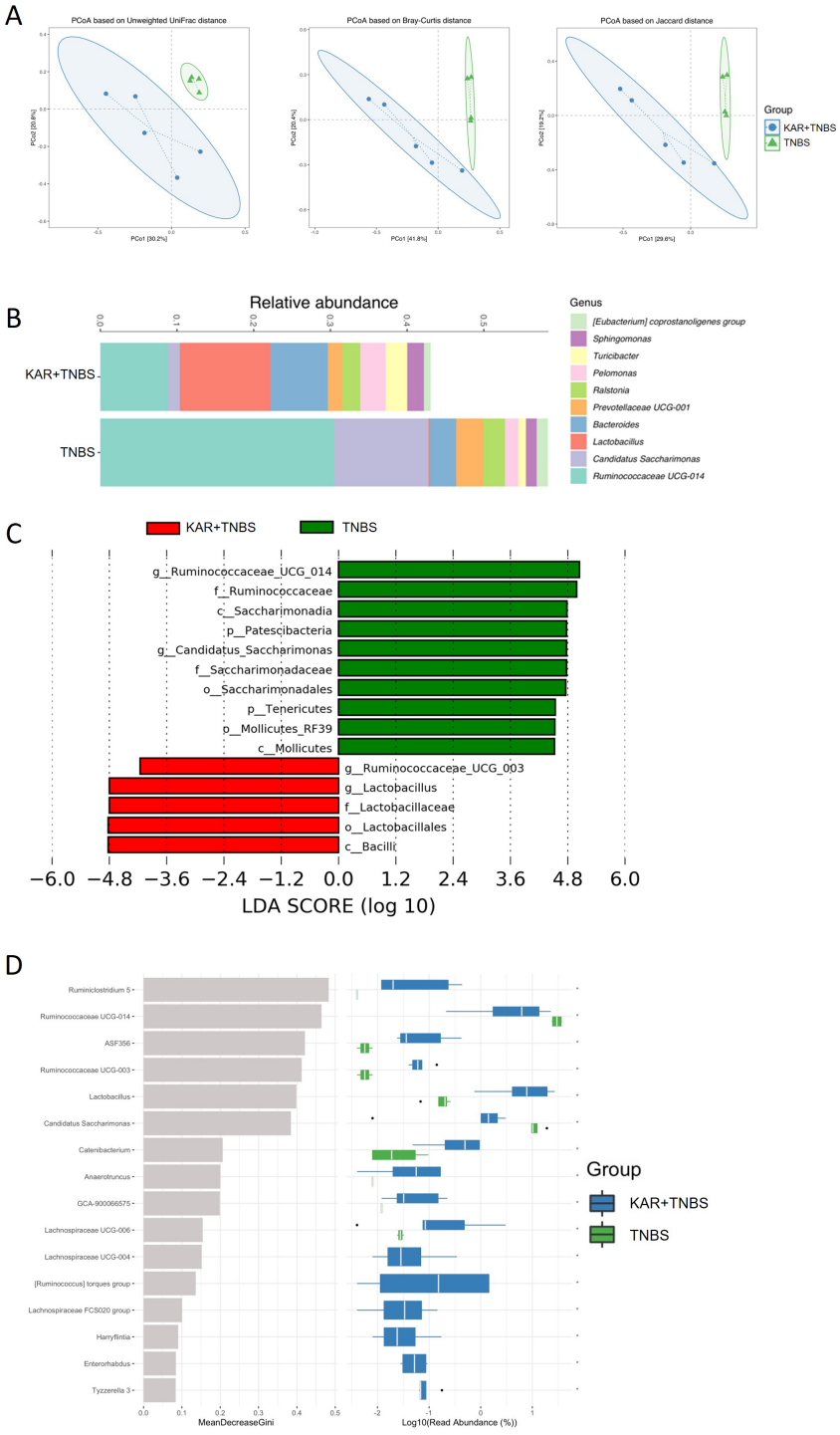

FIGURE 4
KAR regulates the imbalance of gut microbiota. Fecal samples were collected from KAR+TNBS and TNBS groups. Gut microbiota homeostasis was analyzed by 16S rRNA gene sequencing. **(A)** β diversity analysis. **(B)** The bar graph at the genus level of the intestinal microbiota based on 16S sequencing results. Different colors denote different taxa. **(C)** LefSe analysis. **(D)** Random forest analysis.

increased the relative abundances of beneficial microbial taxa such as *Lactobacullus* and *Ruminiclostridium*, while significantly decreasing the relative abundances of Ruminococcaceae UCG-014 and Candidatus Saccharimonas (Figure 4D), indicating that KAR significantly reduced the abundance of harmful microbial taxa such as Mollicutes and Mollicutes_RF39, while restoring the abundance of beneficial microbial taxa (Lactobacullus, Lactobacillales, and Lactobacillaceae).

KAR ameliorates colitis via regulating T cell-mediated immunity

To further elucidate the underlying mechanisms by which KAR ameliorates colitis, we employed a combination of bioinformatics approaches and RNA sequencing. Initially, we identified proteins and pathways associated with KAR treatment through the integration of bioinformatics databases and experimental data. We queried the databases CTD, SwissTargetPrediction, BindingDB, and TargetNet to identify validated targets of KAR from previous studies and predicted potential signaling pathways based on the molecular structure of KAR (Figure 5A). Subsequent KEGG analysis of the predicted genes revealed significant enrichment in pathways related to T-cell

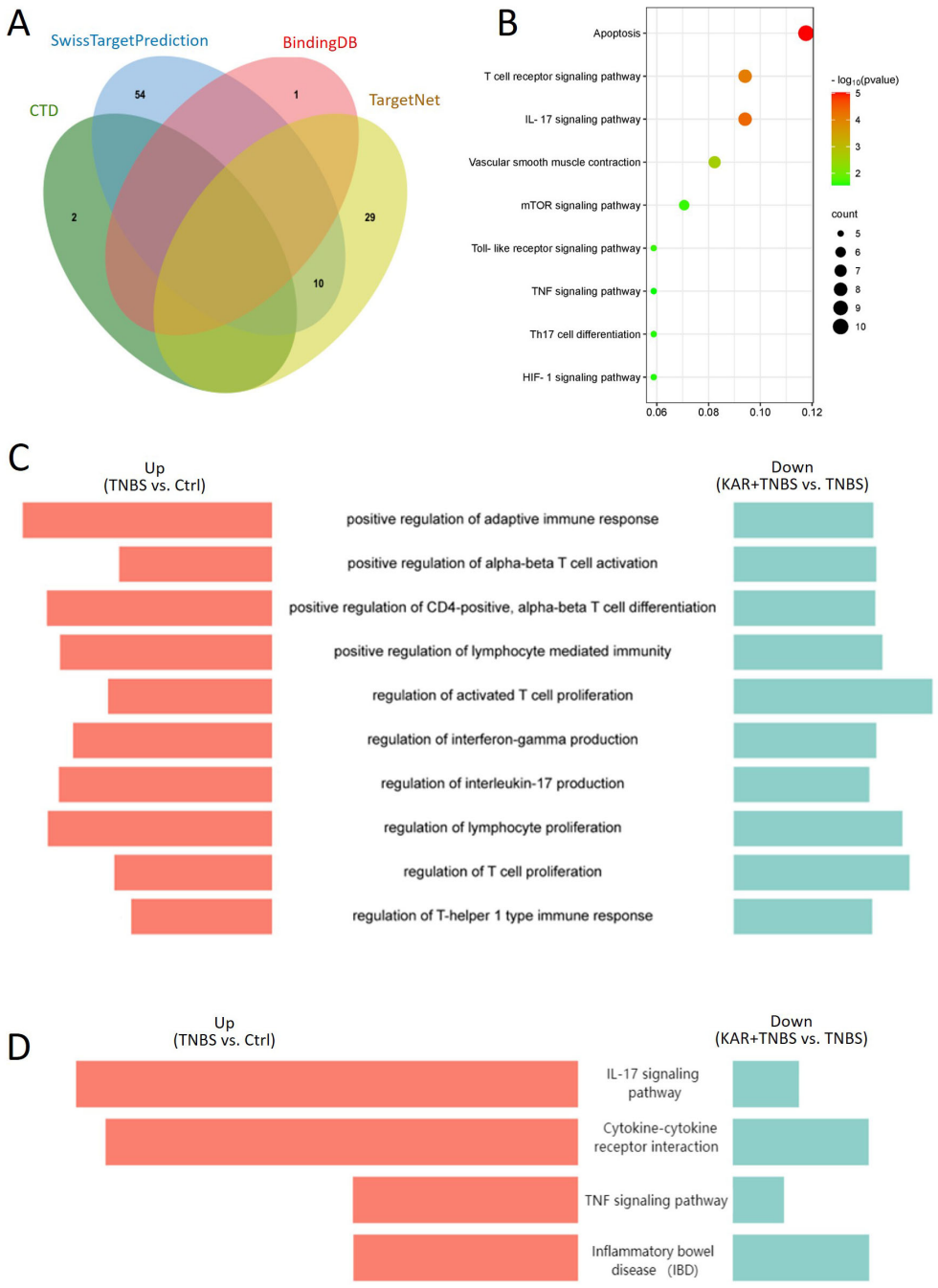

FIGURE 5
KAR mediates T cell immunity. **(A)** Venn diagram of predicted target genes for KAR obtained from the databases CTD, SwissTargetPrediction, BindingDB, and TargetNet. **(B)** KEGG pathway enrichment analysis of the predicted target genes. **(C, D)** Comparative analysis of upregulated genes in the TNBS group versus the control group, and downregulated genes in the KAR+TNBS group versus the TNBS group. The overlapped genes were subjected to **(C)** GO and **(D)** KEGG analysis. Enriched pathways are displayed as butterfly plots.

immunity, including the IL-17 signaling pathway and Th17 cell differentiation, suggesting that T-cell-mediated inflammatory responses might be a potential target of KAR (Figure 5B). Next, we investigated the changes in gene expression within the colonic mucosa of colitis mice following KAR treatment using transcriptome sequencing. To identify genes associated with KAR treatment, we performed a comparative analysis of upregulated genes in the TNBS group versus the control group, and downregulated genes in the KAR+TNBS group versus the TNBS group. The overlapped genes were subjected to GO (Figure 5C) and KEGG (Figure 5D) analysis, which revealed significant enrichment in pathways related to T-cell responses, such as the regulation of IL-17 production, cytokine-cytokine receptor interaction, and IBD. Combined with the bioinformatics predictions, these findings indicated that Th17 cell responses could be a potential target of KAR in IBD.

To further confirm whether KAR modulated Th17 cell immunity, we performed *in vitro* cell culture experiments, demonstrating that KAR could significantly inhibit the differentiation of T cells into Th17 cells. Our previous studies have shown that when Th17 differentiation is inhibited (Figure 6A), the expression of the anti-inflammatory cytokine IL-10 is upregulated (Figure 6B), which is an essential regulatory mechanism for T-cell-mediated mucosal inflammation in IBD (21). Furthermore, *in vitro* inhibition assays confirmed that KAR-treated Th17 cells could exert immunosuppressive effects through IL-10 secretion (Figure 6C). Taken together, KAR ameliorates colitis by inhibiting the differentiation of Th17 cells and upregulating IL-10 secretion, thereby suppressing mucosal inflammation in IBD.

KAR mediates IL-10 expression in Th17 cells via Blimp-1

To further explore the molecular mechanisms underlying KAR-mediated IL-10 production in Th17 cells, we focused on Blimp-1, which has been shown to be crucial for IL-10 expression in Th17 cells in both previous studies and our own research (25–27). Therefore, we investigated whether KAR promotes IL-10 production in Th17 cells through Blimp-1. We used Blimp-1 KO mice and their wild type (WT) littermates to isolate $CD4^+$ T cells from the spleen. These cells were then treated with KAR and induced to differentiate into Th17 cells. Flow cytometry analysis revealed that after KAR treatment, the proportion of IL-10-positive $CD4^+$ T cells in Blimp-1 KO mice was significantly reduced compared to that in WT mice (Figure 7A). Additionally, the inhibitory effect of KAR-treated Th17 cells on T cell proliferation (Figure 6C) was impaired by Blimp-1 deficiency (Figure 7B). Taken together, these findings indicate that Blimp-1 is a key mediator of KAR-induced IL-10 expression in Th17 cells.

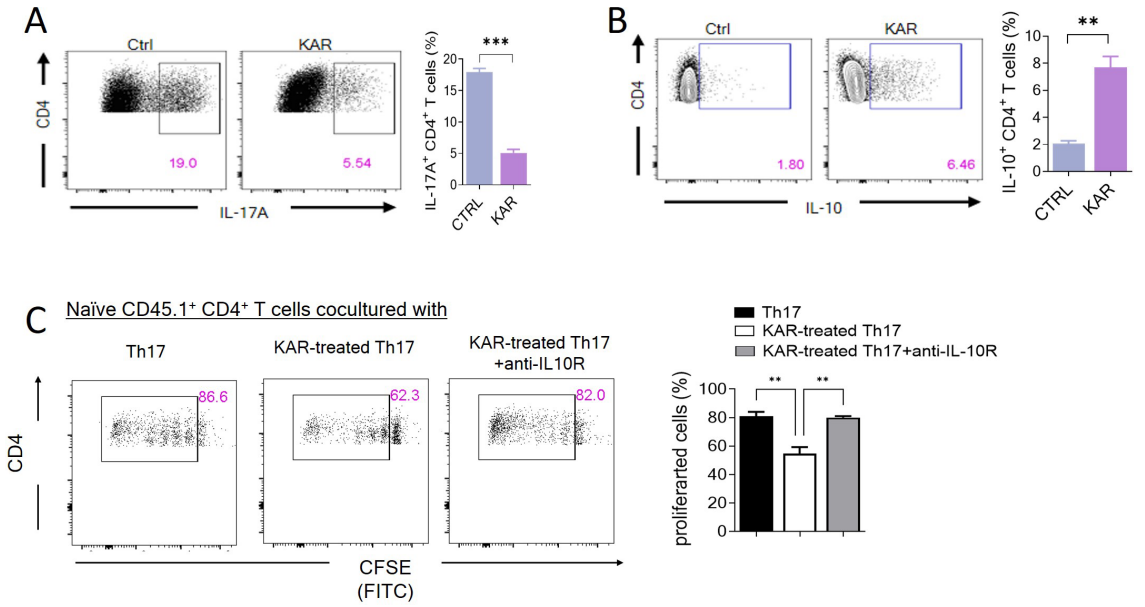

FIGURE 6
KAR facilitates T helper (Th)17 cell production of interleukin (IL)-10. Splenic $CD4^+$ T cells were purified and differentiated under Th17-skewing conditions with or without KAR for 5 days. After harvesting, flow cytometry was performed to analyze $CD4^+$ T cell expression of **(A)** IL-17A and **(B)** IL-10. The frequencies of $IL\text{-}17A^+$ and $IL\text{-}10^+$ $CD4^+$ T cells are shown as a histogram. Unpaired Student's t test, ** $p < 0.01$, *** $p < 0.001$, compared to cells without KAR treatment (ctrl). **(C)** Splenic $CD4^+$ T cells from CD45.2 mice were purified and differentiated under Th17 conditions with or without KAR for 5 days. These cells were then cocultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled splenic naïve $CD4^+$ T cells from CD45.1 mice for 5 days. Anti-IL-10 receptor (IL-10R) antibody was added to block the IL-10 pathway. Flow cytometry was performed to analyze the CFSE intensity (FITC). The frequencies of proliferated cells are shown as a histogram. One way ANOVA, ** $p < 0.01$ compared to naïve CD45.1 cells cocultured with Th17 or KAR-treated Th17 cells plus anti-IL-10R.

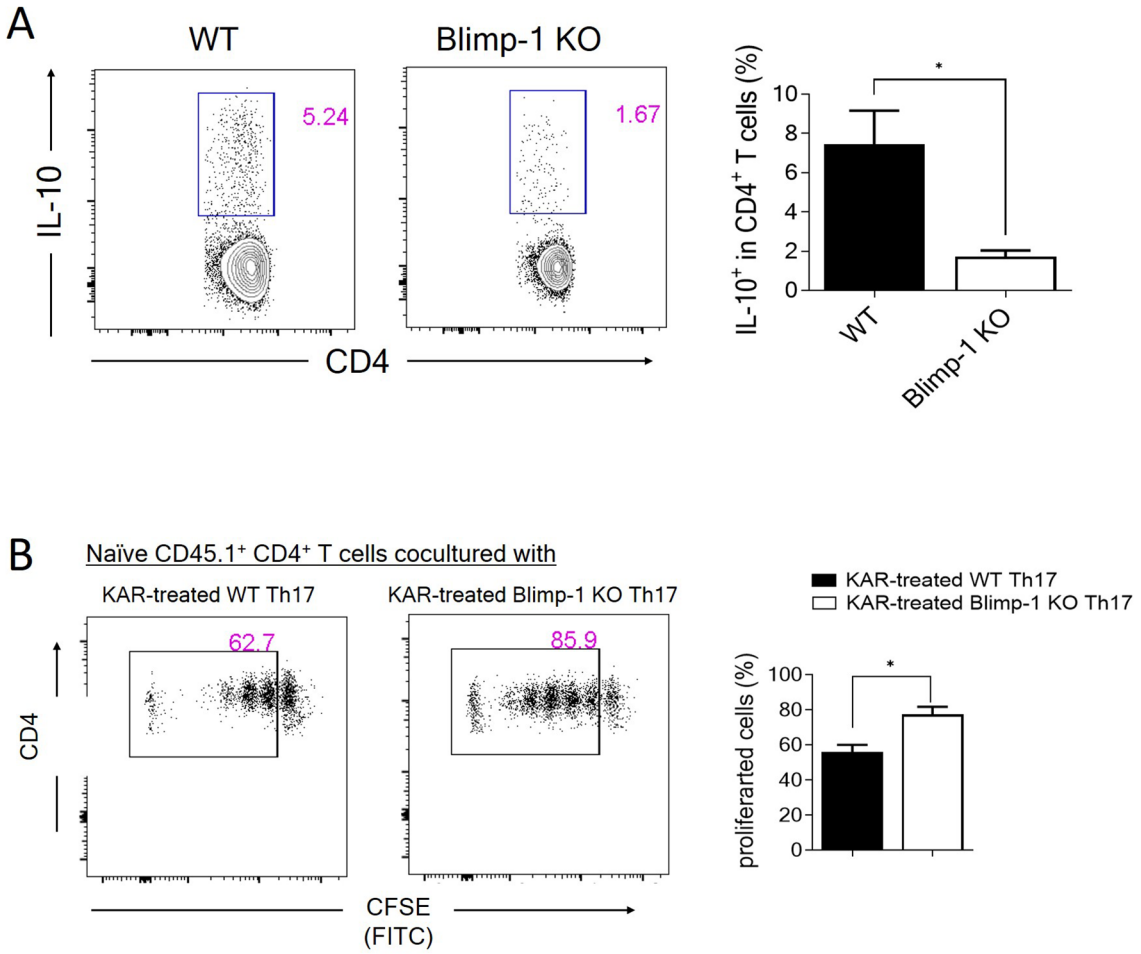

FIGURE 7
KAR mediates IL-10 expression in Th17 cells via Blimp-1. **(A)** $CD4^+$ T cells were purified from wild type (WT) and Blimp-1 knockout (Blimp-1 KO) mice and differentiated under Th17 conditions with or without KAR. $CD4^+$ T cell expression IL-10 was measured by flow cytometric intracellular staining. The frequencies of IL-10^+ $CD4^+$ T cells are shown as a histogram. **(B)** WT and Blimp-1 KO $CD4^+$ T cells were purified and differentiated under Th17 conditions with or without KAR for 5 days. Two groups of cells were then cocultured with CFSE-labeled CD45.1^+ naïve $CD4^+$ T cells, respectively. Flow cytometry was performed to analyze the CFSE intensity (FITC). The frequencies of proliferated cells are shown as a histogram. Unpaired Student's t test, * $p < 0.05$.

Discussion

In this study, we observed that treatment with KAR significantly ameliorated colitis in mice, as evidenced by milder clinical symptoms and reduced colonic tissue damage. Specifically, KAR treatment led to a notable decrease in the infiltration of inflammatory cells, including monocytes/macrophages, neutrophils, and T lymphocytes, particularly reducing the pro-inflammatory subtypes of monocytes/macrophages and dendritic cells. Regarding the intestinal epithelial barrier, KAR treatment resulted in a mild reduction in goblet cells within the colonic epithelium but a significant upregulation of mucin expression compared to the TNBS group. Additionally, KAR treatment increased the expression of tight junction proteins such as Occludin, E-cadherin, and ZO-1, suggesting enhanced intestinal barrier integrity. Furthermore, KAR treatment restored the gut microbiota towards a normal composition. Mechanistically, KAR not only suppressed Th17 cell differentiation, but also upregulated their production of IL-10 via Blimp-1. These findings collectively suggest that KAR has significant potential as a therapeutic agent for IBD.

TCM, a treasure trove of medical wisdom, has garnered increasing attention in modern medical research. Modern pharmacological studies have demonstrated that TCM exerts therapeutic effects through the synergistic actions of multiple components, pathways, and targets. Herbal treatments often possess multiple active compounds with synergistic actions, targeting various pathological pathways involved in IBD (28). Additionally, herbal medicines are generally well-tolerated and may offer a lower risk of adverse effects compared to conventional pharmaceuticals. Despite the complex nature of herbal medicines, advancements in research methodologies have enabled the identification and characterization of their active ingredients, mechanisms of action, and potential therapeutic benefits. In this context, KAR has emerged as a promising compound with demonstrated efficacy in reducing inflammation

and ameliorating colitis symptoms. Further research is warranted to fully elucidate the therapeutic potential of KAR in IBD.

The intestinal mucosal barrier is a complex and dynamic system composed of epithelial cells, tight junction complexes, mucus layers, secretory immunoglobulin A (sIgA), antimicrobial peptides (AMPs), and Paneth cells. Under physiological conditions, this barrier maintains a dynamic equilibrium, with stem cells continuously proliferating to replace shed epithelial cells, thereby preserving the integrity of the epithelial layer. Tight junction proteins such as ZO-1, Occludin, Claudin, and E-cadherin form a robust seal between intestinal epithelial cells. Additionally, goblet cells secrete mucins, particularly Mucin2, which provides an extra protective gel-like mucus layer for the epithelial cells. In IBD, however, many of these components fail, leading to disrupted tight junctions, reduced mucus production by goblet cells, and downregulated mucosal repair capacity. As a result, the intestinal epithelium becomes more permeable (29). Studies have shown that intestinal mucosal barrier dysfunction may occur in the early stages of IBD. For instance, specific defects in intestinal epithelial cells can induce colitis even in the presence of normal immune function and gut microbiota (30). Conversely, pharmacological enhancement of the intestinal mucosal barrier function can alleviate intestinal inflammation in mice (31). These findings highlight that a complete, microbe-impermeable mucosal barrier is essential for maintaining intestinal homeostasis, and improving intestinal mucosal barrier function is an important therapeutic target for UC. Li et al. reported that KAR promotes sIgA secretion, thereby enhancing the regulation of the intestinal mucosal barrier and improving resistance to pathogens (32). In an irritable bowel syndrome (IBS) mouse model, KAR effectively alleviated visceral hypersensitivity and maintained intestinal barrier function (12). In our study, we observed severe disruption of colonic epithelium in TNBS-treated mice, with significant reductions in goblet cells and mucin, as well as decreased expression of tight junction proteins Occludin, ZO-1, and E-cadherin. Following KAR treatment, TNBS-induced mice exhibited better-preserved epithelial integrity, improved goblet cell preservation, enhanced mucus secretion, and increased expression of Occludin, ZO-1, and E-cadherin in colonic tissues. These results suggest that KAR has the potential to repair intestinal mucosal barrier function and offers protective effects on multiple aspects of the intestinal barrier.

The abnormal response of $CD4^+$ T cells to the gut microbiota is a central mechanism in the pathogenesis of IBD. In this context, Th17 cells, a subset of $CD4^+$ T cells producing IL-17, have been closely associated with the progression of IBD. Recent studies on the IL-23/Th17 axis have shown that Th17 cells dominate in the gut of chronic experimental colitis mice (33). Compared to healthy controls, patients with active IBD exhibit significant infiltration of Th17 cells in the intestinal mucosa, with elevated expression of Th17-related cytokines (including IL-17, IL-21, and IL-22) at inflammatory sites and in peripheral blood. These cytokines are positively correlated with disease severity in IBD (34). Furthermore, transferring Th1 and Th17 cells into severe combined immune deficiency (SCID) mice revealed that while Th1 cells induced mild chronic colitis, Th17 cells caused severe intestinal mucosal damage (33). These findings highlight the critical role of Th17 cells in the immunopathology of IBD. In our study, we observed that KAR significantly downregulated Th17 cells in the colonic mucosa of TNBS-induced colitis mice. Consistent with our findings, previous studies have shown that KAR inhibits Th17 cell differentiation and proliferation in the experimental autoimmune encephalomyelitis (EAE) mouse model, reducing the expression of multiple pro-inflammatory cytokines and preventing the infiltration of inflammatory cells into the central nervous system (13). Similarly, Tang et al. reported that KAR treatment significantly reduced the levels of IL-17A and other pro-inflammatory cytokines in the serum and paw tissues of collagen-induced arthritis (CIA) mice (35). These results collectively support the notion that KAR modulates $CD4^+$ T cell responses, thereby attenuating inflammation.

Interestingly, KAR treatment not only downregulated the secretion of IL-17A by Th17 cells but also increased the expression of IL-10. The inhibitory effect of KAR on Th17 cell proliferation was abolished upon IL-10 blockade, suggesting that IL-10 is crucial for KAR-mediated regulation of Th17 cells. IL-10, a key anti-inflammatory cytokine, is essential for the immunosuppressive function of regulatory T cells (Tregs) in IBD and for maintaining mucosal homeostasis in both effector $CD4^+$ T cells and innate immune cells. Li et al. demonstrated that KAR downregulated IL-17A while upregulating TGF-β1 and IL-10, restoring the balance between Th17 and Treg cells and promoting intestinal immune homeostasis in UC (36). These findings align with our results and further highlight the importance of IL-10 in KAR-mediated immunomodulation. Notably, while $CD4^+$ Tregs are the primary source of IL-10, effector $CD4^+$ T cells can also transiently co-express IL-10 as part of an intrinsic negative feedback mechanism to limit inflammatory immune responses (37). This suggests that KAR may enhance the anti-inflammatory properties of Th17 cells by promoting IL-10 expression, thereby contributing to the resolution of intestinal inflammation.

Blimp-1, encoded by the *Prdm1* gene, is a multifunctional transcriptional regulator involved in the modulation of a wide range of genes associated with cell signaling, communication, and survival (38, 39). Previous studies have highlighted the critical role of Blimp-1 in the expression of the immunoregulatory cytokine IL-10 (40). For instance, in the context of IBD, Blimp-1-mediated IL-10 production by $CD4^+$ T cells has been demonstrated to suppress colitis (25, 27). Moreover, genetic variations in *Prdm1* gene have been linked to the susceptibility of IBD (41), further emphasizing the importance of Blimp-1 in immune regulation. In our study, we observed a significant reduction in IL-10-positive $CD4^+$ T cells in Blimp-1 KO mice compared to WT mice following KAR treatment. This finding supports that KAR promotes IL-10 production in Th17 cells at least partly through Blimp-1. This mechanism appears to be crucial for the immunoregulatory effects of KAR in intestinal mucosal inflammation. Given the established role of Blimp-1 in driving the differentiation of immunosuppressive cells and modulating cytokine production, our results suggest that KAR may enhance the regulatory functions of Th17 cells by upregulating Blimp-1, thereby contributing to the resolution of inflammation and maintenance of mucosal homeostasis. Future research should focus on elucidating the specific signaling pathways through which KAR activates Blimp-1

in Th17 cells and exploring the therapeutic potential of targeting this pathway in inflammatory diseases.

Despite the promising findings, this study has several limitations. Firstly, the research was conducted in a mouse model of colitis, which may not fully represent the complexity of human IBD. Therefore, caution should be exercised when extrapolating the results to human patients. Secondly, the mechanisms underlying the effects of KAR on various aspects of IBD pathogenesis require further elucidation. Future studies should aim to explore these mechanisms in more detail. Additionally, long-term effects and potential side effects of KAR treatment need to be investigated to ensure its safety and efficacy as a therapeutic option for IBD.

In conclusion, this study highlights the potential of KAR as a therapeutic agent for IBD. Although additional studies are needed to address the limitations of this research and fully elucidate the mechanisms underlying the therapeutic effects of KAR, our findings contribute to the growing body of knowledge on IBD pathogenesis and offer promising perspectives for the development of novel therapeutic approaches.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by The Animal Care and Use Committee at Sichuan Provincial People's Hospital. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YP: Investigation, Methodology, Writing – original draft, Writing – review & editing. BD: Investigation, Writing – original draft, Writing – review & editing. TW: Methodology, Writing – original draft, Writing – review & editing. ZZ: Data curation, Writing – original draft, Writing – review & editing. JW: Data curation, Writing – original draft, Writing – review & editing. CG: Funding acquisition, Writing – original draft, Writing – review & editing. CH: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Acetylcholine from tuft cells promotes M2 macrophages polarization in Hirschsprung-associated enterocolitis

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Background: Hirschsprung-associated enterocolitis (HAEC) is one of the most severe complications in patients with Hirschsprung's disease (HSCR). Previous research has indicated that acetylcholine (ACH) plays an anti-inflammatory role during inflammation by acting on the α7 nicotinic acetylcholine receptor (α7nAchR) to promote the secretion of anti-inflammatory factors. However, the specific role of ACH in HAEC remains unclear. This experiment aims to explore the sources of ACH in HSCR and its anti-inflammatory mechanisms, thereby identifying new directions for the prevention and treatment of HAEC.

Methods: We analyzed single-cell transcriptome data from HSCR to identify cells that secrete ACH and observed their distribution using immunofluorescence. In *Ednrb-/-* mice, F4/80, iNOS, ARG-1 and CD206 were used to identify and locate M1 and M2 macrophages in different intestinal segments. Western blot, reverse transcription-quantitative polymerase chain reaction, and enzyme-linked immunosorbent assay were used to test the levels of IκBα, tumor necrosis factor-α, interleukin-10, and the macrophage activation pathway proteins JAK2 and STAT3 in different intestinal segments of *Ednrb-/-* mice. Organoid and cell culture techniques were used to verify the anti-inflammatory mechanism of ACH *in vitro* models.

Results: scRNA-seq analysis revealed that tuft cells expressed the CHAT protein. In HSCR, aganglionic segments exhibited heightened cholinergic activity compared with dilated ganglionic segments. In HAEC, inflammation was mainly concentrated in the dilated ganglionic segment and was associated with an increase in M1 macrophages, whereas the aganglionic segment showed less inflammation and was associated with an increase in M2 macrophages. Furthermore, *in vitro* experiments showed that intestinal organoids containing tuft cells promoted an increase in M2 macrophage markers, and ACH promoted M2 macrophage polarization.

Conclusions: Differences in inflammation among various intestinal segments in HAEC may be linked to ACH secreted by tuft cells. Drugs targeting tuft cells have the potential to become important components of HAEC treatment in the future.

KEYWORDS

Hirschsprung-associated enterocolitis, acetylcholine, tuft cells, macrophages, Hirschsprung's disease, *Ednrb-/-* mice, *Ednrb-/-*

1 Introduction

Hirschsprung disease (HSCR) is a rare congenital intestinal condition characterized by the absence of ganglion cells in the distal bowel (1, 2). Abnormalities in the proliferation or apoptosis of colonic neural crest cells can contribute to the development of HSCR (3). The most common and serious complication of HSCR is Hirschsprung-associated enterocolitis (HAEC), which is the major cause of mortality (4). Previous studies have identified multiple factors contributing to the progression of HAEC, including alterations in the intestinal flora, abnormal immunity, and a breached intestinal barrier (5–7). However, these factors only partially elucidate the pathophysiological processes in HAEC.

Interactions between epithelial cells are essential for the function and immune defense of mucosal tissues (8, 9), and many substances, including acetylcholine (ACH), serve as bridges between them (10). Although ACH is secreted by nerve cells (11), non-neuronal sources of ACH have also been reported (12, 13). Among the various types of epithelial cells, tuft cells are the primary source of ACH and play a key role in detecting harmful substances in the mucosal layer (14–16). In the respiratory epithelium, tuft cells induce neuronal inflammation, ion transport in neighboring epithelial cells, and ciliary beat frequency via ACH secretion (17, 18). In the intestinal epithelia, ACH released by tuft cells can act on Paneth cells through paracellular pathways, triggering the release of antimicrobial peptides (19).

Although the interactions between tuft and epithelial cells play a significant role in resisting inflammatory invasion (20), immune cells in the lamina propria cannot be overlooked. Previous studies have shown that IL-25 secreted by tuft cells can act on type 2 innate lymphoid cells (ILC2) in the lamina propria to resist helminth infections (21). However, the effects of ACH secreted by tuft cells on other immune cells have not yet been elucidated.

In this study, we focus on the elevation of ACHE in HSCR. By analyzing scRNA-seq data from HSCR and utilizing immunofluorescence (IF) staining, we explore the reasons for the differences in ACH levels in the intestinal epithelium of HSCR patients. Furthermore, we delve into the anti-inflammatory effects of ACH, aiming to provide new methods for the treatment and prevention of HAEC.

Abbreviations: ACH, Acetylcholine; ACHE, Acetylcholinesterase; α7nAchR, Alpha-7 nicotinic acetylcholine receptor; BZK, Benzalkonium; CHAT, Choline acetyltransferase; DMEM, Dulbecco's modified eagle medium; ENS, Enteric nervous system; ELISA, Enzyme-linked immunosorbent assay; HAEC, Hirschsprung-associated enterocolitis; HSCR, Hirschsprung's disease; HE, staining Hematoxylin and eosin staining; iNOS, Inducible Nitric-Oxide Synthase; IKbα, NF-kappa-B inhibitor alpha; ILC2, Type 2 innate lymphoid cells; IF, Immunofluorescence; IHC, Immunohistochemistry; PBS, Phosphate-buffered saline; RT-qPCR, Reverse transcription-quantitative polymerase chain reaction analysis; scRNA-seq, Single-cell RNA sequencing; TRPM5, Transient receptor potential melastatin 5; UMAP, Uniform manifold approximation and projection.

2 Materials and methods

2.1 Patients and samples

Between August 2023 and December 2024, we collected eight colon samples with a pathological diagnosis of HSCR from Fujian Medical University Union Hospital (4 short-HSCR, 2 common-HSCR, 2 long-HSCR). HSCR was diagnosed using preoperative assessments, including traditional anorectal manometry and barium enema evaluation, and the diagnosis was confirmed using postoperative pathological analysis. This study was approved by the Ethics Committee of Fujian Medical University Union Hospital (Approval Number: 2023KJCX006) and was conducted following the government policies and the principles outlined in the Declaration of Helsinki. The parents of all patients signed informed consent forms. Fresh samples were frozen after surgical processing and stored at −80°C.

2.2 *Ednrb-/-* mice

Mice model of HSCR. A breeding colony of *Ednrb*$^{tm1Ywa/J}$ heterozygote mice (*Ednrb*$^{tm1Ywa/J}$ on a hybrid C57BL/6J–129 Sv background) donated by the Animal Center of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The animal experimental protocol was approved by the Animal Ethics Committee of the Fujian Medical University Experimental Animal Center (Approval Number: IACUC FJMU 2022-0890). The mice were housed in an SPF-grade environment and had free access to food and water. *Ednrb+/-* mice were obtained

through genetic identification after mating with *Ednrb*+/+ mice. Once sexually mature, *Ednrb*+/- female and male mice were crossed to generate *Ednrb*-/- mice. When *Ednrb*-/- mice exhibited signs of abdominal distension, inability to expel feces, lethargy, and significant weight loss, they were euthanized via intraperitoneal injection of an overdose of 1% pentobarbital (0.3 mL/20 g). Their intestines were dissected for further analysis. This experiment involved 18 *Ednrb*-/- mice. Of these, eight developed HAEC (3 weeks old) and were included in the experimental group for further study, Littermate 3-week-old *Ednrb*+/+ mice were used as the control group.

2.3 Benzalkonium-treated rat model

The animal experimental protocol was approved by the Animal Ethics Committee of the Fujian Medical University Experimental Animal Center (Approval Number: IACUC FJMU 2024-0029). Sixteen adult female Sprague–Dawley rats were randomly assigned to two groups: an intervention group and a saline control group with eight rats each. The rats were fasted for 1 day prior to surgery to minimize intestinal contents and reduce the risk of aspiration during anesthesia. On the following day, they were anesthetized with 2% sodium pentobarbital administered via intraperitoneal injection (1.0 mL/400 g). Once the rats lost their muscular reflexes and were fully anesthetized, the abdominal cavity was opened. In the BZK group, filter paper soaked in 0.1% BZK solution was wrapped around the colon, whereas in the control group, filter paper soaked in saline was used. The filter paper was replaced every 5 min for a total of six changes. After the final change, the abdominal cavity was irrigated with saline solution and sterilized. Post-surgery, the rats received intraperitoneal injections of gentamicin sulfate (100 mg/kg body weight) prophylactically to prevent infections. After 2 weeks, the mice were euthanized using 2% pentobarbital sodium (0.4 mL/100 g); dilated and narrow intestinal segments were collected. The location of the ganglia was observed using immunohistochemical (IHC) staining for calretinin. In the BZK-treated group, segments with ganglia were classified as dilated ganglionic segments, whereas those without ganglia were classified as BZK-treated segments. The control group was designated the healthy control.

2.4 Quality control and batch effects correction of scRNA-seq data

We analyzed HSCR single-cell data (22), which included eight aganglionic and five ganglionic intestinal segments from seven patients with HSCR (2 short-HSCR, 1 common-HSCR, 1 long-HSCR, and 3 total colonic aganglionosis), as well as 29 healthy segments from seven healthy donors. In total, we analyzed 92,958 cells, including 59,594 ganglionic segments and 33,364 normal segments.

Gene-barcode matrices were converted into a Seurat object using the "Seurat" R package (version 4.0.2) (23). Each HSCR tissue sample was filtered to remove cells with fewer than three genes expressed, as well as cells that expressed fewer than 500 genes. Cells with mitochondrial and hemoglobin genes were removed to filter out low-quality and potential red blood cells. Subsequently, 13 HSCR tissue sample count matrices were merged. To account for differences in sequencing depth across samples, the SCTransform R package (version 0.3.2) was used to normalize the expression values for the total unique molecular identifier counts per cell. PLCG2 (24), mitochondrial, and ribosomal genes were removed from the highly variable genes, as they contributed to the highest variability in the merged dataset, consistent with a previous study (25).

2.5 Dimensionality reduction and clustering

Clustering was performed and visualized using the uniform manifold approximation and projection (UMAP) algorithm for harmony dimensionality reduction via the Seurat function, RunUMAP. Marker genes described in previous studies were used to categorize the cells into known biological cell types (25, 26). A series of visualization maps were used to locate the expression sites of CHAT.

2.6 Western blot analysis

Colonic tissues were obtained from *Ednrb*-/- and *Ednrb*+/+ mice and ground in RIPA buffer (Epizyme, China) containing 1% protease inhibitor (Epizyme, China) and 2% phosphatase inhibitors (Epizyme, China). The protein concentration was determined using a BCA protein assay kit (Epizyme, China), and the samples were prepared for electrophoresis. The extracted total protein was separated using SDS-PAGE and transferred onto a PVDF membrane. The PVDF membrane was blocked with 5% skim milk at room temperature. After blocking, the membrane was incubated overnight at 4°C with a mixture of diluted primary antibodies, including iNOS (Proteintech, 1:1000), TNF-α (Proteintech, 1:2000), IκBα (Proteintech, 1:5000), p-IκBα (Proteintech, 1:5000), CD206 (Proteintech, 1:2000), CD68 (Proteintech, 1:5000), CHAT (Proteintech, 1:1000), P-STAT1 (CST, 1;2000), P-STAT6 (CST, 1;2000) JAK2 (ABclonal,1;2000), P-JAK2 (Abcam, 1;2000), STAT3 (Abcam, 1;2000), P-STAT3 (Proteintech, 1:1000), GAPDH (Proteintech, 1:50000), and β-actin (Proteintech, 1:50000). The following day, the membrane was incubated with a TBST-diluted secondary antibody (Beyotime, China) for 1 h at room temperature. Post-incubation, it was washed three times with TBST at room temperature for 5 min each time. Protein bands were visualized using Omni-ECL (Epizyme, China), and detected using the ChemiDoc MP multifunctional imaging system (Bio-Rad, USA). Protein band densities were analyzed using the ImageJ version 1.8.0 software (National Institutes of Health).

2.7 Reverse transcription-quantitative polymerase chain reaction analysis

Colonic tissues were obtained from patients, *Ednrb*-/- mice, and healthy control mice (the latter groups were sacrificed prior to tissue

collection) and mechanically milled for homogenization, and total mRNA was isolated using RNAiso Plus (Takara, Japan). The concentration of the isolated total mRNA was measured at 260 nm using a spectrophotometer (Thermo USA), and its integrity was assessed using Agarose gel electrophoresis. Total cDNA was synthesized from the isolated mRNA according to the instructions provided with the PrimeScript™ RT reagent Kit (Takara, Japan). Subsequently, the TB Green Premix EX Taq kit (Takara, Japan) and specific primers for the genes were added to prepare the reaction mixture for quantitative polymerase chain reaction. The quantitative polymerase chain reaction was performed as follows: An initial denaturation step was carried out at 95°C for 2 min, followed by 40 cycles of reverse transcription-quantitative polymerase chain reaction. Each cycle included DNA denaturation at 95°C for 5 s and primer annealing/extension at 60°C for 34 s. The primer sequences are listed in the attached Table 1.

2.8 Enzyme-linked immunosorbent assay

ACH and IL-10 levels were measured using a commercially available ELISA kit (Elabscience, China). Following euthanasia, the colonic tissues were quickly weighed on an ice platform and nine times the volume of cold normal saline was added to generate a 10% homogenate. Subsequently, 0.8 mL of the homogenate was collected, and 1.4 mL of pure water was added, followed by 0.2 mL of 1.54 mmol/L physostigmine sulfate. The sample was centrifuged for 10 min at 4 °C and 3500 r/min, and the supernatant was stored in a refrigerator at -20 °C until testing. The ACH content was determined using Hestrin alkaline hydroxylamine colorimetry (27) according to the manufacturer's instructions, the same thing with macrophage cell line.

2.9 Hematoxylin and eosin staining

The obtained intestinal tissues were embedded and sliced. After dewaxing and hydration, hematoxylin and eosin staining was performed using a hematoxylin and eosin staining kit (Solarbio, China) and observed under a light microscope.

2.10 IHC staining

The tissue sections were dewaxed and rehydrated using a series of xylene washes and an ethanol gradient to remove any waxy substances and gradually restore the hydration state of the tissues. Each section was fixed in 4% paraformaldehyde (Biosharp, Canada) for 30 min. Antigen retrieval was performed using Tris-EDTA buffer (Solarbio, China). Each slide was treated with a peroxidase-blocking solution (Genentech, China) for 30 min. Membranes were permeabilized using 1% Triton X-100 (Solarbio, China). Subsequently, slides were blocked with 10% goat serum (Solarbio, China) for 1 h, and incubated overnight at 4°C with primary antibodies, including Calreticulin (Proteintech, 1:500) and acetylcholinesterase (ACHE) (Proteintech, 1:500). The following day, the corresponding secondary antibody was applied, followed by DAB (Genetech, China) and hematoxylin staining (Solarbio, China). The slides were observed under a microscope, and density analysis was performed using ImageJ version 1.8.0 (National Institutes of Health).

2.11 IF staining

After baking the sections in a 60°C constant temperature oven for 1 h, they were dewaxed and hydrated using a series of xylene and graded ethanol series. Each section was fixed with 4% paraformaldehyde (Biosharp, China) for 30 min and washed with phosphate buffer saline (PBS). Antigen retrieval was performed using Tris-EDTA (Solarbio, China) at 95°C, followed by another PBS wash. Permeabilization was achieved by incubating the sections with 0.1% Triton X-100 (Solarbio, China) for 10 min. Subsequently, the sections were blocked with 10% goat serum (Solarbio, China) for 1 h. The primary antibodies used were CHAT (Proteintech, 1:200), DCLK1 (Proteintech, 1:200), F4/80 (CST, 1:600), iNOS (Proteintech, 1:500), CD206 (Proteintech, 1:500), and α7nAchR (Proteintech, 1:200). These antibodies were incubated with the sections overnight at 4°C. The following day, the sections were washed and stained with fluorescent secondary antibodies (goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 647, and goat anti-rabbit Alexa Fluor 555; 1:200). After DAPI (Sigma, Germany) staining, the sections were mounted using an anti-fade mounting medium and imaged using a super-resolution confocal microscope (TCS SP8).

2.12 Flow cytometry

Mouse colonic tissue was rinsed in PBS, cut into small pieces, and placed in RPMI 1640 medium containing 0.2 mg/mL collagenase IV and 0.05 mg/mL DNAase I. The tissue fragments and digestive enzymes were transferred into a centrifuge tube and incubated in a shaking water bath at 37°C for 40 min. Digestion was stopped by supplementing the medium with 5% FBS. After filtering through a 40-μm cell strainer and washing twice with PBS (400 g, 4 min), a mouse colon single-cell suspension was obtained. The cells were blocked with 2% BSA at 4°C for 30 min, washed with PBS, and incubated with antibodies F4/80 (Biolegend APC) and CD206 (Biolegend FITC) in the dark for 45 min. After centrifugation (400g, 4 min), the cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 for 10 min, then incubated with antibody iNOS (Biolegend PE) in the dark for 45 min. Finally, the cells were washed and resuspended in 200 μL PBS for flow cytometry analysis.

2.13 Organoid culture

C57BL/6 mice aged 6–8 weeks were euthanized with an intraperitoneal injection of 1% pentobarbital sodium (0.5 mL/20

g). Once respiratory and cardiac arrest were confirmed and muscle reflexes ceased, the upper one-third of the cecum was excised for subsequent organoid experiments. The intestinal tissue was placed in a culture dish on ice, the connective tissue was removed, the lumen was opened longitudinally, and the tissue was washed with cold DPBS (Gibco, USA) to remove the luminal contents. The tissues were cut into pieces and washed with cold DPBS containing 1% double antibiotics. Tissue fragments were incubated with 0.02% EDTA cell dissociation solution (Beyotime, China) on ice for 15 min. After removing EDTA, the tissue fragments were resuspended in pre-cool DPBS and filtered through a 70 μm cell strainer to isolate the crypts. Following centrifugation at 300 g for 5 min, the crypts were resuspended in Matrigel (Corning, USA). The crypts and Matrigel mixture were seeded at 50 μL per well in a 24-well plate, and 500 μL of organoid culture medium was added to each well. The culture medium contained Advanced DMEM/F12 (Gibco, USA), L-Glutamine (1x, Yeasen, China), Hepes (0.01M, Procell, China), Penicillin (10 kU/mL)-streptomycin (10 mg/mL) (1x, Procell, China), N2 supplement (1x, Yeasen, China), B27 supplement (1x, Yeasen, China), n-Acetylcysteine (1.25 mM, Yeasen, China), EGF (0.05 μg/mL, Yeasen, China), R-spondin1 (500 ng/mL, Yeasen, China), Noggin (100 ng/mL, Yeasen, China), and Wnt3a (100 ng/mL, Yeasen, China), IL-13 protein (20ng/ml, MCE, USA) Subculture was performed after the organoids grew to a diameter exceeding 400 μm.

2.14 Cell culture

RAW264.7 cells (Procella China) were cultured in DMEM medium (Procella China) containing 10% Fetal Bovine Serum (Vivacell China) and 1% Penicillin(10 kU/mL)-streptomycin(10 mg/mL) (Procella China). The cells were maintained at 37°C in a 5% CO_2 incubator. Once cells reached 80% confluence, they were routinely passaged. After three passages, the cell morphology was observed under a microscope to ensure stability before proceeding with the experiments. RAW264.7 cells were pretreated with LPS (Sigma, 0.5 μg/mL) for 2 h, followed by stimulation with α7nAchR stimulant (MCE, 10 μM), ACH (Sigma, 1 μM), and α7nAchR inhibitor (Tocris, 10 μM) for 22 h.

2.15 Immunocytochemistry

RAW264.7 cells (0.05×10^6) were seeded in polylysine-coated culture dishes, followed by drug treatment. The cells were treated with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 10% goat serum for 1.5 h. Primary antibodies, including CD206 (Proteintech,1:200) and iNOS (Proteintech,1:200), were incubated with the cells overnight at 4°C. The following day, fluorescent secondary antibodies (Abcam, goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 647, 1:200) were added, followed by DAPI (Sigma, USA) staining. Images were captured using a confocal laser scanning microscope.

2.16 Statistical analysis

Data analysis and plotting were performed using GraphPad Prism 8.4 software. First, perform a normality test. If the data followed a normal distribution, paired t-tests were used to compare intestinal tissues from *Ednrb-/-* mice with those from healthy control mice, as well as to compare ganglionic dilated segments with aganglionic segments in patients with HSCR. Statistical significance was set at $p < 0.05$. If not, apply Wilcoxon signed-rank test, Statistical significance was set at $p < 0.05$. Additionally, cell experiment data were analyzed using Mann-Whitney test, with the significance level set at $p < 0.05$.

3 Results

3.1 Cholinergic system in intestinal epithelium of aganglionic segments is active

HSCR was previously diagnosed by observing positive expression of ACHE in aganglionic segments through IHC staining, indicating the presence of numerous abnormally activated cholinergic systems in the aganglionic segments (28–30). To determine the cholinergic distribution, we performed similar experiments related to ACHE. In patients with HSCR, the expression of ACHE was significantly elevated in aganglionic segments compared to that in ganglionic dilated segments, and was abundantly present in the epithelial layer of the aganglionic segments (Figures 1A, B). To verify whether the same phenomenon occurs in animal models, we performed ACHE IHC staining. We found that abundant positive ACHE expression in the intestinal epithelium of the aganglionic segments of BZK rats (Figures 1C–E) and *Ednrb-/-* mice (Figures 1F–H), compared with ganglionic dilated segments and sham controls.

3.2 Cholinergic activity in intestinal epithelium of aganglionic segments is associated with tuft cells

Cholinergic activity is associated with ACH (31). To investigate the ability of intestinal epithelial cells to synthesize ACH, we analyzed single-cell data from five ganglionic segments and eight aganglionic segments derived from seven patients with HSCR (2 short-HSCR, 1 common-HSCR, 1 long-HSCR, and 3 total colonic aganglionosis), as well as 29 healthy intestinal segments from seven healthy donors. After quality control and doublet exclusion, a total of 92,958 cells from patients with HSCR and 64,045 cells from healthy donors were analyzed. Using UMAP for dimensionality reduction, and classic gene markers for cell annotation, we successfully visualized the distribution of cell clusters in HSCR single-cell RNA-seq (Figures 2A–C). By localizing CHAT, a key enzyme for ACH synthesis, in the single-cell RNA-seq of patients with HSCR, we found that

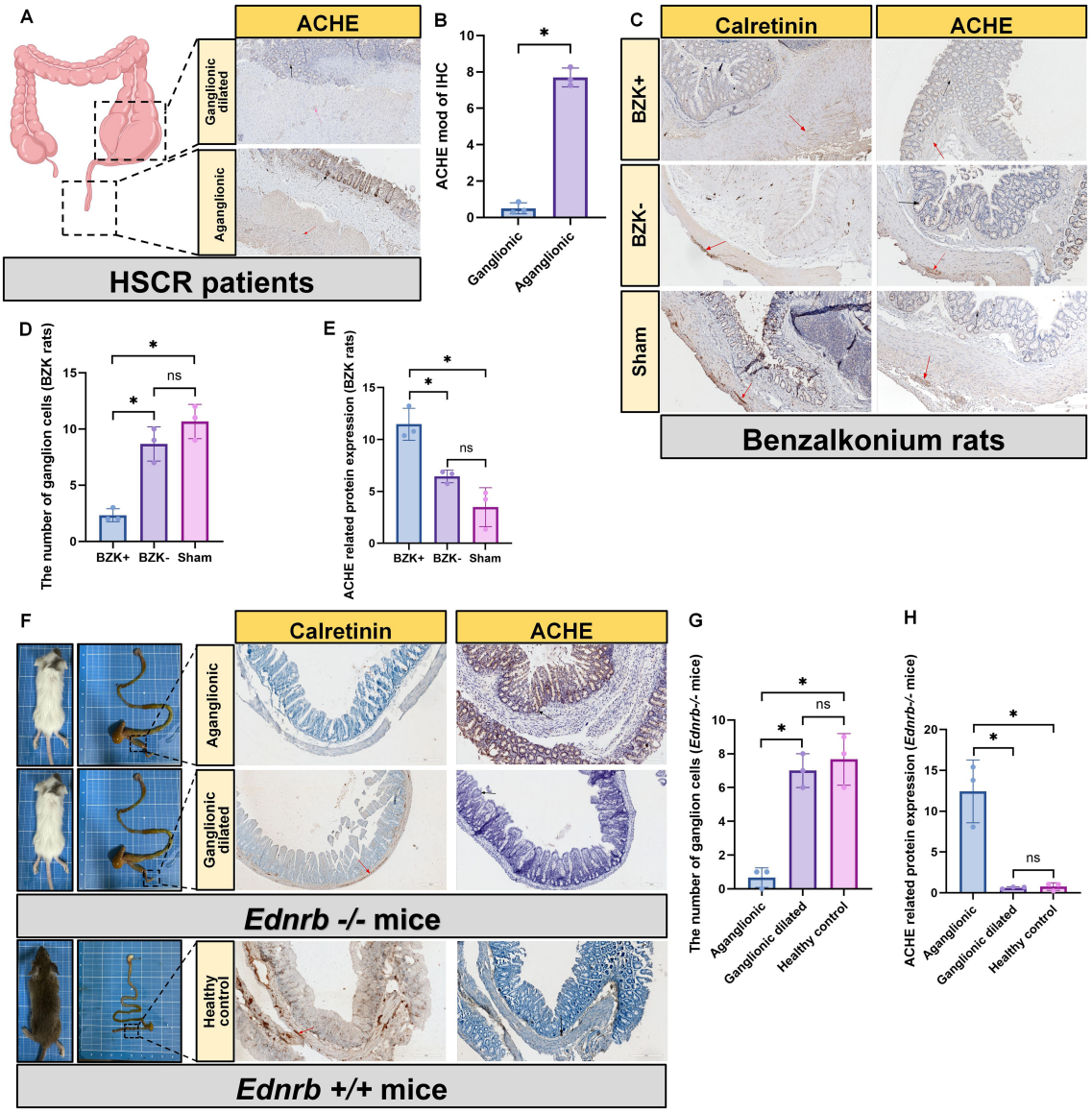

FIGURE 1
Increased cholinergic activity in the intestinal epithelium of aganglionic segments. **(A)**: IHC staining for the detection of ACHE in ganglion dilated segments and aganglionic segments in HSCR (n=3). Scale bar: 200 μm. Black arrow: the location of ACHE, Red arrow: the location of ganglion cell. **(B)**: Statistical analysis of intestinal tissues from patients with HSCR (n=3) by ACHE IHC. The data conform to a normal distribution. Paired t-test (*: P<0.05). **(C)**: IHC staining for the detection of calretinin and ACHE in BZK untreated segments (BZK-) and BZK treated segments (BZK+) in rats and Sham group (n=3), scale bar: 200 μm. Black arrow: the location of ACHE, Red arrow: the location of ganglion cell. **(D)**: Statistical analysis of ganglion cell counts in BZK rats (n=3) using calretinin IHC. The data conform to a normal distribution. Paired t-test (*: P<0.05, ns: P>0.05). **(E)**: Statistical analysis of intestinal tissues from BZK rats (n=3) by ACHE IHC. The data conform to a normal distribution. Paired t-test (*: P<0.05, ns: P>0.05). **(F)**: IHC staining for the detection of calretinin and ACHE in ganglion dilated segments and aganglionic segments in *Ednrb-/-* mice (n=3). Arrows: ganglia c ells, scale bar: 200 μm. Black arrow: the location of ACHE, Red arrow: the location of ganglion cell. **(G)**: Statistical analysis of ganglion cell counts in *Ednrb-/-* mice (n=3) and *Ednrb+/+* mice (n=3) using calretinin IHC. The data conform to a normal distribu tion. Paired t-test (*: P<0.05, ns: P>0.05). **(H)**: Statistical analysis of intestinal tissues from *Ednrb-/-* mice (n=3) and *Ednrb+/+* mice (n=3) by ACHE IHC. The data conform to a normal distribution. P aired t-test (*: P<0.05, ns: P>0.05).

CHAT was only expressed in tuft cells of the intestinal epithelial cells (Figures 2D–F). Moreover, in patients with HSCR, *Ednrb-/-* mice, and BZK-treated rats, tuft cells were predominantly scattered at the basal part of the intestine, particularly in aganglionic segments (Figures 2G–L).

3.3 Inflammation in HAEC mainly occurs in ganglionic segments

To explore the relationship between tuft cells and other immune cells in HAEC, we investigated the sites of inflammation. Because

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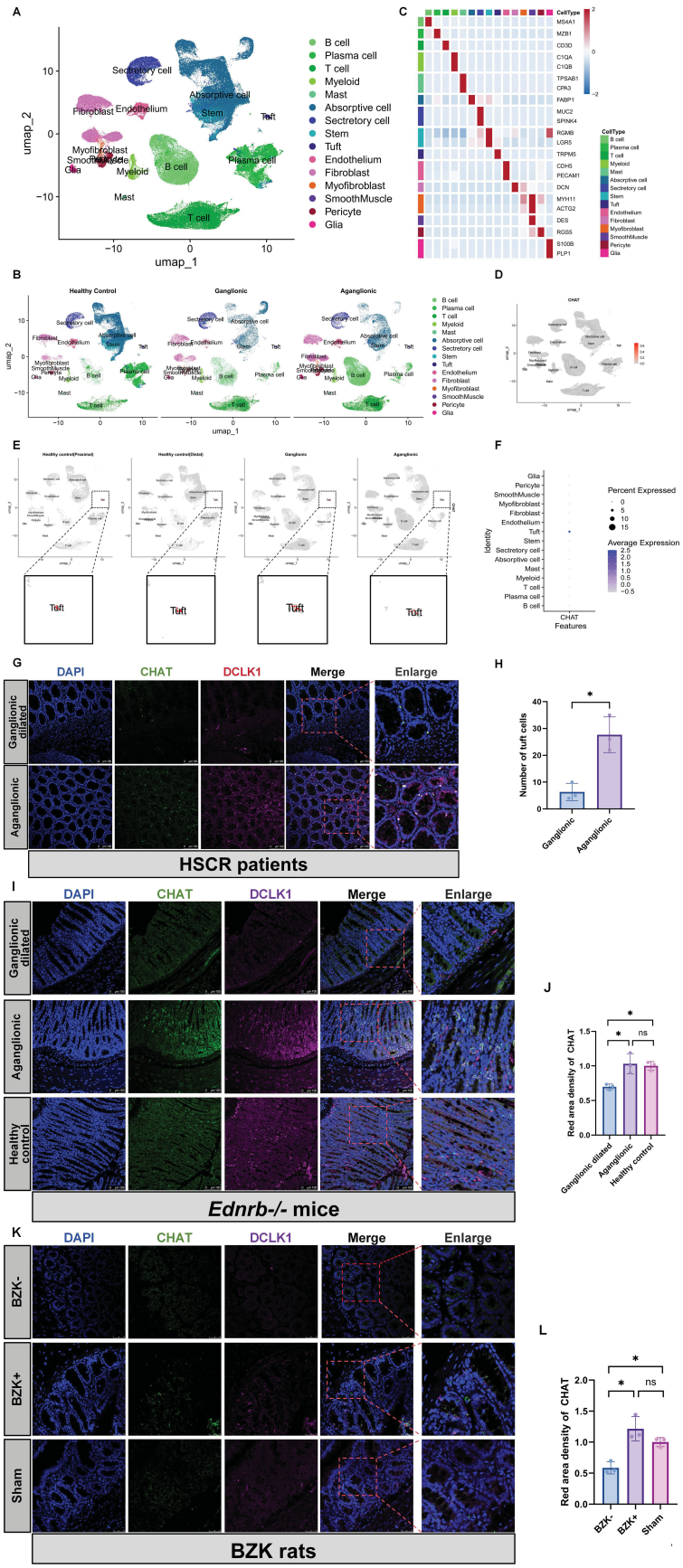

FIGURE 2 (Continued)

FIGURE 2 (Continued)
Increased ACH in aganglionic segments is associated with tuft cells. **(A, B)**: Perform UMAP projection of scRNA-seq data comparing patients of HSCR (n=5) and healthy controls (n = 7). Display separately the scRNA -seq samples from healthy controls, ganglionic segments, and aganglionic segments. **(C)**: Create a Heatmap showing the relative expression of marker genes in merged datasets of cells, with color bars corresponding to different cell-type groups. **(D, E)**: The localization of CHAT within cell clusters across different groups. **(F)**: A bubble chart was used to localize the expression levels of CHAT within different cell clusters. **(G)**: Localization of DCLK1 and CHAT proteins in the aganglionic(n=3) and ganglionic-dilated (n=3) segments of the patience of HSCR. Red, DCLK1; Gre en, CHAT; Blue, DAPI. Scale bars: 100 μm. **(H)**: Statistical analysis of number of tuft cells in intestinal tissues of patients with HSCR. The data conform to a normal distribution. Paired t-test (*: P<0.05). **(I)**: Localization of DCLK1 and CHAT proteins in the aganglionic(n=3) and ganglionic dilated(n=3) segments of the *Ednrb-/-* mice and *Ednrb+/+* mice colo n. Red, DCLK1; Green, CHAT; Blue, DAPI. Scale bars: 100 μm. **(J)**: Statistical analysis of CHAT IF optical density in intestinal tissues of *Ednrb-/-* mice and *Ednrb+/+* mice. All fluorescence intensities across the groups have been normalized to DAPI. Optical density values from the aganglionic(n=3) and ganglionic dilated (n=3) segments were normalized against those of the healthy control group. The data conform to a normal distribution. Paired t-test (*: P<0.05). **(K)**: Localization of DCLK1 and CHAT proteins in the BZK+(n=3) and BZK-(n=3) segments of the BZK rats and healthy control. Pink, DCLK1; Green, CH AT; Blue, DAPI. Scale bars: 50 μm. **(L)**: Statistical analysis of CHAT IF optical density in intestinal tissues of BZK rats. All fluorescence intensities across the groups have been normalized to D API.. Optical density values from BZK+ and BZK- groups were normalized against those of the healthy control group. The data conform to a normal distribu tion. Paired t-test (*: P<0.05).

Ednrb-/- mice typically develop enterocolitis in the third week, we chose them for subsequent research. In the third week, significant immune cell infiltration and the appearance of crypt abscesses were observed in the ganglionic dilated segment of *Ednrb-/-* mice. However, these features were absent in the aganglionic segments and the healthy control group (Figure 3A). Pathological scoring (32) of inflammatory damage to intestinal tissues revealed that the small bowel colitis score was highest in the dilated colon (Figure 3B), Furthermore, the expression of the inflammation-related gene and protein Iκbα was increased in the ganglionic dilated segment but decreased in the aganglionic segment (Figures 3C–E). Conversely, the anti-inflammatory factor, IL-10, showed increased expression in the aganglionic segment and decreased expression in the dilated ganglionic segment (Figure 3F). Notably, ACH expression increased in the aganglionic segment (Figure 3G). Our findings indicate that inflammation in HAEC was mainly concentrated in ganglionic dilated segments, whereas inflammation in aganglionic segments was relatively mild and correlated with ACH expression.

3.4 Inflammation in HAEC is related to the polarization of intestinal macrophages

The relationship between HAEC and macrophages was confirmed (33, 34). Our study results revealed that macrophages in the dilated ganglionic segment were predominantly of the M1 type, whereas those in the aganglionic segment were mainly of the M2 type (Figures 4A, B), Flow cytometry analysis also revealed increased expression of M2 macrophages (marker: CD206) in aganglionic bowel segments (Q2+Q4 quadrants), while M1 macrophages (marker: iNOS) were elevated in dilated ganglionic segments (Q2+Q4 quadrants)(Figure 4C). Given the high expression of ACH in the aganglionic segment, we assessed the expression of α7nAchR in this segment. The result showed that the expression level of α7nAchR was significantly high in the aganglionic segment compared to that in the ganglionic dilated segment, and it co-localized with macrophages (Figures 4D–F). Furthermore, the JAK2-STAT3 signaling pathway was activated in the aganglionic segment (Figures 4G–I).These findings suggest that macrophages in aganglionic segments receive ACH signals, activating the JAK2-STAT3 signaling pathway and subsequently promoting M2 polarization.

3.5 Tuft cells promote the increase of M2 macrophage markers

To explore the relationship between tuft cells and macrophages, we first analyzed their interactions within HAEC. Our findings revealed that they were in proximity to each other (Figure 5A). Subsequently, we isolated crypts from C57 mice to culture intestinal organoids, followed by their identification (Figures 5B–D). Previous studies have shown that IL-13 promotes the differentiation of tuft cells in intestinal organoids (20, 35). Using IL-13 supplements, we successfully cultured intestinal organoids containing tuft cells (Figure 5E), Concurrent CHAT protein expression analysis revealed substantially elevated activation levels subsequent to IL-13 administration(Figure 5F). When co-cultured with three different types of macrophages, intestinal organoids containing tuft cells, which were cultured with IL-13 supplements, promoted an increased expression of M2 macrophage markers compared to intestinal organoids without IL-13 supplements (Figures 5G–I). Additionally, we observed elevated expression of IL-10, an anti-inflammatory cytokine(Figure 5J). These findings suggest that tuft cells not only influence type 2 innate lymphoid cells in the intestine but also affect macrophages.

3.6 ACH can promote macrophage polarization towards the M2 phenotype

To explore the anti-inflammatory effects of ACH, we evaluated the expression of genes encoding pro-inflammatory mediators secreted by RAW264.7 cells. The results demonstrated that both ACH and α7nAchR agonists augmented the gene expression of anti-inflammatory factors, including IL-10, and significantly decreased the protein levels of iNOS, along with the expression levels of *TNF-α* genes (Figure 6A). Further examination revealed that this anti-inflammatory mechanism was mediated by Activating the JAK2-STAT3 signaling pathway (Figures 6B–E). Simultaneously, we observed that STAT1, a polarization marker

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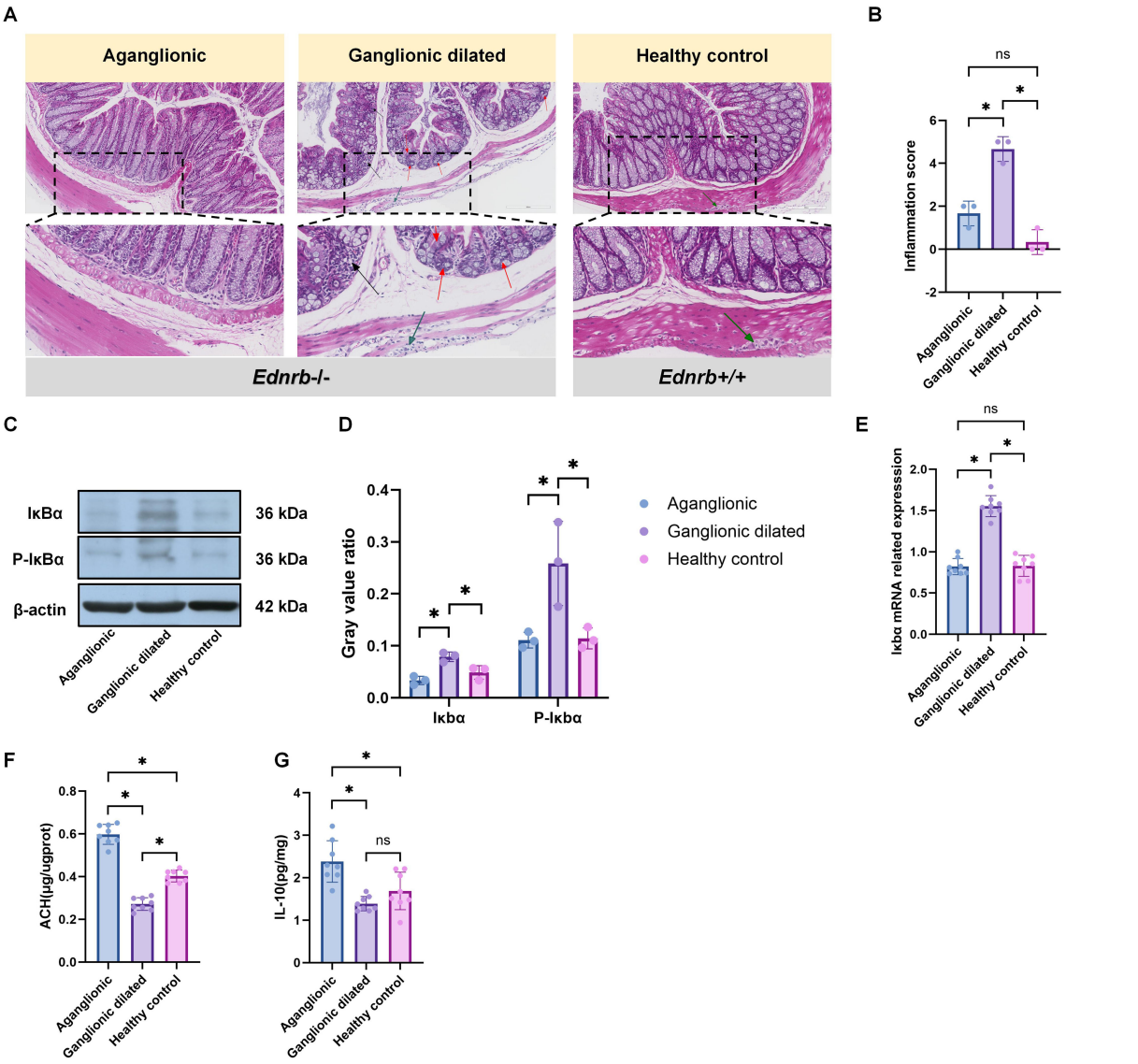

FIGURE 3
The inflammation in HAEC is mainly concentrated in the dilated ganglion segment. **(A)**: HE staining images corresponding to different intestinal segments (n=3). Red arrows: crypt abscess, black arrows: immune cell infiltration, green arrows: ganglion cell. Scale bars: 200 μm. **(B)**: Statistical analysis of intestinal inflammation scores in HE staining. Data shown represent results from 3 independent experiments. The data do not conform to a normal distribution. Wilcoxon Test (*: P<0.05). **(C)**: Expression of inflammation-related proteins, including Ikba and p-Ikbo, in different intestinal segments(n=3). **(D)** Semiquantitative analysis of protein expression levels, with each protein being normalized to β-actin. The data conform to a normal distribution. Paired t- test (*: P<0.05). **(E)**: Gene expression related to *Iκbα* in different intestinal segments of *Ednrb-/-* mice (n=8). The data do not conform to a normal distribution. Wilcoxon Tes t(*: P<0.05, ns: P>0.05). **(F)**: ELISA experiment to detect the expression levels of IL-10-related proteins in different intestinal segments of *Ednrb-/-* mice (n=8). The data do not conf orm to a normal distribution. Wilcoxon Test (*: P<0.05, ns: P>0.05). **(G)**: ELISA experiment to detect the expression levels of ACH in different intestinal segments of *Ednrb-/-* mice (n=8). The data do not conform to a normal distribution. Wilcoxon Test (*: P<0.05).

of M1 macrophages, was activated in the LPS- and α7nAChR inhibitor-treated groups, whereas STAT6, a polarization marker of M2 macrophages, was activated in the α7nAChR agonist- and ACH-treated groups (Figures 6F, G). Additionally, an increase was observed in the expression levels of markers associated with M2 macrophages in the groups treated with ACH and α7nAchR agonists. Conversely, we observed a decrease in the expression of the markers associated with M1 macrophages (Figures 6H, I). These findings indicated that ACH exerts cholinergic anti-inflammatory effects on macrophages.

4 Discussion

In this study, inflammation predominantly occurred in the dilated ganglionic segments of HAEC. We observed an abnormally hyperactive cholinergic system in the intestinal epithelium of aganglionic segments. Specifically, through IHC staining, we established that ACHE was abnormally elevated in the intestinal epithelium of the aganglionic segments. Subsequently, we verified that ACH levels were increased in the aganglionic segments of *Ednrb-/-* mice. Finally, by analyzing single-cell RNA data from

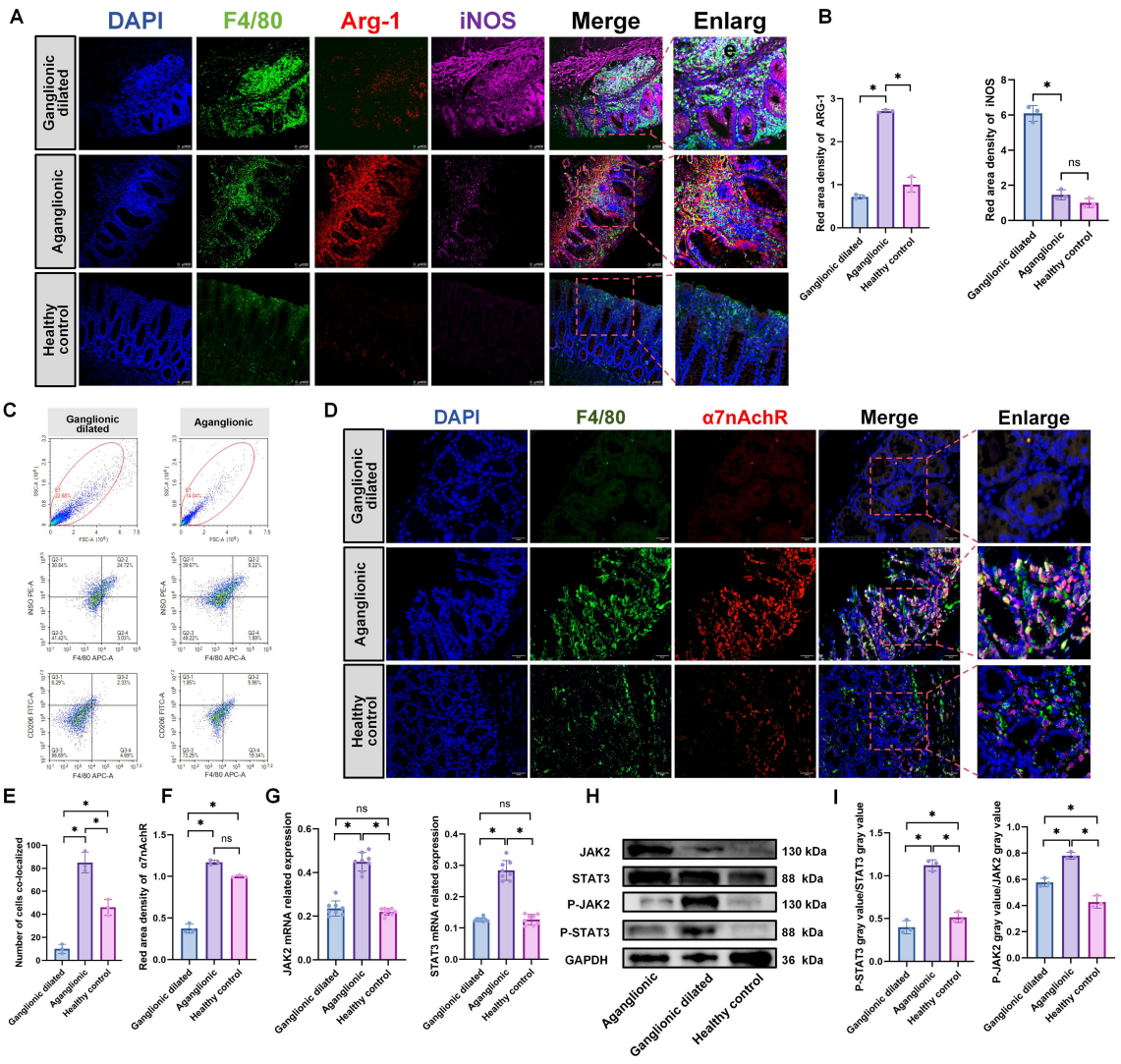

FIGURE 4
M2 macrophages is elevated in the aganglionic intestinal segment of 3-week-old *Ednrb-/-* mice. **(A)**: IF staining of F4/80, CD206, and iNOS to identify M1 and M2 macrophage in different intestinal segments of *Ednrb-/-* mice (n=3). Green, F4/80; Red, ARG-1; Pink, iNOS; Blue, DAPI. Scale bars: 100 μm. **(B)**: Statistical analysis of ARG-1 and iNOS IF optical density in intestinal tissues of *Ednrb-/-* (n=3). All fluorescence intensities across the groups have been normalized to DAPI. Optical density values from the aganglionic(n=3) and ganglionic dilated(n=3) segments were normalized against those of the healthy control group. The data conform to a normal distribution. Paired t-test (*: P<0.05). **(C)**: Flow cytometry analysis of distinct macrophage subset proportions in the intestines of *Ednrb-/-* mice. APC: F4/80, PE: iNOS, FITC: CD206 **(D)**: IF staining of α7nAChR and F4/80 to colocalizate in macrophages in different intestinal segments (n=3). Red, α7nAChR; Green, F4/80; Blue, DAPI. Scale bars: 50 μm. **(E)**: Count the number of cells co-localizing F4/80 and α7nAchR. The data conform to a normal distribution. Paired t-test (*: P<0.05). **(F)**: Statistical analysis of α7nAchR IF optical density in intestinal tissues of *Ednrb-/-* (n=3). All fluorescence intensities across the groups have been normalized to DAPI. Optical density values from the aganglionic(n=3) and ganglionic dilated(n=3) segments were normalized against those of the healthy control group. The data conform to a normal distribution. Paired t-test (*: P<0.05). **(G)**: The mRNA levels of *JAK2* and *STAT3* by RT-qPCR in different intestinal segments of *Ednrb-/-* mice (n=8). The data do not conform to a normal distribution. Wilcoxon Test (*: P<0.05, ns: P>0.05). **(H, I)**: Western blot analysis of colonic proteins in different intestinal segments(n=8). Semiquantitative analysis of protein expression levels, with each protein being normalized to GAPDH, and compare the grayscale of phosphorylated protein to total protein to observe phosphorylation levels. Data shown represent results from 3 independent experiments. The data do not conform to a normal distribution. Wilcoxon Test (*: P<0.05, ns: P>0.05).

patients with HSCR, we found that tuft cells were the only intestinal epithelial cells that had the ability to synthesize ACH. Additionally, compared to ganglionic dilated segments, the expression of tuft cell markers was higher in aganglionic segments by IF staining. Considering the established connections between tuft and immune cells in the lamina propria, we investigated whether there were interactions between other immune cells and tuft cells. Moreover, given that the role of macrophages in HAEC has been defined, we believe that a relationship exists between ACH secreted by tuft cells and macrophages. By co-culturing organoids containing tuft cells with three different macrophage lines, we observed an increased expression of M2 macrophage markers, particularly in RAW264.7. This finding is consistent with the phenomenon of elevated M2 macrophage marker expression in the aganglionic intestinal segments. Furthermore, we found that the expression of α7nAchR receptors was significantly increased in the aganglionic intestinal segments of *Ednrb-/-* mice and

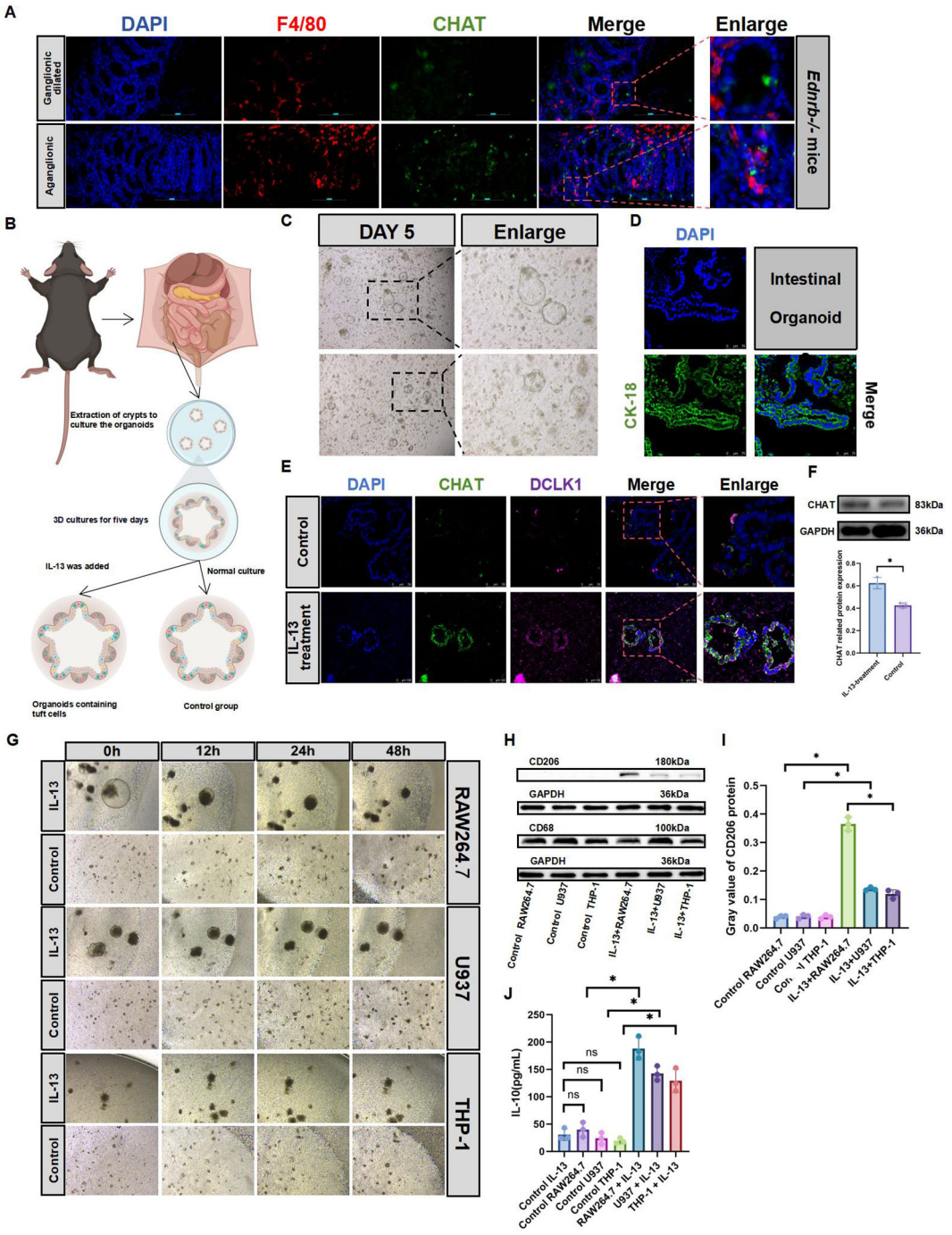

FIGURE 5
Tuft cells promote macrophage M2 polarization. **(A)**: IF staining of F4/80 and CHAT to identify tuft cell and macrophage (n=3). Red, F4/80; green, CHAT; blue, DAPI.Scale bars: 100 μm. **(B)**: Diagram of the organoid culture model. Addition of IL-13 on the fifth day promoted organoid differentiation into tuft cells **(C)**: Culture the organoids normally, and passaged them when vacuoles were > 400μm for further experiments. Day 5 of organoid culture: diameter >100μm. Scale bars: 100 μm. **(D)**: IF identification of organoids on the fifth day of normal culture. Green, Cytokeratin-18 (CK-18); blue, DAPI. Scale bars: 75 μm. **(E)**: IF identification of tuft cell expression in intestinal organoids treated with IL-13 (20 ng/mL) for 3 days. Red, DCLK1; green, CHAT; blue, DAPI. Scale bars: 100 μm. **(F)**: Expression of CHAT protein in IL-13-treated organoids. Semiquantitative analysis of protein expression levels, with each protein being normalized to GAPDH. The data conform to a normal distribution. Paired t-test (*: P<0.05). **(G)**: On Day 5 of intestinal organoid culture, IL-13 is added. On Day 8, macrophages are co-cultured for 2 days without removing the Matrigel. Observations of macrophage aggregation and the status of intestinal organoids are conducted at Oh, 12h, 24h, and 48h. RAW264.7 uses DMEM culture medium; U937 and THP-1 use 1640culture medium. **(H, I)**: Western blotting analyzed CD206 and CD68 expression in co-cultured macrophages. Semiquantitative analysis of protein expression levels, with each protein being normalized to GAPDH. Data shown represent results from 3 independent experiments. The data do not conform to a normal distribution. Wilcoxon Test (*: P<0.05, ns: P>0.05). **(J)**: ELISA analysis of IL-10 secretion in co-culture supernatants. The data do not conform to a normal distribution. Wilcoxon Test (*: P<0.05, ns: P>0.05).

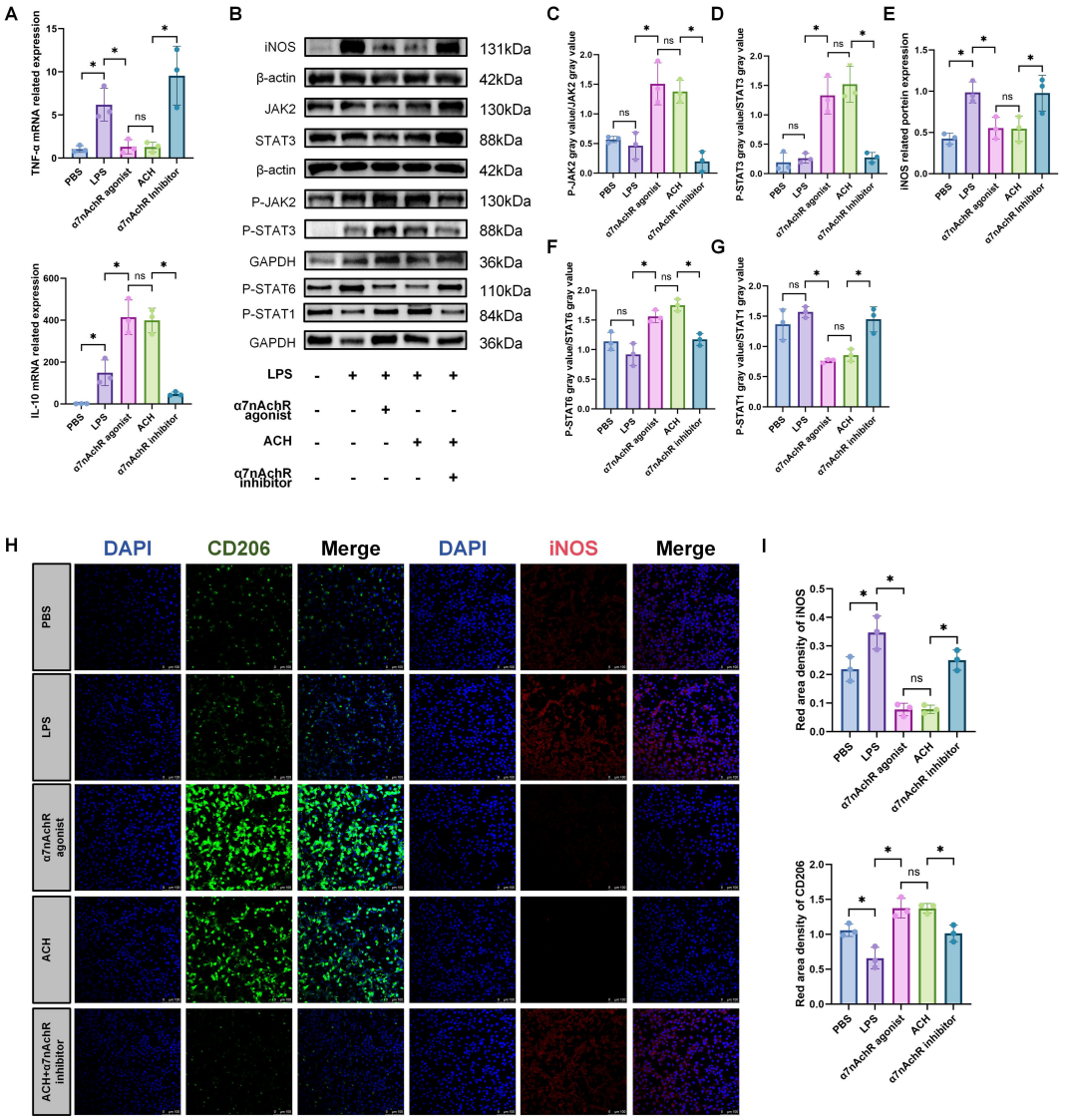

FIGURE 6
ACH promotes M2 polarization of macrophages to exert an anti-inflammatory effect. **(A)**: In vitro experiments validate the anti-inflammatory effects of ACh on RAW264.7 cells. RAW264.7 cells were pretreated with α7nAchR agonist (10 μM), ACH (1 μM), and α7nAchR inhibitor (10 μM) for 2 hours, followed by stimulation with LPS (0.5 μg/mL) for 22 hours. qRT-PCR was used to validate the expression of inflammation-related genes *TNF-α* and *IL-10*(n=3). The data do not conform to a normal distribution. Mann-Whitney (*: P<0.05, ns: P>0.05). **(B)** Western blotting was performed to assess the activation of inflammation-related proteins iNOS(n=3) and the JAK2-STAT3(n=3) signaling pathway. **(C, D).(E–G)**: Western blotting protein band intensity quantification(n=3). The data do not conform to a normal distribution. Mann-Whitney (*: P<0.05, ns: P>0.05). **(H)**: IF was used to observe the expression levels of M1 macrophage marker (iNOS) and M2 macrophage marker (CD206) in RAW264.7 cells after treatment with LPS, α7nAchR inhibitor, agonist, and ACH(n=3). Red, iNOS; Green, CD206; Blue, DAPI. Scale bars: 100 μm. **(I)**: Statistical analysis of CD206 and iNOS IF optical density in different groups (n=3). All fluorescence intensities across the groups have been normalized to DAPI. The data do not conform to a normal distribution. Mann-Whitney (*: P<0.05, ns: P>0.05).

that these receptors co-localized with macrophages. *In vitro* experiments revealed that ACH and α7nAchR agonists promoted the phenotypic switch of macrophages from M1 to M2. Collectively, these results indicate that in HAEC, ACH in the intestinal epithelium of aganglionic segments is mainly expressed by tuft cells. Tuft cells transmit harmful information from the intestinal lumen to macrophages in the lamina propria by secreting ACH, promoting the M2 polarization of macrophages and thus exerting an anti-inflammatory effect in aganglionic segments.

Pathological examination for ACHE, which indicates the presence of an abnormally hyperactive cholinergic system (36–38), was traditionally established as the gold standard for the diagnosis of HSCR (28). We confirmed this using IHC staining in patients with HSCR, *Ednrb-/-* mice, and BZK-treated rats. Additionally, ELISA experiments performed on *Ednrb-/-* mice revealed a significant increase in ACH expression in the aganglionic segments. Previous studies have demonstrated that RET mutations in HSCR can lead to an increase in CHAT expression in the intestine (39), which partially explains the high expression of ACHE in aganglionic segments. Our findings also revealed a hyperactive cholinergic system in the intestinal epithelial layer. Through analysis of single-cell RNA data from patients with

HSCR, we found that tuft cells in the intestinal epithelium were the only cells that could synthesize ACH. Therefore, we believe that ACH of epithelial origin is mainly secreted by tuft cells.

Notably, the BZK rat model has recently emerged as a viable animal model for HSCR (40). Studies have demonstrated elevated ACHE expression in BZK-treated intestinal segments, consistent with the pathological features of HSCR (41). Therefore, the BZK-treated rat model was incorporated into our study as one of the HSCR models. To confirm the success of the BZK rats model, we performed calretinin IHC staining on BZK-treated intestinal segments to check for ganglion cell. Calretinin, a calcium-binding protein, is widely present in the ganglion cells and nerve fibers of the ENS and serves as a neuronal marker (42). In normal intestinal segments, Calretinin exhibits normal staining of ganglion cells and nerve fibers. However, in the aganglionic segment of the bowel affected by HSCR, ganglion cells are absent (43, 44). In this study, the intestinal segments treated with BZK exhibited an absence of ganglion cells, while the distal colon of *Ednrb-/-* mice also displayed aganglionosis. These findings indicated that both models have the same pathological state with HSCR.

Tuft cells are classified as intestinal epithelial secretory cells and are significant members of the intestinal epithelial immune system (26, 45, 46). They are equipped with a series of key components for taste signal transduction, including transient receptor potential melastatin 5 (TRPM5), gustducin, and phospholipase β2 (47–49). These components play central roles in the response of tuft cells to intestinal microbial infections. Therefore, tuft cells represent another major type of intestinal epithelial cell with immune function, alongside goblet cells and Paneth cells (35, 47, 50). Recently, it was found that CHAT, which synthesizes ACH, is expressed in tuft cells of both mice and humans (19). Consequently, tuft cells are regarded as the only epithelial cell type that could synthesize ACH (15, 51). Tuft cells have been detected in patients with HSCR, and compared to ganglionic intestinal segments, the tuft cell marker gene, DCLK1, has been shown to have increased expression in aganglionic segments. This finding aligns with the results of tuft cell IF staining observed in our experiment.

Macrophages play a crucial role in inflammatory progression by polarizing into different types (52, 53). The role of macrophages in HAEC has been well-established; they drive the occurrence of HAEC by promoting polarization towards the M1 phenotype in the dilated segment (54, 55). In our study, we observed an increasing number of M1 macrophages in the ganglionic dilated segment, whereas M2 macrophages were predominant in the aganglionic segment of *Ednrb-/-* mice. Simultaneously, we also observed that the expression of inflammatory factors was predominantly concentrated in the ganglionic dilated segment, with a corresponding decrease in the aganglionic segment. Additionally, we also found that a large number of immune cell infiltrations and crypt abscesses were present in the intestinal epithelium of the ganglionic dilated segment. Therefore, we believe that the inflammation associated with HAEC was mainly concentrated in the dilated ganglionic segment, which is consistent with the findings of previous studies (54, 55). Notably, under normal conditions, IκBα binds to NF-κB and prevents its entry into the nucleus, maintaining NF-κB in an inactive state. When cells receive external stimuli, the IKK complex gets activated and subsequently phosphorylates IκBα at serine residues 32 and 36 (56). Following IκBα phosphorylation, the released NF-κB translocates into the nucleus where it binds to the promoter regions of various inflammation-related genes (57). Therefore, in this study, we performed protein detection of phosphorylated IκBα and revealed that the phosphorylated IκBα expression level was significantly increased in ganglionic dilated intestinal segments.

Studies on tuft cells have primarily focused on their ability to resist helminth infection by promoting the proliferation of ILC2 in the lamina propria mucosa (58). However, in our experiments, IL-13 treatment led to increased tuft cell numbers within the intestinal organoids and upregulated CHAT, co-culturing organoids containing tuft cells with three different macrophage lines resulted in increased expression of M2 macrophage markers, indicating that tuft cells can affect other immune cells. We also observed an increase in IL-10 expression, which was an anti-inflammatory cytokine that could counteract the production of pro-inflammatory factors such as IL-12/23 p40, IL-6 and TNF by macrophages (59), indicating that organoids containing tuft cells could promote M2 macrophage polarization and exert anti -inflammatory effects. IF staining revealed that they were in close proximity to macrophages in *Ednrb-/-* mice. Moreover, the ACH receptor α7nAchR was highly expressed in *Ednrb-/-* mice and co-localized with macrophages. We believe that the tuft cells could promote the polarization of M2 macrophages in the aganglionic segment. Our *in vitro* revealed that ACH increased the expression of M2 macrophage markers, It also promoted the expression of IL-10 gene. ACH activated the JAK2/STAT3 signaling pathway in macrophages, promoting their polarization towards the M2 phenotype, which was consistent with the observations made in the aganglionic segments of *Ednrb-/-* mice.

Concerning the main strengths of this study, we explored the reasons for cholinergic activation in the epithelium of the aganglionic segment and unraveled an acetylcholine-related anti-inflammatory mechanism. However, this study also comprises several limitations. First, we have not explored the acetylcholine synthesis process in tuft cells. Yet, we discovered that IL-13 stimulation boosts acetylcholine synthesis-related CHAT protein expression in organoids. In the future, we will delve deeper into analyzing the acetylcholine synthesis mechanism in tuft cells. Second, though postoperative HAEC exhibits certain incidence after surgery, no such cases occurred in our collected samples. Therefore, our study does not involve postoperative HAEC research. Finally, the organoid experiments in this study were based on normal C57 mice to extrapolate the physiological changes in the intestines of *Ednrb-/-* mice. Given the complexity of the intestinal conditions in *Ednrb-/-* mice, further research is necessary. In the future, we will also focus on developing HSCR-derived intestinal organoids and further elucidating the mechanisms underlying the initiation and progression of HAEC.

In summary, our findings revealed that in HAEC, the hyperactive cholinergic system in the aganglionic segments was

associated with increased ACH secretion, which occurred not only in the muscular layer but also in the epithelial layer. Additionally, tuft cells were the only cells in the epithelial layer capable of synthesizing ACH. Therefore, we believe that the elevation of ACH in the epithelial layer of the aganglionic segments is related to the tuft cells. Examination of inflammation in different intestinal segments of HAEC revealed that inflammation in HAEC was predominantly concentrated in the mucosal layer of dilated ganglionic segments. This difference was associated with a reduction in ACH secretion from tuft cells. This reduction potentially hindered the polarization of macrophages into the M2 phenotype. Consequently, it may have contributed to inflammation accumulation and the development of HAEC. Targeting tuft cells to regulate the levels of ACH within the epithelial layer and influence macrophage polarization may become a primary choice for the treatment of HAEC.

Data availability statement

The scRNA-seq data supporting the findings of this study from HSCR intestinal samples have been deposited at Genome Sequence Archive (GSA)(GSA: HRA002993). Any additional information related to the data analysis in this work paper is available from the lead corresponding authors on reasonable request.

Ethics statement

The studies involving humans were approved by Ethics Committee of Fujian Medical University Union Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. The animal study was approved by Animal Ethics Committee of the Fujian Medical University Experimental Animal Center. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZZ: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft. LL: Funding acquisition, Writing – review & editing. HL: Methodology, Writing – original draft. JZ: Validation, Writing – original draft. ZW: Validation, Writing – review & editing. YW: Supervision, Writing – review & editing. JC: Supervision, Writing – review & editing. CL: Supervision, Writing – review & editing. RL: Supervision, Writing – review & editing. ZS: Supervision, Writing – review & editing. MZ: Supervision, Writing – review & editing. CX: Supervision, Writing – review & editing. YC: Supervision, Writing – review & editing. XZ: Supervision, Writing – review & editing. ZG: Supervision, Writing – review & editing. RD: Investigation, Resources, Supervision, Visualization, Writing – review & editing. SH: Funding acquisition, Project administration, Visualization, Writing – review & editing. FC: Data curation, Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cimifugin ameliorates ulcerative colitis-related lung injury by modulating the JAK1/STAT1 signaling pathway and macrophage M1 polarization

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Introduction: Ulcerative colitis (UC)-related lung injurys is a commonly overlooked extraintestinal manifestation and there are currently no drugs with definitive efficacy available. Cimifugin has been found to inhibit aberrant inflammation and oxidative stress, but its efficacy in UC-related lung injurys has not yet been demonstrated.

Methods: This study explored the effects of Cimifugin on UC-related lung injurys using RNA-seq in combination with 16S rRNA sequencing.

Results: Cimifugin significantly ameliorated symptoms and attenuated colon and lung injury in a UC mouse model, restored the integrity of the intestinal and lung epithelial barriers, and suppressed lung inflammation, which was achieved by inhibiting the JAK1/STAT1 pathway and the M1 macrophage-mediated inflammatory state in the colon and lungs, as well as by improving the homeostasis of the intestinal microbiota.

Discussion: Cimifugin ameliorates UC-associated lung injury by modulating the JAK1/STAT1 pathway and macrophage M1 polarization.

KEYWORDS

cimifugin, ulcerative colitis-related lung injurys, JAK1/STAT1, gut microbiota, macrophage

1 Introduction

Inflammatory bowel diseases (IBDs) are a chronic digestive disease that afflicts millions of people worldwide and consists of Crohn disease and ulcerative colitis (UC) (1, 2). UC is more common than Crohn disease, and in addition to causing damage to the colon and rectum, 40-50% of patients often have skeletal muscle, skin, lung, and eye complications,

which are called "extraintestinal manifestations" (3–6). Lung injury is an parenteral manifestation that was often overlooked in the past, but existing evidence suggests that lung injury occurs much more frequently than previously recognized, mainly because most patients do not notice their respiratory symptoms (7, 8). Lung injury in patients with UC can occur in the airways, lung parenchyma, and interstitium, but airway injury characterized by bronchodilatation and airway inflammation is the most prevalent type. The pathogenesis of UC-related lung injury is currently unknown, and the possible causes include systemic chronic inflammation triggered by immunodeficiency, dysregulation of protease activity, and impairment of intestinal barrier function (9–11). However, all these hypotheses need to be further confirmed.

Clinical treatments for UC include aminosalicylates, immunosuppressants, and biologics. However, the long-term efficacy of these drugs is mediocre and associated with varying degrees of side effects. Importantly, there is no evidence that they attenuate UC-related lung injury.These factors lead to a reduced quality of life for patients, prompting many UC patients to seek complementary and/or alternative therapies (12).Cimifugin is a coumarin derivative isolated from *Saposhnikovia divaricata*, a plant traditionally used in the treatment of digestive disorders, skin diseases, and immune disorders. Recent studies have confirmed that Cimifugin can inhibit inflammation and oxidative stress (13–15). In an irritable bowel syndrome disease model, cimifugin regulates the NRF2/HO-1 signaling pathway by up-regulating the expression of SIRT1, which effectively maintains intestinal barrier integrity and intestinal function (16). Meanwhile, cimifugin also reduced the separated gap between airway epithelial cells by regulating the expression of tight junction proteins (17).These results suggest the potential value of cimifugin application in UC-related lung injury, but have not been explored yet.

In this study, we constructed a classical UC mouse model using dextran sulfate sodium (DSS) and investigated for the first time the therapeutic effect of cimifugin on UC-related lung injury (18, 19). We further analyzed the potential mechanism of action of Cimifugin using RNA-seq and 16S rRNA sequencing, and found that cimifugin mainly affected the JAK1/STAT1 signaling pathway. In addition, we explored the possibility that cimifugin prevents macrophage M1 polarization to maintain lung and colon immune homeostasis. The findings provide a reliable data resource for further understanding of the pathogenesis of UC-related lung injury, as well as a scientific basis for the clinical application of cimifugin.

2 Materials and methods

2.1 Reagents

Cimifugin (LOT: S02HB193600, HPLC ≥98%) was purchased from Shanghai Yuanye Biotechnology Co. Mesalazine was purchased from Heilongjiang Tianhong Pharmaceutical Co. DSS (MW 36,000–50,000) was sourced from MP Biochemicals. The above reagents were stored according to the manufacturer's recommendations and used at the specified concentrations.

2.2 Animal experiments and sample collection

Male C57BL/6 J mice aged 6-8 weeks were purchased from Pengyue Experimental Animal Breeding Co., LTD. (Jinan, China). The mice were kept in conditions free of specific pathogens with a humidity of 40-80%, a temperature of 24 ± 1°C, and a 12-hour light/dark cycle. The study was conducted in accordance with the Declaration of Helsinki, all animal testing procedures were approved by the Experimental Animal Ethics Committee of Shandong Academy of Traditional Chinese Medicine (No. SDZYY20230621005). After 7 days of adaptive feeding, the mice were randomly divided into 6 groups. Group 1 (normal control group): no DSS exposure and no special treatment; Group 2 (model group): DSS exposure and the same volume of normal saline intragastric treatment; Group 3 (positive control group): DSS exposure and reference treatment with 500 mg/kg mesalazine; Groups 4-6 were high (CIM-H), medium (CIM-M), and low (CIM-L) dose groups: DSS exposure and treatment with 50mg/kg, 25mg/kg, and 12.5 mg/kg of cimifugin, respectively (20). Induction of the UC model was achieved by administering 3% DSS to mice for 7 consecutive days. In short, the DSS was dissolved to a 3% concentration in drinking water and the mice were allowed to drink freely. The DSS solution is changed once a day and the corresponding medication is administered at the same time as the DSS exposure. The weight and fecal characteristics of the mice were monitored during the experiment. The Disease Activity Index (DAI) scoring in mice was performed using the following equation, according to the method of Bang et al (21). DAI = (weight loss score + stool trait score + blood in stool score)/3. After the experiment, the mice were euthanized, the colon length was measured and photographed, and the colon contents were collected for 16 S rRNA gene sequencing. Liver, kidney, part of colon and lung tissue were immobilized in 4% paraformaldehyde for 24 hours for pathology testing, while the rest of colon and lung were rapidly frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

2.3 Histomorphological analysis

The tissue was fixed with paraformaldehyde, then paraffin embedded, cut into 4 μm thick slices, and stained with hematoxylin and eosin (H&E). According to the methods provided in the literature, colon tissue and lung tissue were respectively scored for inflammation (22, 23).

2.4 Immunofluorescence

Anti-F4/80 rabbit pAb and anti-iNOS rabbit pAb were purchased from Solarbio Science & Technology Co., Ltd.

(Wuhan, China). Remove wax from lung and colon tissue slices and perform antigen repair. After cooling the slices to room temperature, apply 3% bovine serum albumin for 30 minutes and incubate the primary antibody overnight at 4 °C. The primary antibodies used include F4/80 and iNOS (diluted 1:100). After washing with phosphate buffered saline, the slices were incubated with goat anti rabbit secondary antibody (diluted 1:200) at 37 °C for 50 minutes. The nucleus was re-stained with 4', 6-diamidino-2-phenylindole, and an autofluorescence quenchant was added. The film was washed and sealed, and images were observed under a fluorescence microscope. We used Image J software to quantitatively analyze the immunofluorescence images.

2.5 Western blot

Rabbit anti-JAK1 antibody (GB115719), rabbit anti-P-JAK1 antibody (GB115604), rabbit anti-P-STAT1 antibody (GB115605), rabbit anti-iNOS antibody (GB113965), rabbit anti-IL-1β antibody (GB115752), rabbit anti-Arg1 antibody (GB115724), rabbit anti-Occludin antibody (GB111401), rabbit anti-ACTIN antibody (GB15003), rabbit anti-ZO-1 antibody (GB111402) and goat anti-rabbit IgG antibodies (GB23303) were purchased from Servicebio Technology Co., Ltd (Wuhan, China). Rabbit anti-STAT1 antibody (10144-2-AP) was obtained from Sanying Biotechnology Co., Ltd (Wuhan, China). Rabbit anti-CD206 antibody (DF4149) was obtained from Affinity Biosciences Co., Ltd (Jiangsu, China). Following total protein extraction, protein concentration was determined using the BCA method. Equal amounts of protein samples were subjected to gel electrophoresis and membrane transfer. After blocking, the membranes were incubated overnight at 4°C with primary antibodies against JAK1, P-JAK1, STAT1, P-STAT1, iNOS, IL-1β, Arg1, CD206, Occludin, and ZO-1 (diluted 1:1,000) in Tris-buffered saline containing Tween 20 (TBST) supplemented with 5% non-fat milk. Subsequently, a secondary goat anti-rabbit antibody (diluted 1:200) was applied and incubated at room temperature for 1.5 hours. The membranes were then washed four times with TBST to remove excess antibodies, and protein bands were visualized on the membrane. The gray values of the protein bands were detected using AlphaEase FC software.

2.6 16S rRNA gene sequencing and analysis

After isolation of genomic DNA of bacteria from fecal samples, PCR amplification of the V3-V4 region was performed with sequencing analysis and species identification. Operational taxonomic units (OTUs) obtained from the standard analysis were screened for samples and species, and the Alpha diversity index of the samples was calculated using Qiime software (Version1.9.1). The OTUs in the samples were sorted by relative abundance (or number of sequences contained) from largest to smallest to obtain the corresponding sort number, and then the Rank Abundance curve was plotted with the sort number of the OTUs as the horizontal coordinate and the relative abundance in the OTUs as the vertical coordinate. Beta diversity analysis was performed with Qiime software (Version 1.9.1) and non-metric multidimensional scaling (NMDS) was performed using the vegan package of R. LDA Effect Size (LEfSe) analysis was used for biomarker discovery and revealing macrogenomic features. Spearman correlation analysis was performed on all samples to obtain the species correlation coefficient matrix and then remove the connections with correlation coefficients < 0.6 to build a genus-level species correlation network diagram. Finally, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was performed based on the gene information on Greengene database and OTUs, i.e., to construct the predicted gene function of microorganisms in the samples. In other words, we constructed gene function profiles of the microorganisms in the samples and "mapped" the composition of the sequenced colonies into the database to analyze the metabolic functions of the colonies.

2.7 RNA-seq analysis

RNA was extracted with TRIzol reagent and assayed on a 1% agarose gel. Total RNA samples were assessed for purity, concentration and integrity before further analysis. High-throughput sequencing of multiple samples was performed using the Illumina Hiseq sequencing platform.The raw reads were filtered by removing adapter and poly-N sequences and inferior quality reads from the raw reads. HISAT2 software was used to quickly and accurately compare the clean reads with the reference genome to obtain the localization information of the reads on the reference genome. The levels of quantitative gene expression were estimated by determining the fragments per kilobase of transcript per million fragments mapped. Limma was used to identify differentially expressed genes (DEGs) in different groups. $P < 0.05$ and $|\log2FC| > 0.5$ were defined as significant differences. Gene ontology (GO) function, Kyoto encyclopedia of genes and genomes (KEGG) pathway Enrichment Analysis and Gene Set Enrichment Analysis (GSEA) were performed using DEGs.

2.8 Single-cell RNA sequencing data resources and analysis methods

The UC-associated single-cell transcriptome dataset GSE182270 was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) (24). Data quality control was based on gene counts > 200 and < 2500 for each cell, and a percentage of mitochondrial genes < 5%. Subsequently, the "vst" method was used in the FindVariableFeatures function to select the top 2000 highly variable expression genes. The principal component analysis (PCA) and clustering analysis of data were achieved through the uniform manifold approximation and projection (UMAP) of RunPCA, FindClusters, and RunUMAP functions. In addition, we applied Single R V1.4.1 and CellMarker 2.0 to annotate cell types (25). Finally, single-cell functional enrichment analysis and intercellular

communication analysis were performed using R package "irGSEA" and "Cellchat", respectively (26). Functional scoring of cimifugin related regulatory genes obtained from transcriptome was performed using "AUCell" and "irGSEA" packages to obtain cimifugin related target cells and visualize them.

2.9 Cell culture and treatment

Mouse monocyte-macrophage RAW 264.7 cells were procured from Pricella (Wuhan, China). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, USA) supplemented with 10% Fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Solarbio, Beijing, China). Culturing was conducted at 5% CO2 and 37°C in a humidified incubator. The experimental design proceeded as follows: the LPS group was treated with 1 μg/mL LPS (Solarbio) for 24 h. The control group was treated with PBS (Gibco). The cimifugin intervention group was divided into three groups according to the concentration gradient (50mg/L, 100mg/L, 200mg/L), and each group was treated with the above concentrations of LPS and cimifugin for 24 hours. For the pathway inhibition assay, cells were treated with 8 μM Upadacitinib (a selective JAK1 inhibitor, Cayman) for 1 hour before administration.

2.10 Enzyme-linked immunosorbent assay

Colon and lung tissues were prepared as homogenates and the concentration of total protein was determined by BCA protein assay kit. Culture supernatants of cells corresponding to each group in the *in vitro* study were also collected. The concentrations of IL-6 (88–7064), IL-1β (88–7013), and TNF-α (88–7324) in the above samples were determined using a commercial ELISA kit according to the manufacturer's instructions (Thermo Fisher Scientific Inc.).

2.11 Statistical analyses

Statistical analysis was conducted using GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA). Data are presented as the mean ± Standard error of mean (SEM). Significant differences between two groups were evaluated using a two-tailed unpaired Student's t-test, whereas the differences among multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* multiple comparison's test. All results were considered statistically significant at $P < 0.05$.

3 Results

3.1 Cimifugin ameliorates colitis symptoms and lung injury

In this study, the most widely used colonic length, body weight change and DAI score were selected to confirm the ameliorative effect of cimifugin in UC mice. Colon length was significantly shorter in the UC group of mice compared to normal mice, while this effect was significantly suppressed in the CIM-H and CIM-M groups (Figure 1A). The body weight of DSS-treated mice was significantly decreased, and this trend was significantly attenuated by high and medium doses of cimifugin compared to the UC group, with no difference between the two, but the effect of low doses of cimifugin was not significant (Figure 1B). In addition, DAI scores were elevated in the UC group compared with the normal group, indicating successful induction of the UC model. Both high and medium doses of cimifugin reduced the DAI scores of mice, which was more significant in the CIM-M group, but mesalazine and low-dose cimifugin had no significant effect on this (Figure 1C). Histopathology showed that the intestines of mice in the UC group exhibited extensive epithelial damage, inflammatory cell infiltration, abnormal crypts and localized ulceration. More importantly, the present study similarly observed that UC mice developed significant lung inflammation and tissue damage, including widened alveolar septa, interstitial edema and alveolar collapse, and the presence of inflammatory cell infiltration centered on the airways. As expected, high and medium doses of cimifugin inhibited DSS-induced disruption of the intestinal mucosa, and both intestinal inflammation and epithelial barrier were ameliorated to varying degrees, airway inflammation and alveolar septal widening were also significantly ameliorated, and histopathological scores of both lungs and colon were significantly reduced (Figures 1D–G). In addition, quantitative analysis of several key inflammatory factors (IL-6, IL-1β, and TNF-α) also showed that both high and medium doses of cimifugin significantly down-regulated their concentrations in the lungs and colon, with non-significant differences between groups (Figures 2A, B). It is worth mentioning that both high and medium doses of cimifugin did not cause significant pathological damage to the liver and kidney of mice, indicating a high safety profile (Supplementary Figures S1A, B). In summary, we focused primarily on the pharmacologic effects of medium-dose cimifugin, taking into account health economic costs and potential drug side effects. Occludin and ZO-1 are two tight junction-associated proteins that play important roles in maintaining the integrity of intestinal and lung epithelia. The expression levels of these proteins were both reduced in the UC group compared with the control group, and cimifugin significantly reversed the DSS-induced decrease in protein expression (Figures 2C, D). In lung tissues, cimifugin mainly down-regulated the mRNA expression levels of classical inflammatory factors such as CCR5, CCR8 and CXCL10 (Figures 2E–G). In conclusion, the above results suggest that cimifugin significantly improved the severity of ulcerative colitis and its associated lung injury.

3.2 Cimifugin ameliorates gut flora dysbiosis in DSS-induced UC models

Considering the significant contribution of intestinal flora dysbiosis to the progression of UC, we assessed the effect of

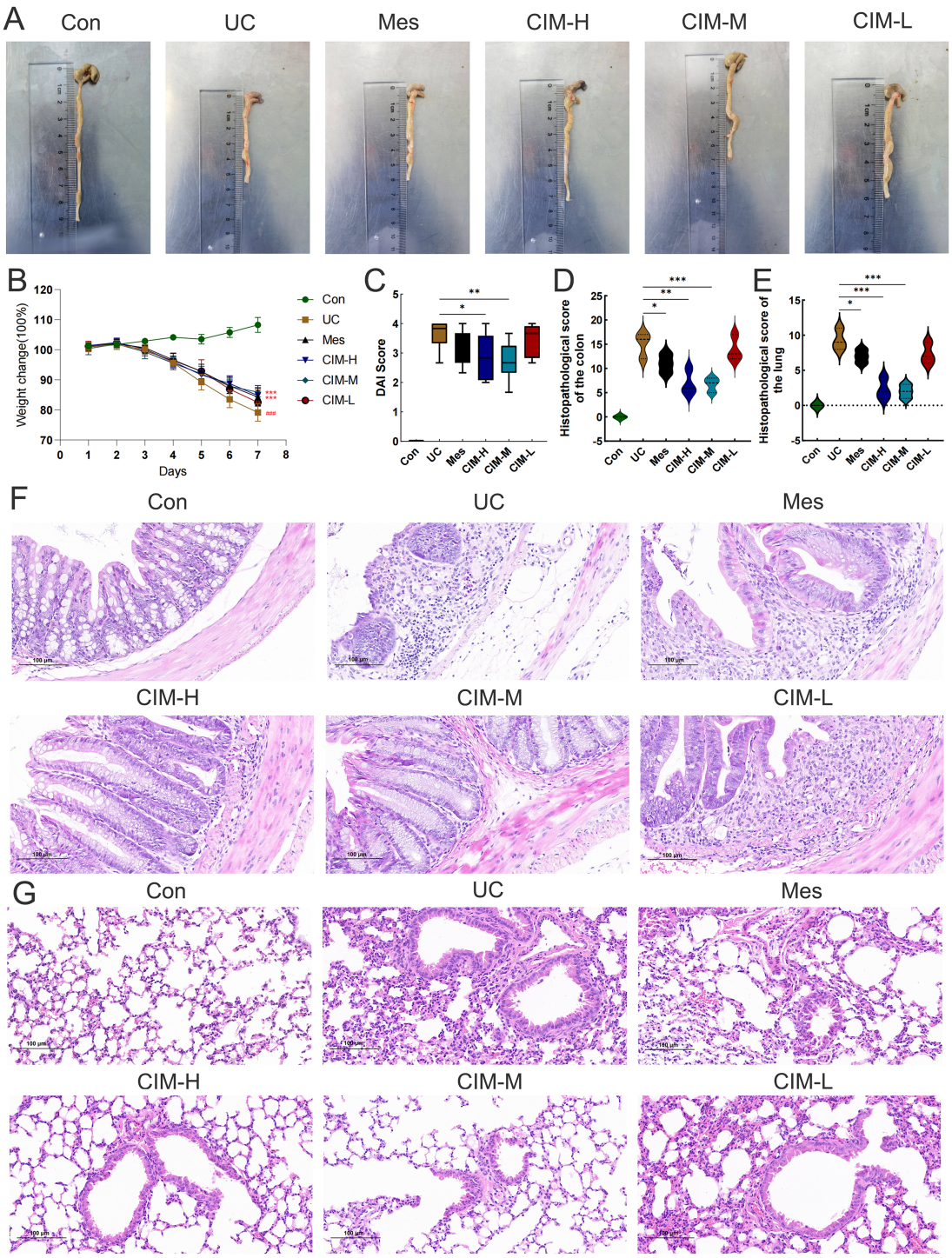

FIGURE 1
Cimifugin ameliorates DSS-induced colitis and lung injury. **(A)** Differences in colon length between different groups (n = 6). **(B)** Changes in body weight of mice in different groups. **(C)** DAI scores of mice in different groups. **(D, E)** Differences in histopathologic scores of colon and lung between different groups. **(F)** Representative images of HE staining of the colon in different groups of mice (n = 3). **(G)** Representative images of HE staining of lungs in different groups of mice (n = 3). Con, Control group; UC, Ulcerative colitis group; Mes, Mesalazine group; CIM-H, High-dose cimifugin group; CIM-M, Medium-dose cimifugin group; CIM-L, Low-dose cimifugin group. $^{###}P < 0.001$, compared with Con group. $^{*}P < 0.5$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with DSS group.

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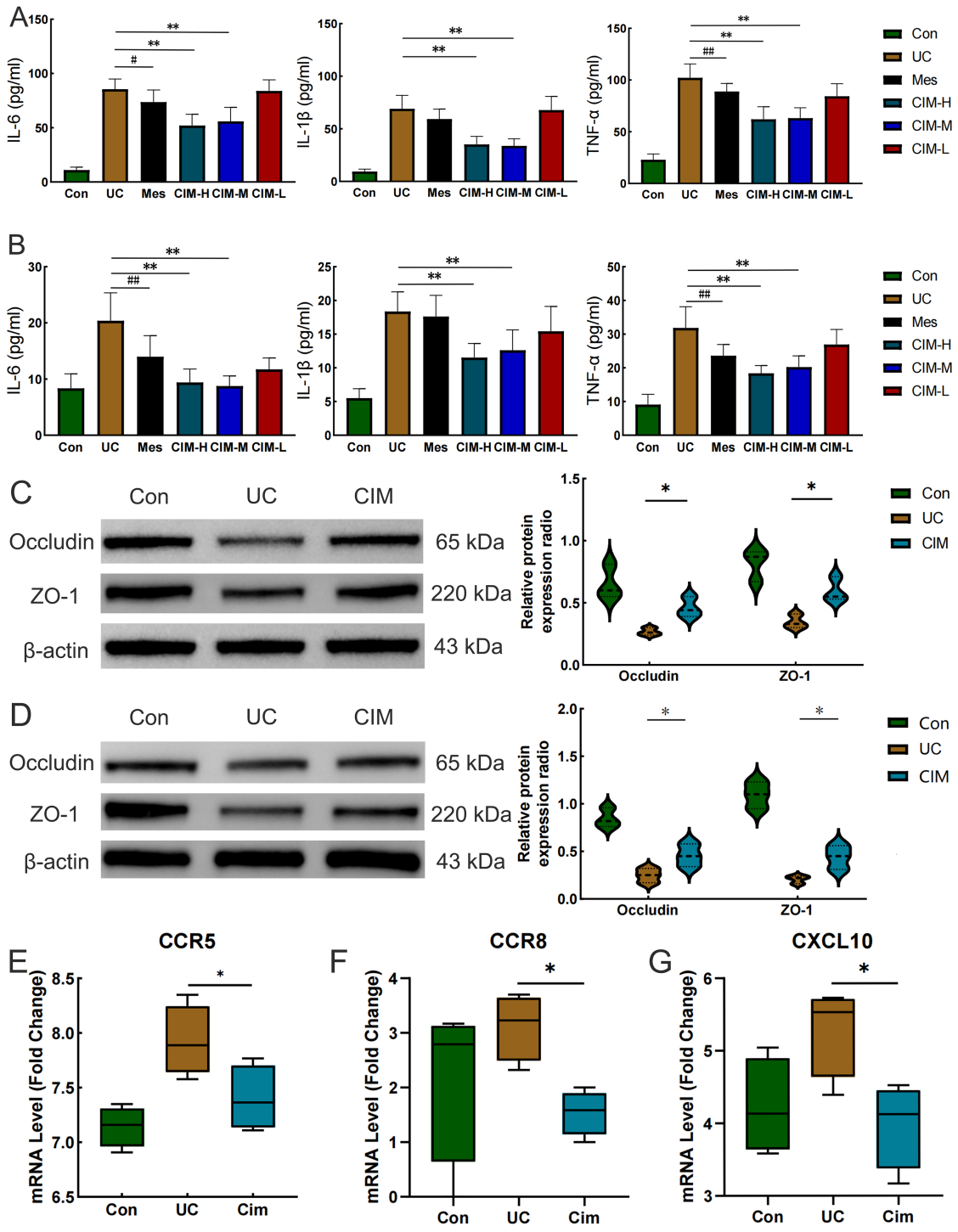

FIGURE 2
Cimifugin ameliorates DSS-induced colonic epithelial barrier damage and lung inflammation. **(A)** Effect of Cimifugin on IL-6, IL-1β, and TNF-α levels in colonic tissue (n = 6). **(B)** Effect of Cimifugin on IL-6, IL-1β, and TNF-α levels in lung tissues (n = 6). **(C)** Expression levels of Occludin and ZO-1 proteins in the colon of different groups of mice (n = 3). **(D)** Expression levels of Occludin and ZO-1 proteins in the lung of different groups of mice (n = 3). **(E–G)** The mRNA expression levels of CCR5, CCR8 and CXCL10 in lung tissues of different groups of mice (n = 4). Con, Control group; UC, Ulcerative colitis group; Mes, Mesalazine group; CIM-H, High-dose cimifugin group; CIM-M, Medium-dose cimifugin group; CIM-L, Low-dose cimifugin group; IL-6, Interleukin - 6; IL-1β, Interleukin-1 beta; TNF-α, Tumor necrosis factor-alpha; ZO-1, Zonula Occludens-1; CCR5, C-C chemokine receptor type 5; CCR8, C-C chemokine receptor type 8; CXCL10, C-X-C motif chemokine ligand 10. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with Mes group. $^{*}P < 0.5$; $^{**}P < 0.01$, compared with DSS group.

cimifugin on the diversity and composition of intestinal flora by 16S rRNA amplicon sequencing. A total of 643 OTUs were obtained in the normal control, UC and cimifugin-treated groups, of which 404 were common to all 3 groups. There were 116, 78, and 45 unique OTUs in the normal control, UC, and cimifugin-treated groups, respectively (Figure 3A). cimifugin administration attenuated the DSS-induced decrease in bacterial diversity and abundance as shown by the α-diversity index represented by observed-species, Chao1, and ACE (Figure 3B). Indeed, PCoA analysis revealed that the cimifugin-treated group had a more similar microbial community structure to the normal control group (Figure 3C), which was confirmed by the Rank Abundance curve, i.e., the CIM group had a longer and smoother span of the curve relative to the UC group, suggesting higher species richness and a more homogeneous species distribution (Figure 3D). Comparison of beta diversity indices between groups confirmed that cimifugin significantly improved the beta diversity of the samples, and NMDS also found that the CIM group was closer to the normal group (Figures 3E, F). In terms of species composition, LEfSe analysis showed that the UC and CIM groups had different taxa and suggested that *Muribaculum*, *Marvinbryantia*, *Alloprevotella*, *Prevotellaceae*, and *Muribaculaceae* might mediate the interfering effects of cimifugin on UC (Figure 3G). Specifically, at the phylum level, cimifugin inhibited the increase in the number of *Planctomycetota*, *Myxococcota*, *Acidobacteriota*, *Cyanobacteria*, *Gemmatimonadota*, *Desulfobacterota*, and *Firmicutes* and increased abundance of *Bacteroidota* (Figures 3H, I). Figure 3J demonstrates the differences in dominant species at the phylum level among the three groups, with *Acidobacteriota* identified as the dominant species in the UC group and *Proteobacteria* as the dominant species in the CIM group. At the genus level, cimifugin suppressed the abundance of *Lactococcus*, *Ligilactobacillus*, *Blautia*, *Bacteroides*, *Parabacteroides*, and *Romboutsia* and increased the abundance of *Lactobacillus* (Figures 4A, B). T-test revealed that the abundance of Bacteroides was significantly lower and Muribaculum was significantly higher in the CIM group compared to the UC group ($P < 0.05$, Figure 4C). Figure 4D demonstrates the differences in dominant species at the genus level among the three groups, where *Bacteroides* was identified as the dominant species in the UC group, while *Prevotellaceae*, *Alloprevotella*, and *Enterobacter* were the dominant species in the CIM group. After cimifugin treatment, *Parasutterella*, *Butyricoccus*, *Anaeroplasma*, and *Akkermansia* were identified as dominant species in the CIM group that dominated the interactions and played unique as well as important roles in maintaining the stability of microbial community structure and functioning in this environment (Figure 4E). Meanwhile, cimifugin also significantly affected the genomic function of the colony. cimifugin down-regulated the activity of ion coupled transporters, gene transcription processes, and glycolysis, and up-regulated the enrichment of processes such as energy metabolism, amino acid metabolism, and homologous recombination (Figure 4F). Interestingly, alterations in metabolism-related pathways were an important feature of the CIM group. cimifugin significantly enhanced cysteine, methionine, riboflavin, niacin, and nicotinamide metabolism in the colony and down-regulated carbohydrate metabolism processes ($P < 0.05$, Figure 4G). Taken together, cimifugin may attenuate DSS-induced intestinal dysbiosis by altering intestinal microbiota composition and metabolic functions.

3.3 Cimifugin targets JAK1/STAT1 signaling pathway to improve ulcerative colitis-related lung injury

To further investigate the signaling mechanism of cimifugin to improve ulcerative colitis-related lung injury, we performed RNA-seq analysis on colon and lung tissues separately. Transcriptome analysis showed that 228 DEGs existed between colon tissues of CIM and UC groups (Figure 5A), and GO analysis revealed that DEGs were mainly concentrated in mitochondrial translation, CXCR3 chemokine receptor binding, motile cilium, carboxylic acid transmembrane transporter activity, transition metal ion binding, chemokine receptor binding, cytokine binding, and other processes closely related to UC (Figure 5B). Compared with normal mice, cell cycle checkpoints, mitotic spindle checkpoints, and other pathways related to cell division were upregulated in the UC group, while collagen formation was downregulated in the CIM group (Figure 5C). In lung tissues, 605 DEGs existed between the CIM and UC groups (Figure 5D), and GO analysis revealed that DEGs were mainly focused on response to lipid, cellular response to chemical stimulus, hormone activity, collagen trimer and regulation of cell death, while regulation of signaling receptor activity, inflammatory response, metal ion homeostasis and carboxylic acid transport are biological processes shared with the colon, suggesting that the above processes may be common target processes for cimifugin to ameliorate ulcerative colitis-related lung injury (Figure 5E). GSEA similarly validated these results and found that FCεRI and NF-κB-mediated signaling processes were up-regulated in lung tissues of UC mice compared to normal mice, whereas cimifugin mainly down-regulated the activity of collagen synthesis and chemokine signaling pathways (Figure 5F). In the colon, cimifugin mainly acts on cytokine-cytokine receptor interaction, cysteine and methionine metabolism, Hippo signaling pathway, TGF-β signaling pathway, cellular senescence, glycolysis, and JAK-STAT signaling pathway (Figure 6A), whereas in the lung, cimifugin mainly affected cytokine-cytokine receptor interaction, PI3K-Akt signaling pathway, TNF signaling pathway, JAK-STAT signaling pathway, IL-17 signaling pathway, *Staphylococcus aureus* infection, cellular senescence, PPAR signaling pathway (Figure 6B). Considering that the JAK-STAT signaling pathway was mentioned in both tissues and that tofacitinib, which is a pan-Janus kinase inhibitor, was also shown to be effective in UC, we explored the effect of cimifugin on the JAK1/STAT1 signaling pathway (27). The expression of both JAK1 and STAT1 was up-regulated in lung and colon tissues in the DSS group compared with the control group, whereas the expression of both JAK1 and STAT1 proteins was significantly suppressed by the administration of cimifugin (Figures 6C–E).

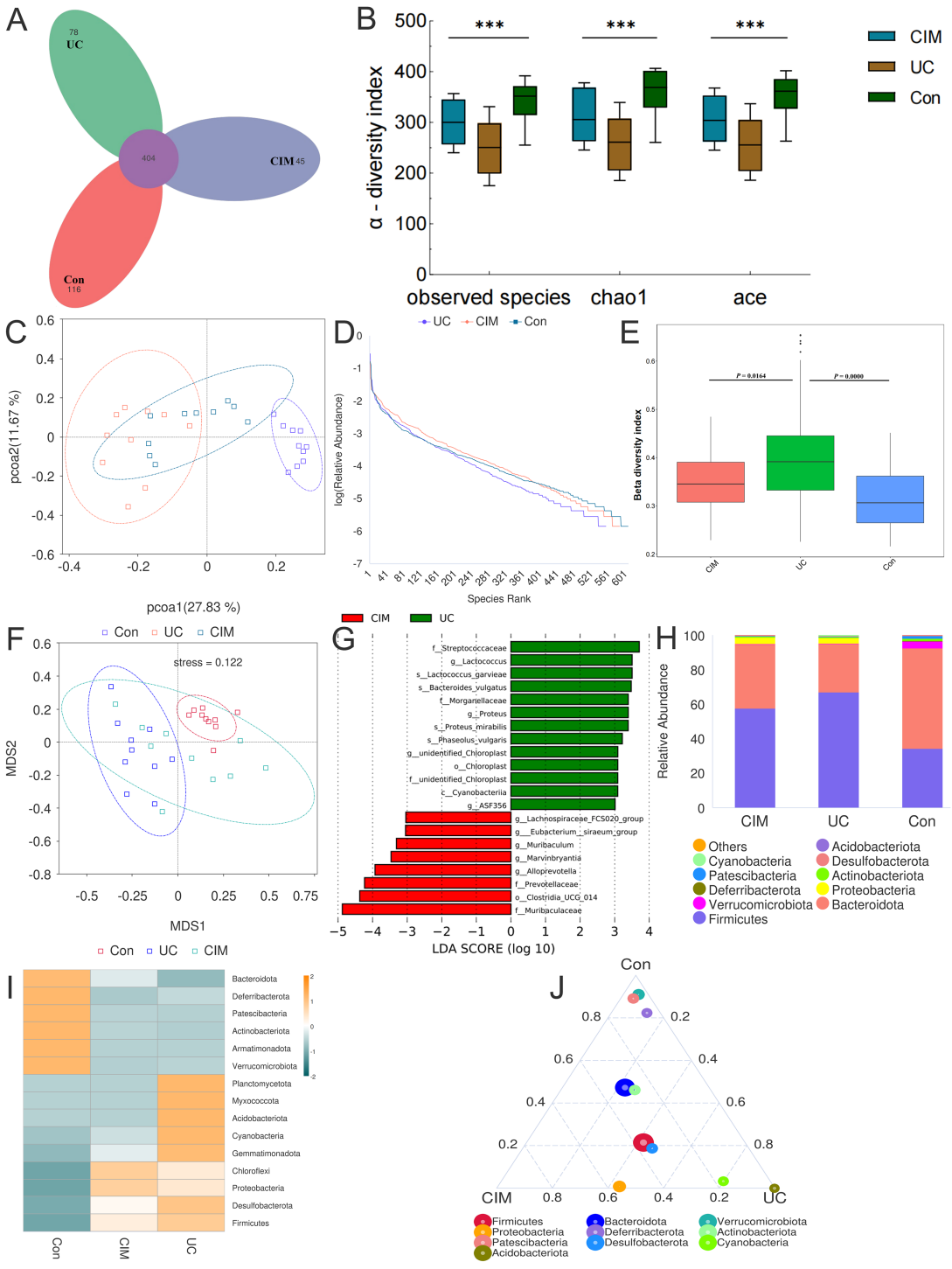

FIGURE 3
Cimifugin remodeled the gut microbiota composition in mice with DSS-induced ulcerative colitis. **(A)** Petal plot demonstrating the difference in the number of OTUs between the 3 groups. **(B)** α-diversity analysis based on multiple methods such as observed-species, Chao1, and ACE. **(C)** Bray-Curtis principal coordinate analysis (PCoA) analysis of gut microbiota based on the OTU level among three groups. **(D)** Three groups' Rank Abundance curves. In the horizontal direction, species richness is reflected by the width of the curve, the higher the species richness, the larger the span of the curve on the horizontal axis; in the vertical direction, the smoothness of the curve reflects the homogeneity of the species in the samples, the flatter the curve, the more homogeneous the species distribution. **(E)** Between-group comparison of β-diversity indices. **(F)** NMDS based on the differences in gut microbiota among the three groups. **(G)** Histogram of the distribution of LDA values in the CIM and UC groups. **(H)** Classification and composition ratio of the gut microbiota of mice in the three groups at the phylum level. **(I)** Based on the species annotation and abundance information of the intestinal microbiota of mice in the three groups at the phylum level, the heat map was obtained by clustering using the maximum ranking method. **(J)** Select the top 10 species with average abundance in the three groups of samples at the phylum classification level, and generate a ternaryplot reflecting the difference of dominant species among the three groups of samples. Con, Control group; UC, Ulcerative colitis group; CIM, Cimifugin group. N = 10, ***$P < 0.001$.

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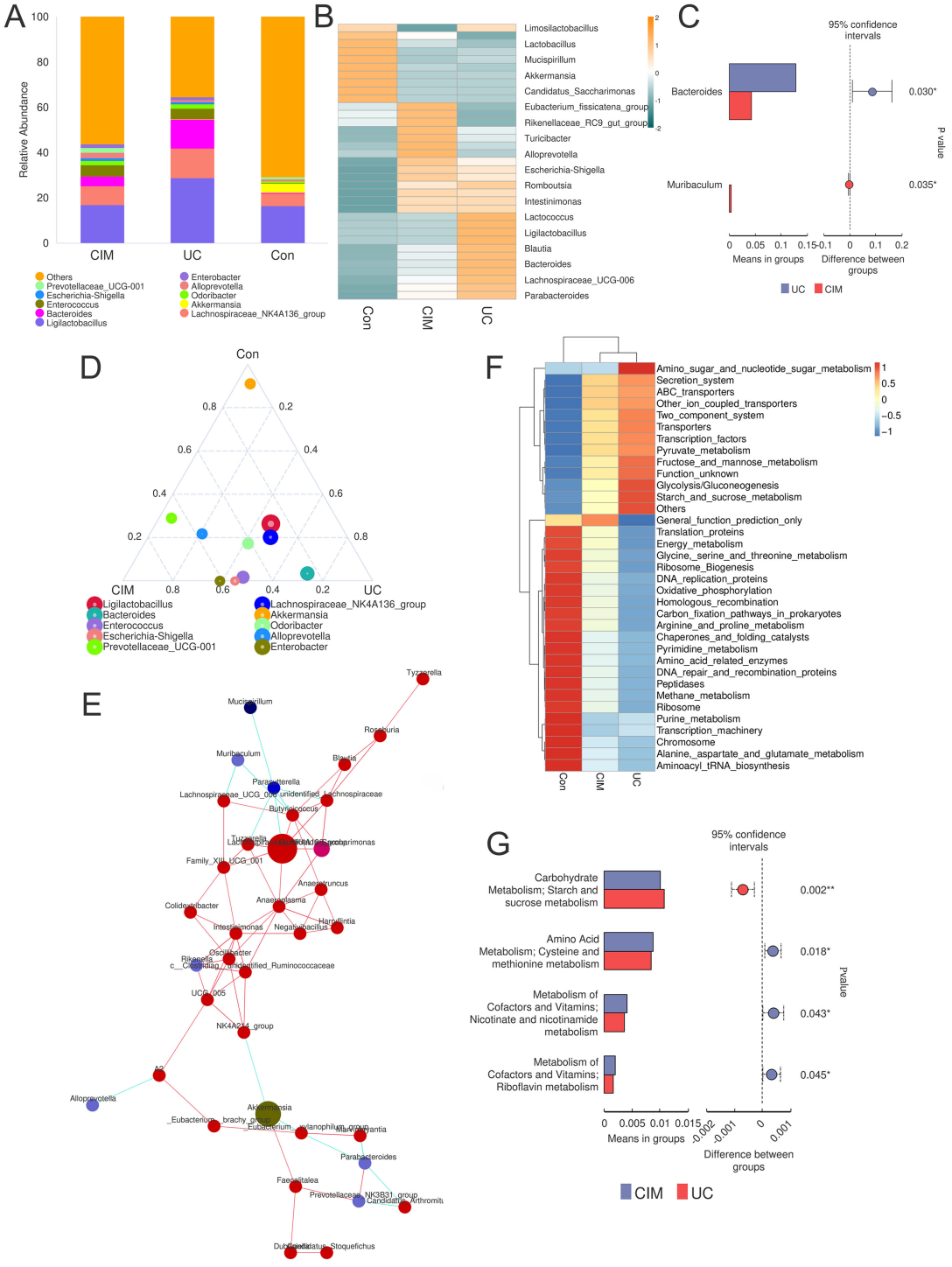

FIGURE 4
Cimifugin alters the genomic function of the gut microbiota. **(A)** Taxonomic and compositional ratios of the gut microbiota of mice in the three groups at the genus level. **(B)** Heatmap obtained by clustering the gut microbiota of mice in the three groups based on species annotation and abundance information at the genus level using the maximum value ranking method. **(C)** Species with significant differences at the genus level between the UC and CIM groups. **(D)** Species that ranked in the top 10 in average abundance at the genus classification level for the three groups of samples were selected to generate a ternaryplot reflecting the differences in dominant species among the three groups of samples. **(E)** Species correlation network plot showing dominant species that dominate under the action of cimifugin and the clusters of species that are closely related to them. **(F)** Heatmap of the relative abundance of microbial genome functions among different groups obtained based on PICRUSt analysis. **(G)** Metabolic processes in the presence of significant differences between the UC and CIM groups. Con, Control group; UC, Ulcerative colitis group; CIM, Cimifugin group. N = 10, *$P < 0.5$; **$P < 0.01$.

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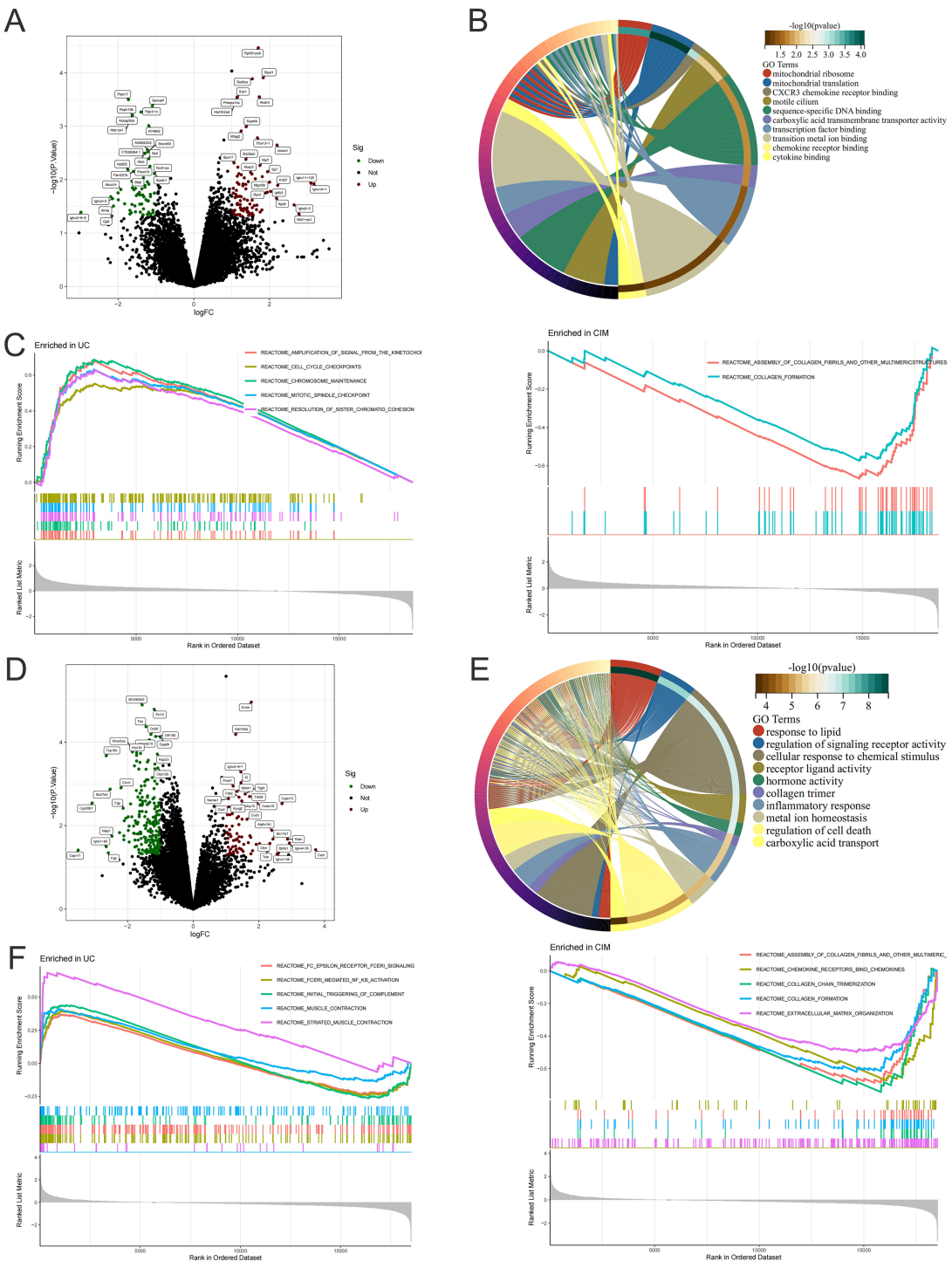

FIGURE 5
Cimifugin alters gene expression in colon and lung tissue. **(A)** Volcano plot demonstrating up- and down-regulated genes in the colon of the CIM group compared to the UC group. **(B)** GO enrichment analysis of DEGs in the colon. **(C)** GSEA analysis of DEGs in the colon of both groups. **(D)** Volcano plots demonstrating up- and down-regulated genes in lung tissues of the CIM group compared with the UC group. **(E)** GO enrichment analysis of DEGs in lung. **(F)** GSEA analysis of DEGs in lung tissues of both groups. N = 4.

3.4 Cimifugin inhibits macrophage polarization to M1 in the colon and lungs

UC is typically an immune-disordered disease, so we combined scRNA-seq data from the colon of UC patients with RNA-seq data from the colon tissue of the CIM group to look for immune cells that might be targeted by cimifugin. Gene expression markers were used to classify all cells into eight major subpopulations, including plasma cells, B cells, $CD4^+$ T cells, macrophage, fibroblast, $CD8^+$ T cells, smooth muscle cells, and epithelial cells (Figure 7A). The

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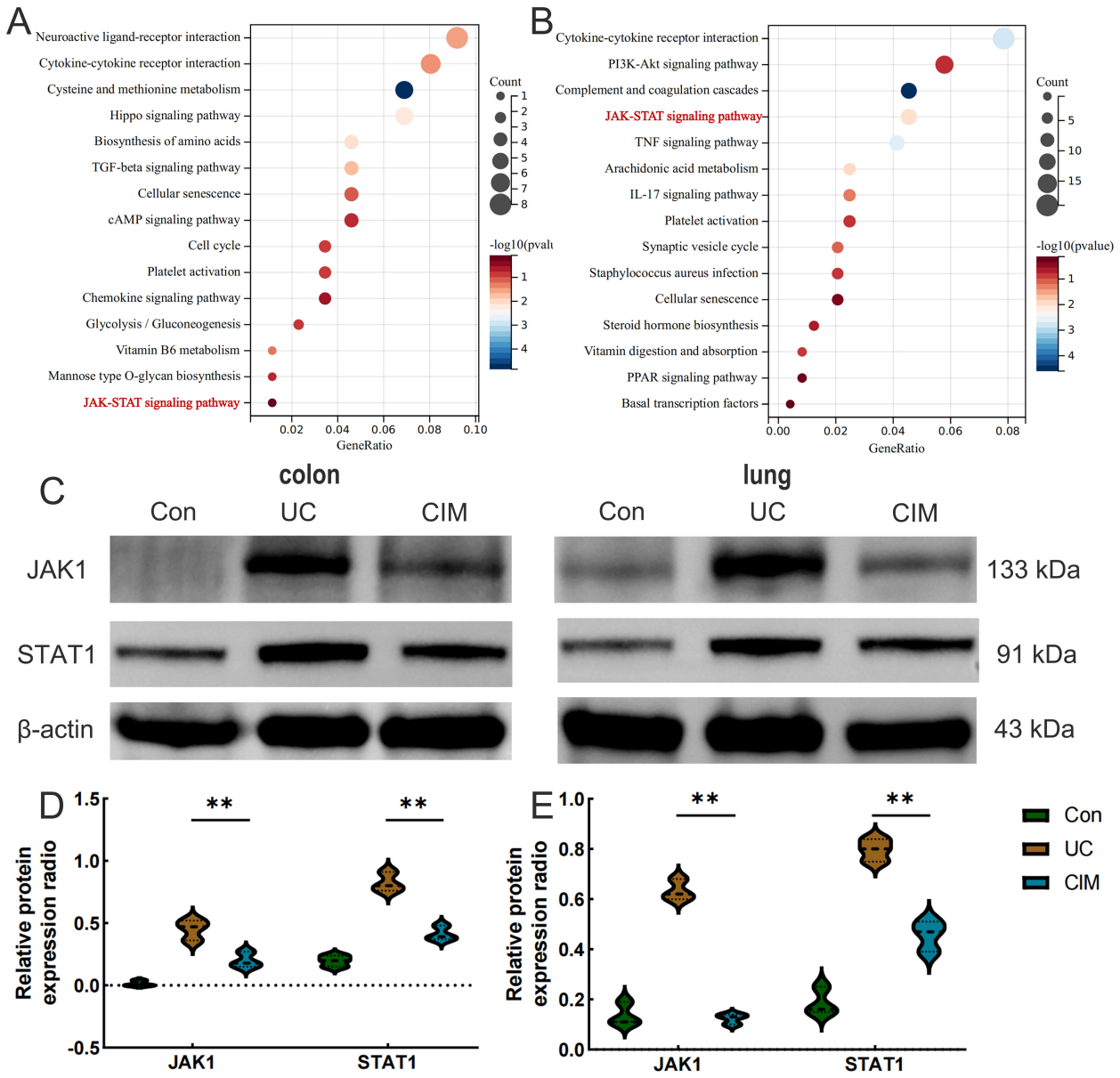

FIGURE 6
Cimifugin affects JAK1/STAT1 protein expression in colon and lung tissues of UC mouse models. **(A)** KEGG enrichment analysis of DEGs in colon. **(B)** KEGG enrichment analysis of DEGs in lung tissues. **(C–E)** Expression levels of JAK1 and STAT1 proteins in colon and lung tissues of different groups of mice (n = 3). Con, Control group; UC, Ulcerative colitis group; CIM,: Cimifugin group; JAK1, Janus kinase 1; STAT1, Signal Transducer and Activator of Transcription 1. $^{**}P < 0.01$.

cimifugin-related regulatory genes obtained from transcriptome analysis were functionally scored and found to mainly fit the gene transcriptional changes in plasma cell, B cell, $CD4^+$ T cell and macrophage (Figure 7B). Among them, macrophage had the most significant UC-related features, such as inflammatory response and cell death, which were mainly reflected in the activation of TNF-α/NF-κB signaling pathway, γ-interferon signaling pathway, and apoptosis, etc., and there was a higher intensity of intercellular communication between macrophage and other cell types (Figures 7C, D). Most importantly, among the eight major subpopulations, JAK/STAT was activated only in macrophage, suggesting that M1 macrophage, which exhibits a pro-inflammatory phenotype, may be a target cell for cimifugin. Immunofluorescence showed that compared with normal mice, the infiltration of M1 macrophages in the colon tissue of the UC group was significantly increased, while cimifugin downregulated the abundance of M1 macrophages. More importantly, this synchronous phenomenon was also found in lung tissue (Figures 8A, B). These findings are consistent with previous analysis of scRNA seq, suggesting that macrophages may be the target cells mediating the protective effect of cimifugin on UC and its associated lung injury (Figures 8C, D).

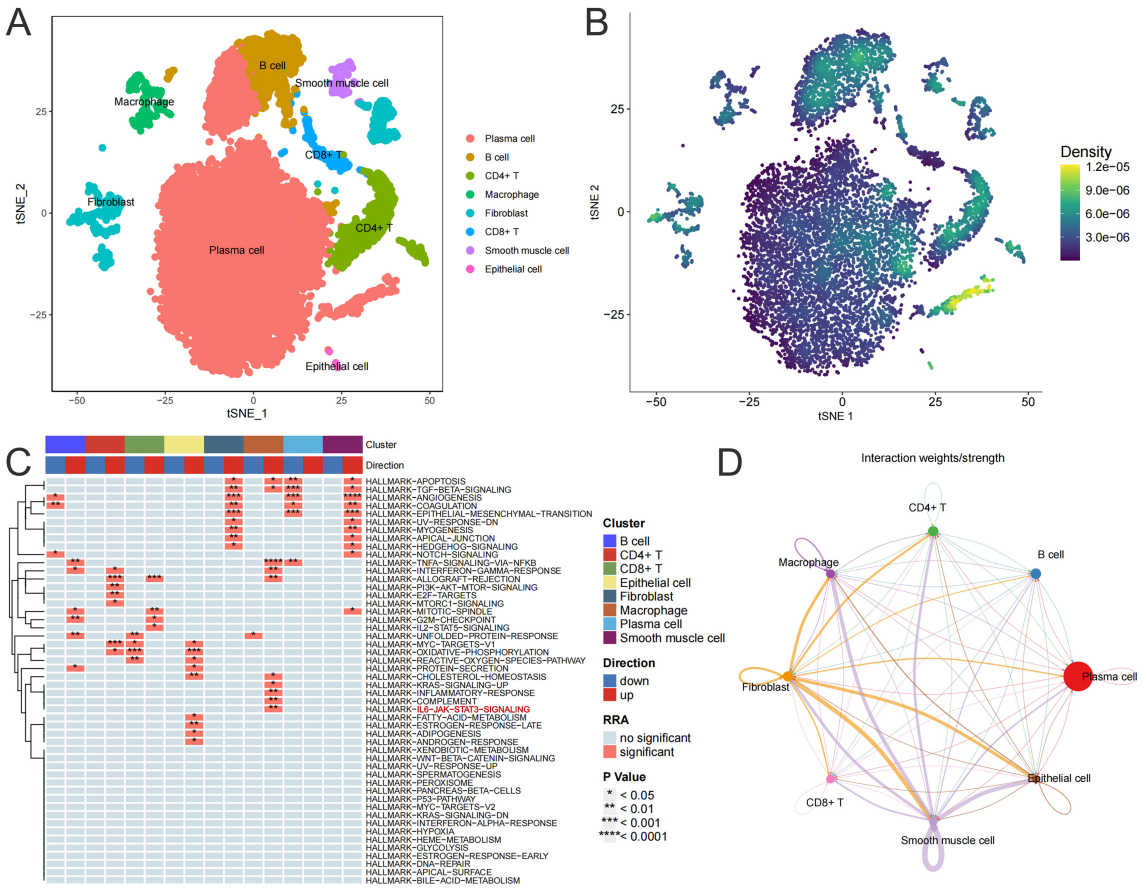

FIGURE 7
M1 macrophage may be a target cell for cimifugin intervention in UC. **(A)** TSNE used for dimensionality reduction, cell classification, and definition. **(B)** Scoring the regulatory genes related to cimifugin and mapping them to transcriptional changes in various types of cells to obtain the target cells most likely to be intervened by cimifugin. **(C)** Changes in the enrichment pathways of marker gene sets for each cell subpopulation in UC. **(D)** Inference of intercellular communication network strength in UC sample colon tissue, with the thickness of the lines representing the strength.

3.5 Cimifugin regulates macrophage polarization to resist inflammation by inhibiting JAK1/STAT1 pathway activation

Next, we investigated how cimifugin regulates macrophage polarization. Considering that lung and colon are co-enriched for the JAK1/STAT1 pathway at the transcriptional level, we investigated the effect of cimifugin on the activation of the JAK1/STAT1 pathway during macrophage polarization. As expected, LPS promoted the phosphorylation of JAK1 and STAT1 in macrophages, whereas cimifugin inhibited this process (Figures 9A, B). Considering that there was no significant difference between the effects of treatment at 100 mg/L and treatment at 200 mg/L on the expression of p-JAK1 and p-STAT1 in macrophages, we chose to observe the correlation between activation of the JAK1/STAT1 pathway and cimifugin-regulated macrophage polarization under the intervention of the 100 mg/L concentration. *In vitro*, LPS promoted M1 polarization in macrophages. However, cimifugin treatment down-regulated the expression of M1 polarization-related markers (IL-1β, iNOS) and up-regulated M2 polarization-related markers (CD206, Arg1), respectively. The modulation of macrophage polarization-related proteins by cimifugin was counteracted after specific inhibition of JAK1 activation using Upadacitinib (Figures 9C–G). Specifically, the LPS + CIM + Upadacitinib group exhibited higher levels of iNOS and IL-1β expression, as well as lower levels of Arg1, CD206 expression, than the LPS + CIM group. In addition, the concentrations of IL-6 and TNF-α detected by ELISA showed expression trends consistent with those of M1 polarization-related markers after Upadacitinib treatment (Figures 9H, I).

4 Discussion

UC is an IBD characterized by intestinal inflammation, abdominal pain, and blood in the stool.Even in the absence of respiratory symptoms, patients with UC may have abnormalities on pulmonary function tests, radiology, and histopathology (28, 29). These untreated airway inflammations can cause irreversible destruction of the airways and therefore require stable, effective long-term pharmacologic interventions (30). In this study, we demonstrated the therapeutic efficacy of cimifugin in UC-related

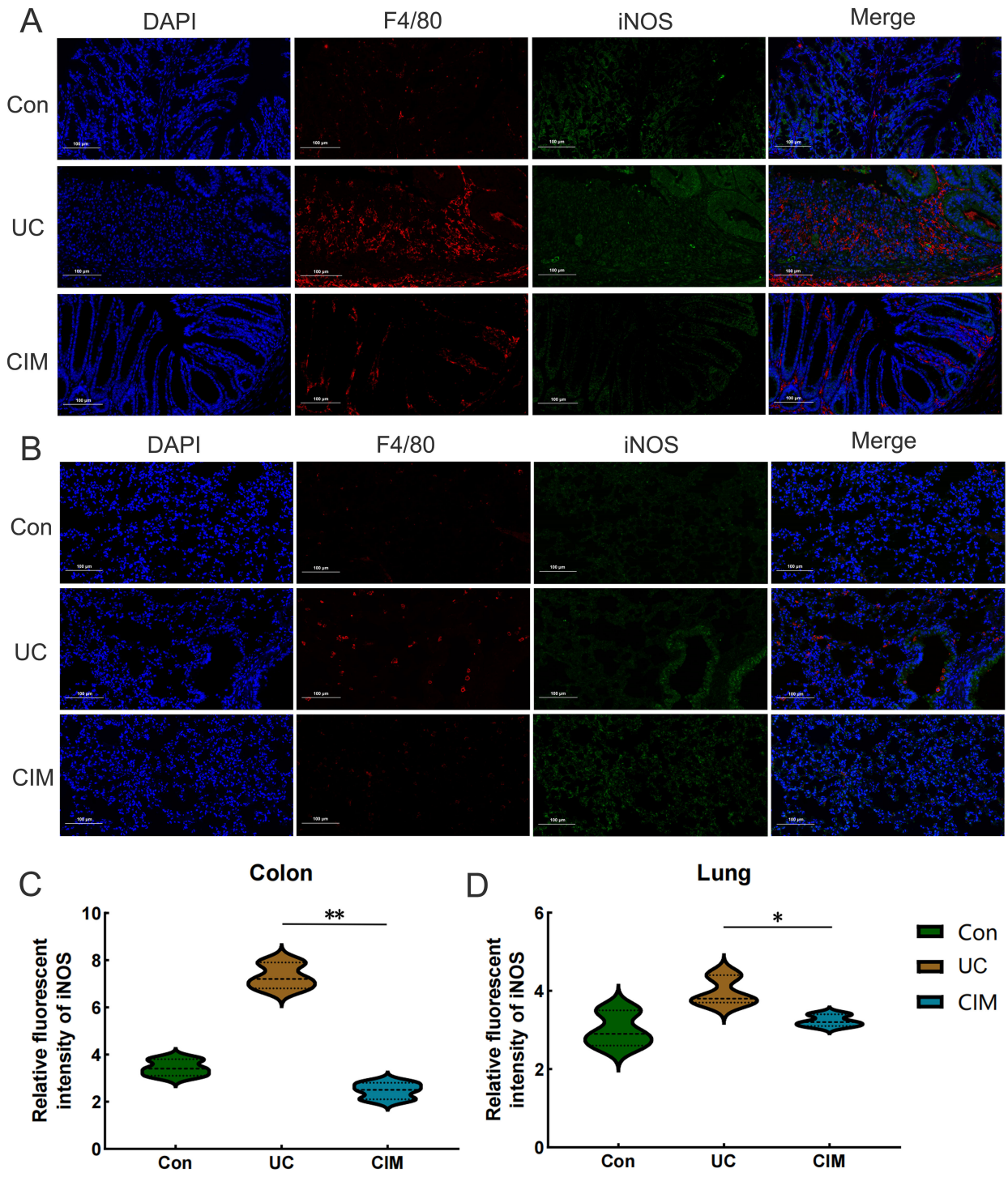

FIGURE 8
In vivo experiments confirmed the centrality of M1 macrophages in Cimifugin intervention in UC and its associated lung injury. **(A)** Immunofluorescence revealed the degree of M1 macrophage infiltration in the colon of different groups of mice. **(B)** Immunofluorescence revealed the degree of M1 macrophage infiltration in the lungs of different groups of mice. **(C, D)** Quantitative analysis results of immunofluorescence in colon and lung tissues (n = 3). Con, Control group; UC, Ulcerative colitis group; CIM, Cimifugin group; iNOS, inducible nitric oxide synthase; DAPI, 4′,6-diamidino-2-phenylindole. *$P < 0.5$; **$P < 0.01$.

lung injury. By combining RNA-seq and 16S rRNA sequencing of both organs, these findings enabled us to construct a comprehensive network from a more holistic perspective, which in turn enhanced our understanding of the deeper mechanisms of cimifugin ameliorating UC-related lung injury and further demonstrated that its mechanism is related to the inhibition of the JAK1/STAT1 pathway and M1 macrophage-mediated inflammatory states in the colon and lungs, as well as ameliorating gut microbiota disruption.

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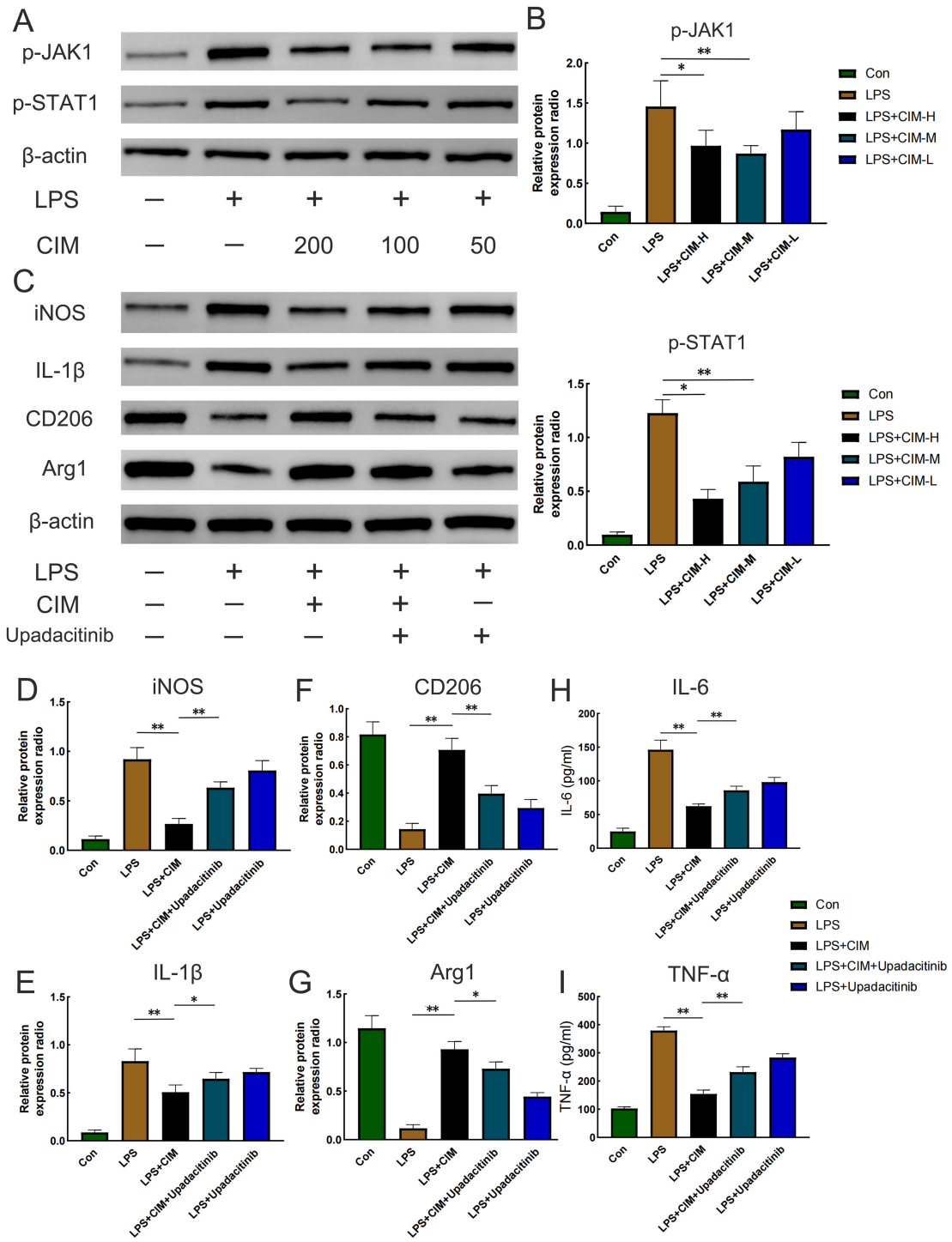

FIGURE 9
Cimifugin regulates M1 polarization in macrophages by inhibiting activation of the JAK1/STAT1 pathway. **(A)** Effects of different concentrations of cimifugin on the phosphorylation of JAK1 and STAT1 in an *in vitro* model. **(B)** Analysis of grayscale values corresponding to p-JAK1 and p-STAT1. **(C)** Expression levels of the corresponding markers after treatment with cimifugin and Upadacitinib. **(D–G)** Analysis of grayscale values corresponding to various markers (iNOS, IL-1β, Arg1, CD206). **(H, I)** Concentrations of IL-6 and TNF-α in different groups. Con, Control group; LPS, Lipopolysaccharide; Cim, Cimifugin; CIM-H, High-dose cimifugin group; CIM-M, Medium-dose cimifugin group; CIM-L, Low-dose cimifugin group; IL-6, Interleukin - 6; IL-1β, Interleukin-1 beta; TNF-α, Tumor necrosis factor-alpha; JAK1, Janus kinase 1; STAT1, Signal Transducer and Activator of Transcription 1; iNOS, inducible nitric oxide synthase; CD206, Cluster of Differentiation 206; Arg1, arginase 1. N = 3, $^{*}P < 0.5$; $^{**}P < 0.01$.

The respiratory tract and gastrointestinal tract are both rich in epithelial cells, goblet cells, submucosal glands, and lymphoid tissue, and the same embryological origin has led researchers to consider the possibility that both may suffer the same type of damage (31). Whether it's an antigen, cytokine storm, or a specific pathogen, all may play a potential role in UC-related lung injury. The breakdown of the intestinal epithelial barrier is a prominent feature of UC. The injury of the intestinal mucosa and intestinal inflammation make the tight connection between the epithelium fail, and the microbial antigens in the intestinal cavity leak, thus promoting the abnormal immune response of the whole body (32). For example, memory T cells are exposed to specific antigens in the inflamed intestinal mucosa and are induced to express large amounts of C-C chemokine receptor 3 and C-X-C chemokine receptor 5 (33). Subsequently, they can migrate to broncho-associated lymphoid tissue, where they express more chemokine receptors. Leakage of microbial antigens into the peripheral circulation also induces activation of tissue-resident macrophages, which induce neutrophil-mediated inflammation by secreting a range of cytokines (34). Indeed, current studies on UC-related lung injury have also focused on exploring the disruption of immune homeostasis by M1 macrophage (3). In our study, CXCL10 was found for the first time to be a differentially expressed chemokine between lung tissues of the UC and normal groups, whereas $CXCL10^{+}$ macrophage has been shown to be enriched in the colon of patients with UC, exhibiting pro-inflammatory gene expression characterized by interferon-related responses (35). CCR5 has also been shown to enhance macrophage migration and infiltration in the lung (36). Although there are no studies on the effects of cimifugin on lung diseases, demonstrated that cimifugin reduced the expression of the above chemokines and their receptors, inhibited the enrichment of M1 macrophage in the colon and lungs, and upregulated the expression of tight junction proteins, such as ZO-1 and Occludin, suggesting that cimifugin restores the structure and function of the epithelial barrier and maintains immune homeostasis in both organs.

Although pan-JAK-inhibitor tofacitinib, also known as IBD, was effective in UC patients but not in Crohn's patients, suggesting that the JAK/STAT signaling pathway may induce more severe inflammation in UC. From published data, JAK inhibitors provide significant clinical benefit in both acute exacerbation and maintenance phases of UC (37). JAK inhibitors show better therapeutic efficacy compared to traditional drugs such as TNF-α monoclonal antibodies and mesalazine (38). JAK inhibitors provide benefit even if the patient has already been treated with a TNF-α monoclonal antibody (39). Adverse events associated with JAK inhibitors are generally controllable and dose-dependent, so the lowest effective dose should be used during the maintenance phase of treatment (40). Although cimifugin has not yet been used in clinical practice, in light of this principle, an appropriate dose range should be explored for cimifugin, which is also a JAK inhibitor. The expression and activation levels of STAT1 in colon tissues of UC patients were significantly increased, which was mainly attributed to infiltrating peripheral neutrophils and monocytes/macrophages (41). In fact, JAK/STAT signaling plays a decisive role in the differentiation of monocytes into macrophages, and macrophage activation is thought to be a driver of intestinal inflammation (42). Studies have shown that inhibition of the JAK1/STAT1 signaling pathway causes macrophages to shift from a pro-inflammatory M1-like phenotype to an M2-like phenotype that inhibits inflammation, ultimately leading to an accelerated recovery of colon inflammation. Based on previously published studies, this study selected three doses within the dose range of 12.5-50 mg/kg as the research subjects and found that both concentrations of 25 mg/kg and 50 mg/kg of cimifugin could significantly inhibit the activation of the JAK1/STAT1 pathway. It indicates that it is a promising inhibitor of the JAK/STAT target (17, 43, 44). Considering that the four different molecules of the JAK family and the seven members of the STAT family have great plasticity and complexity in UC and its associated lung injury, future research should focus on identifying the contribution of each member to further develop highly selective targeted drugs (45, 46).

Many studies have shown that gut flora plays a key role in the pathogenesis of UC, and that improvements in gut flora composition and metabolic patterns can lead to disease remission or even cure (47, 48). In the present study, we demonstrated that cimifugin significantly reversed the intestinal flora disruption in a mouse model of UC, suggesting that the protective effect of cimifugin may be related to its modulation of the intestinal microbiota. Notably, cimifugin treatment significantly increased the abundance of *Lactobacillus* and *Butyricoccus* was identified as the dominant species in the CIM group. Previous studies have shown reduced colonization of *Lactobacillus* as a potential anti-inflammatory bacterium in a mouse model of UC. *Lactobacillus* can alleviate colitis by specifically increasing the proportion of intestinal macrophages and IL-10 secretion through a mechanism that promotes the conversion of macrophages to M2 macrophages and release of IL-10 (49). At the same time, this effect was associated with the promotion of protective acetate production by *Lactobacillus johnsonii* (50). *Butyricicoccus* is a butyrate-producing clostridial cluster IV genus whose numbers are reduced in the stool of UC patients (51). *Butyricoccus* can reverse the inflammation-induced elevation of CLDN1 protein levels by secreting butyrate, which in turn maintains the integrity of the epithelial barrier. In addition, these *Butyricoccus*-derived butyric acids were able to promote the expression of IL-10, ARG1, and CD206 in macrophages in UC and induced their polarization to M2 (52). These connections between microbiota and their metabolites, which have been intensively studied, undoubtedly provide a powerful note for understanding the triad of cimifugin, the JAK1/STAT1 pathway, and macrophage differentiation. We also observed a significant reduction in the abundance of pathogenic *Bacteroides* after cimifugin treatment. A cohort study including 73 patients with UC found that patients with active UC had high levels of *Bacteroides*-derived proteases in their intestines, and these proteases directly induced barrier dysfunction (53). In addition, cimifugin significantly enhances the metabolism of cysteine, methionine, riboflavin, niacin and nicotinamide in the intestinal microbiota. Among them, supplementation of methionine has been proven to alleviate the severity of the disease and inhibit the expression of inflammation-related genes (54). The mechanism

may be related to the abnormal activation of M1 macrophages and the alteration of the production pattern of short-chain fatty acids (55). Riboflavin, on the other hand, attenuates colitis by inhibiting neutrophil infiltration, modulating oxidative stress, and ameliorating DNA damage, as evidenced by a reduction in the ulcer index and the inflammation index (56, 57). niacin has been suggested to protect mice from DSS-induced colitis in a D prostanoid receptor 1-dependent manner, partly through the mechanism of inhibition of pro-inflammatory gene expression in macrophages (58). Together, these results suggest that cimifugin may maintain the stability of the internal environment by increasing the abundance of potentially beneficial bacteria and decreasing pathogenic bacteria.

The study has several flaws. First, due to the lack of lung single-cell transcriptome sequencing samples from UC-related lung injury, this study could not confirm the key target cells of cimifugin in human lung tissue. Second, although cimifugin has been identified as a key species in improving gut microbiota dysregulation, its regulation of microbial metabolic patterns remains unclear. Moreover, UC can often turn into a chronic course, and the composition of the gut microbiota, metabolic patterns, and immune disordered states during the stable phase may be different from those during the acute phase, which needs to be identified in future studies. Finally, other possible mechanisms of action of cimifugin in the intervention of UC-related lung injury suggested by transcriptomics still need further experimental verification.

5 Conclusions

Taken together, our work suggests that oral administration of cimifugin ameliorates DSS-induced colitis and its associated lung inflammation, which is associated with its inhibition of chemokine release, reduction of M1 macrophage infiltration and down-regulation of JAK1/STAT1 protein expression. On the other hand, cimifugin may ameliorate the imbalance of the gut microbiota by regulating the colonization of specific microorganisms. Our findings may contribute to a better understanding of the molecular events and immune microenvironment of UC-related lung injury and highlight the therapeutic potential of cimifugin in related diseases.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by Experimental Animal Ethics Committee of Shandong Academy of Traditional Chinese Medicine. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GW: Data curation, Methodology, Software, Writing – original draft, Writing – review & editing. FY: Data curation, Methodology, Software, Writing – original draft, Writing – review & editing. GZ: Data curation, Methodology, Software, Writing – original draft, Writing – review & editing. NS: Formal Analysis, Methodology, Resources, Writing – original draft, Writing – review & editing. LH: Data curation, Formal Analysis, Methodology, Software, Writing – original draft, Writing – review & editing. ZM: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. ML: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1551892/full#supplementary-material

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