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The gut-immune axis: A complex training ground impacting inflammatory pathologies

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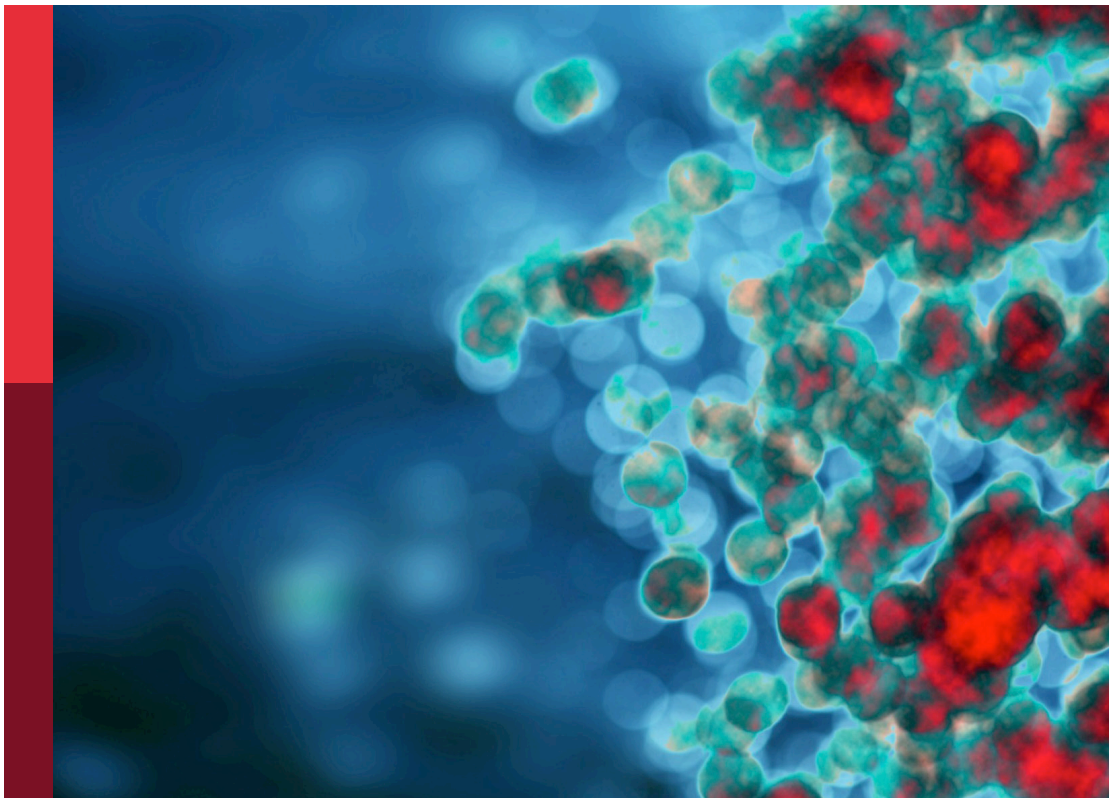

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The gut-immune axis: A complex training ground impacting inflammatory pathologies

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Table of contents

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Editorial: The gut-immune axis: a complex training ground impacting inflammatory pathologies

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gut, inflammation, genetics, immune repertoire, microbiome

Editorial on the Research Topic

The gut-immune axis: a complex training ground impacting inflammatory pathologies

Recently, the role of the gut in the development and training of the immune system has been increasingly recognised. The gut-immune axis is key in the pathogenesis of immune mediated inflammatory diseases (IMIDs), bridging genetic susceptibility and environmental factors. However, the specific mechanisms that connect changes in the gut and its microbiome with their impact at distant locations is far from being understood. Therefore, the identification of the genetic and environmental factors that operate through this axis and the mechanisms that predispose or protect from disease is an active area of research, addressed by several articles in this Research Topic. Elucidating these pathways and the alterations that lead to complex immune pathologies is essential to define their aetiology, progression and avenues for successful therapeutic intervention.

In this topic, research by Ma et al. shows that autoimmunity can predispose to leaky gut, as treatment with a TLR7/8 agonist led to decreased gut barrier integrity in lupus prone mice, but not in congenic healthy controls. This was linked to a reduced frequency in NKp46^{+} cells, critical for gut barrier integrity maintenance. Reduced intestinal permeability may secondarily lead to exacerbation of autoimmunity, by enhancing immune reactivity or inducing changes in the microbiome (dysbiosis).

The potential of studying the microbiota composition as diagnostic tool for altered gut-immune axis related pathologies is supported by Zhou et al. in the context of primary biliary cholangitis (PBC). Their study identifies six amplicon sequences as optimal biomarkers of PBC (Serratia, Oscillospirales, Ruminococcaceae, Faecalibacterium, Sutterellaceae, and Coprococcus), and functionally associates dysbiotic changes with altered lipid metabolism, highlighting the implications of this mechanism in PBC pathology.

Dysbiosis underlies the pathogenesis of multiple conditions, which can be explained by the microbiome as operator of genetic susceptibility. Cross-study analysis using bioinformatic tools, such as mendelian randomization (MR), and public datasets pairing GWAS and microbiome profiling allow to identify causal relations: i.e. genetic loci influencing a differential bacterial abundance that confers risk to a particular disease, whilst excluding cofounding variables with pleiotropic effects. Here, Cao et al. used MR to highlight the causal role of microbiome changes as operators of Sjogren's Syndrome susceptibility loci. Both positive and negatively correlated bacterial taxa were identified, as well as the microbiome-related genes ARAP3, NMUR1, TEC and SIRPD, thus highlighting targets for genetically triggered microbiome alterations.

Environmental factors, such as diet, can underlie dysbiosis and its disease association. Zhao et al. have evidenced this in a novel model of diet-induced gut inflammation through arecoline supplementation. In this model, increased susceptibility to intestinal permeability and inflammation is ascribed to arecoline influencing the host metabolism through modulation of the microbiota.

Likewise, immune modulation can also be achieved through dietary interventions, as shown by Lo Conte et al. in type-1 diabetes NOD mice, where an anti-inflammatory diet, enriched in inulin and omega 3-PUFA, protected from autoimmunity and improved the metabolic profile, leading to gut barrier integrity restoration and changes in the microbiome (namely, an increase of mucus-degrading bacteria such as Akkermansia muciniphila and Akkermansia glycaniphila). In this model, characterised by the generation of islet-reactive T cells, the anti-inflammatory diet induced an expansion of $FoxP3^+$ regulatory T cells and IL- 10^+ Tr1 cells at the expenses of effector Th1/Th17 cells in the intestine, pancreatic lymph nodes and intra-islet lymphocytes. This study also provides proof of concept of gut-driven extra-intestinal pathology.

Importantly, dietary-induced immunomodulation is also possible without involving the microbiome. The gut immune system and microbiome composition varies along its longitudinal axis, with the colon being the primary site of bacterial colonization and the small intestine (SI) standing out for its nutritional role. However, the SI potential to modulate the immune system is not withheld. This anatomical location is the focus of the review by Bodmer et al. The authors describe its tolerogenic role sensing luminal contents and highlight the potential of SI targeted therapies, which induce atypical Tregs generation without altering the microbiome and/or compromising immunity to pathogens, an approach that could be exploited therapeutically to resolve auto-inflammatory pathologies.

Evidence of the gut-immune axis driving extra-intestinal pathology is also provided by He et al., who explain the impact of intestinal inflammation in neurological function through genetically-determined alteration of this axis. By MR analysis, they investigated inflammatory bowel disease genetic signature leading to morphological changes in the cerebral cortex, which may trigger neuropsychiatric disorders. They identified an interconnected network highlighting the top 10 variant-matched genes (STAT3, FOS, NFKB1, JAK2, STAT4, TYK2, SMAD3, IL12B, MYC, and CCL2) involved in neuroinflammation-induced damage, impaired neurological function, and persistent nociceptive input, ultimately leading to cortical reshaping.

Considering the breath of connections of the gut-immune axis, addressing the specificity of regulatory pathways is crucial to prevent unwanted off-site effects. In this sense, Peng et al. have performed an up-to-date revision of the role of the nuclear factor erythroid 2-related factor 2 (Nrf2) in ulcerative colitis (UC). The Nrf2 pathway regulates the intestine's development and function and influences oxidant stress and inflammatory responses, being involved in the development of UC and UC-related intestinal fibrosis and carcinogenesis. The relevance of specific signalling pathways is also highlighted in Sun et al.'s revision in the context of knee osteoarthritis (OA), also influenced by disturbances in the host-microbiome equilibrium. Here, they advocate for the identification of the signalling pathways activated by defined pathogens or specific microbiome changes as priorities for future research.

Targeted approaches like these, beyond the detection of intestinal permeability and dysbiosis underlaying multiple conditions, would help identify tissue/disease-specific alterations and their mechanisms in connection with the gut-immune axis, which will ultimately inform targeted therapeutic interventions. As these progress, future studies may be able to connect disease-associated changes in specific bacterial species with the immune repertoire, identifying species carrying bacterial/human cross-reactive epitopes as well as clonal populations associated with autoimmunity. Thus, whilst brief, this series of articles show the potential of interdisciplinary research to advance our understanding of the gut-immune axis, which warrants exciting discoveries ahead.

Author contributions

JG-M: Conceptualization, Writing – original draft, Writing – review & editing. NG-M: Writing – review & editing. JG: Writing – review & editing.

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Harnessing the small intestinal axis to resolve systemic inflammation

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This Perspective presents the potential of the Small Intestinal Axis, a subdivision of the Gut-immune Axis, to modulate systemic inflammation based on sensing contents of the gut lumen. Gut mucosal immunity regulates tolerance to food and gut contents and is a significant factor in maintaining systemic homeostasis without compromising immunity to pathogens. This is achieved through anatomical structures and signaling pathways that link the tolerogenic potential of the proximal small intestine to systemic immunity. Non-live preparations of microbes isolated from human small intestinal mucosa, and the extracellular vesicles (EVs) which they shed, can resolve systemic inflammation without systemic exposure after oral delivery. The mechanism involves primary interactions with pattern recognition receptors followed by trafficking of immune cells through mesenteric lymph nodes. This generates in the periphery a population of circulating $CD4^+$ T cells which have regulatory function but an atypical $FoxP3^-$ phenotype. There is no modification of the resident gut microbiome. Discoveries using this novel approach of targeting mucosal microbial elements to the tolerogenic proximal regions of the small intestine are revealing some of the mysteries of the relationship between the gut and immune system.

KEYWORDS

immunity, small intestine, mucosa, oral tolerance, T-cell, medicines

Introduction

Since ancient times the intestines have been considered integral to human well-being. The ancient Egyptians removed intestines before mummification and placed them in canopic jars protected by the falcon-headed God, Qebehsenuef, one of the four sons of Horus (1, 2). In modern times, we possess a growing scientific understanding of a profound influence of the gut on the function of the immune system. The large mucosal surface of the small intestine, in particular, functions as an immune sensory organ which relays information and instructions throughout the body based on molecular sensing of luminal content, including food and microbes (3).

Inferences of the gut's influence on immune homeostasis and pathology have focused on the association of changes in composition of gut microbiota measured in stool. This represents the microbial content of the colon and is distinct from that of the small intestine. The colonic microbiota are widely considered as a vital organ for health, despite the observation that individuals who have a total colectomy are generally healthy. This Perspective presents an alternative view centered on a sub-division of the gut-immune axis, the small intestinal axis ("Sintax"), that links small intestinal mucosal immunity to systemic immunity. We describe the therapeutic potential of the small intestinal axis based on an understanding of gut mucosal immunity and of harnessing its links to the periphery.

Recent studies exploring oral agents that modulate peripheral inflammation *via* the small intestinal axis (4, 5) have provided unexpected insights that bridge the fields of oral tolerance, mucosal immunity, and the gut microbiome. This raises the possibility of medicines which induce resolution of systemic inflammation using mechanisms similar to those essential to prevent inflammatory responses to the high burden of daily exposure to food antigens in the proximal small intestine (6).

Pabst and Mowat (7) noted that, "There is an important difference between tolerance to gut bacteria and tolerance to food proteins: whereas tolerance to food protein induced *via* the small intestine affects local and systemic immune responses, tolerance to gut bacteria in the colon does not attenuate systemic responses." We are seeking to bridge this gap with the systemic effects of mucosa-resident organisms on mechanisms of oral tolerance in the proximal small intestine.

On the evolutionary origins of the gut-immune axis

Theodosius Dobzhansky stated that, "Nothing in biology makes sense except in the light of evolution" (8).

The last common ancestor (LCA) of all animals is thought to have been a worm-like creature, possibly *Ikaria wariootia*, found in the Ediacaran of South Australia and estimated to be ~550 million years old (9). The possibility that a worm is the LCA is consistent with predictions based on phylogenetic analysis of modern animals such as *Platynereis dumerilii* (10).

The significance for the gut-immune axis arises from the striking anatomical similarity between worm and small intestine, which is an evolutionary descendent of the LCA. During embryogenesis the proto-gut forms in the first few cell divisions and all other tissues of the body arise from gut epithelial cells. The worm/gut can be regarded as the core ancestral element of the organism, deriving the nutrients from the environment required to drive the energetics of life (11). This evolutionary core must control the myriad tissues and organs that have evolved to enable diverse animals to adapt to widely varied habitats. The control systems are the anatomical immune, neural and metabolic networks which radiate from the gut throughout the body.

With this evolutionary perspective it becomes intuitive to understand that the small intestine can exert physiological control of bodily function. Indeed, it must, as it is a central force of life within all animals. This insight informs our understanding of the small intestinal axis and the creation of new types of medicines.

The conundrum of the gut microbiome

The gut microbiome has been characterized as an essential organ for good health (12). Enormous amounts of data have been generated on the content and diversity of the gut microbiota (13, 14). Associations of gut microbial variation with disease have been reported across most areas of medicine (15, 16). Given ease of access, the majority of data on gut microbiome composition is from stool samples. The colon has a high microbial abundance, several orders of magnitude greater than the upper parts of the intestine (17, 18).

There is, however, compelling evidence that the colonic microbiome may not be generally essential to health. This is most evident in adult patients who have had a total colectomy. Case-control longitudinal follow-up studies after surgery show no adverse effects on health related to the loss of the colon and its microbiota (19). Two large cohort studies may suggest even a reduced risk of cardiovascular disease (20) and type 2 diabetes (21) after colectomy. This is inconsistent with the assumption that a colonic microbiome is essential to good health but remains compatible with the notion that dysbiosis can lead to adverse effects on health reflecting the overgrowth of organisms with which the host has not evolved to co-exist.

Small intestine resection is less commonly performed. However, observations of patients with concomitant inflammatory diseases following Roux-en-Y surgery for obesity suggest that alterations in function of the small intestine can result in inflammation resolution in peripheral tissues (22). Although the mechanism of this effect is unknown, it suggests that the proximal small intestine can influence systemic inflammation.

Evidence that modulating gut mucosal immunity can lead to tolerance and reduced systemic inflammation is long-standing (23). Two related ideas prompted us to think in a different way about the gut-immune axis and how to modulate it therapeutically:

Firstly, there are multiple microbiomes within the GI tract. The colonic and small intestinal mucosal microbiome are distinct, with a density about six orders of magnitude lower in

the duodenum and three orders lower in the distal ileum compared to the colon (24). Microbes resident in the small intestine mucosa are more likely to influence the small intestinal axis than those in stool (25) due to proximity to host cells in the gut epithelium and their evolutionary requirement to adapt to survive in the host environment.

Secondly, it may be possible to select individual microbes or microbial elements which trigger tolerogenic sensory systems in the proximal small intestine for control of systemic inflammation using similar mechanisms to those of food tolerance (6, 26).

We propose a new avenue for the discovery of orally delivered medicines which explores the intersection between mechanisms of oral tolerance and small intestinal mucosal microbiota. This hypothesis led to identification of agents which harness tolerogenic gut-immune networks resulting in broad spectrum systemic inflammation resolution arising from direct interaction with host cells in the small intestine, unrelated to content or change in the microbiota.

The role of small intestinal mucosal immunity

The intestinal mucosal immune system must permit safe nutrient absorption and offer protection from pathogens. Much of the protective function arises from physical, chemical and biochemical barriers in the GI tract (27), ensuring that pathogens never enter the body. A primary requirement of gut mucosal immunity is prevention of adverse inflammatory effects of food consumption (7) and exposure to innocuous microbes whilst still allowing immunity to pathogens.

We have probed the systemic impact of the small intestinal axis using EDP1867 and EDP1815, two preparations of single strains of small intestine mucosa-derived bacteria and the extracellular vesicles which they naturally shed. These can be orally delivered with inflammation resolving effects which match the efficacy of a wide range of comparator anti-inflammatory drugs in animal models.

Ramani et al. in 2022 (4) published the first report of the pharmacology and mechanisms of EDP1867 on the small intestinal axis. EDP1867 is a preparation of a monoclonal strain of the bacterium, *Veillonella parvula*, isolated from the ileum of a healthy human donor who had a total colectomy 25 years earlier. The preparation comprised a mixture of bacterial cells and their extracellular vesicles (EVs). It was γ-irradiated to ensure that the mechanism of action was due to direct action on host cells in the gut rather than GI colonization or modification of the resident microbiota. EDP1867 was therapeutically effective after oral administration in a range of animal models covering Th1, Th2 and Th17 inflammation.

This systemic effect of oral tolerance with a small intestine mucosa-derived microbial preparation has also been shown with EDP1815, prepared from a strain of *Prevotella histicola* isolated from a duodenal biopsy of a human donor (28). EDP1815 is an obligate anaerobe which is killed by exposure to oxygen following anaerobic fermentation. It has shown efficacy in a variety of inflammatory models affecting a range of organs and tissues (29–31). Both EDP1867 and EDP1815 were shown to be gut-restricted after oral administration, demonstrating that the effects in the periphery were due to the transmission of an immunological signal that originated in the gut rather than to systemic absorption of the drug.

Immunogeography of the intestines

The distribution of immune cells, epithelial cells and gene expression throughout the gut is highly non-uniform (32, 33) suggesting differential functions along its length. There is anatomical segregation of the types of dendritic cells (DCs) that migrate to mesenteric lymph nodes draining the small intestine and the colon (34). The mechanisms that mediate oral tolerance, linking the gut to control of systemic inflammation, appear to be prevalent in the proximal small intestine where food is first encountered in the intestines.

Prevention of inflammatory responses to this xenobiotic load requires that initial interactions are regulatory, consistent with the distribution of immune cells along the intestine. The duodenum and upper parts of the jejunum contain substantial numbers of $CD103^{+}CD11b^{+}$ DCs (32), a class of DC unique to the intestine with a role in the generation of regulatory T cells (35).

Esterhazy et al. (36) used direct injection of antigen into proximal and distal regions of the gut to determine the responses generated in the associated draining lymph nodes (LNs). They reported proximal small intestine-draining LNs preferentially giving rise to tolerogenic responses and the distal LNs to pro-inflammatory T cell responses.

We have unpublished results showing a related phenomenon with EDP1815. In animal experiments on EDP1815 formulation, 1mm tablets small enough to be dosed orally to mice were given polymer coatings that released the contents either in the proximal small intestine or the distal ileum/colon. The results complemented those of Esterhazy et al. (36). Only the proximal release formulation of EDP1815 resulted in a significant systemic anti-inflammatory effect in a T cell driven delayed-type hypersensitivity (DTH) model of inflammation. In contrast to Esterhazy et al. there was no pro-inflammatory effect of distal release.

Orally delivered microbial preparations showed similar efficacy to parenterally administered dexamethasone, suggesting the significant control that the proximal small intestinal can exert on systemic inflammation.

Mechanisms of inflammation resolution *via* the small intestinal axis

Descriptions of the phenomenon of systemic tolerance after oral administration of antigens date to the early 1900s. In 1911 Wells and Osborne investigated biological reactions to vegetable proteins (23). Whilst their primary interest was in anaphylactic reactions to intraperitoneally administered proteins, they commented that, "Experiments showed that continuous feeding with a vegetable protein rendered guinea-pigs immune to this protein, so that they could not be sensitized to it." Thus, over 110 years ago it was known that peripheral and gut mucosal immune responses to the same antigen were quite different. Not only did exposure in the gut by feeding not induce anaphylaxis, but it actually prevented sensitization, similar to an observation about LPS noted below (37).

Nearly 100 years later Parameswaran et al. (38) reported that oral tolerance to OVA also elicited a protective effector response to a recombinant OVA-expressing strain of *Salmonella enterica*. The mechanism is not understood but suggests that there are intestinal mucosa immune mechanisms which can simultaneously elicit systemic tolerance and effector responses depending on the context in which the antigen is seen by the host.

The host recognition contacts with foreign matter are pattern recognition receptors (PRRs), first postulated by Janeway in 1989 (39). We now know that there are dozens of PRRs which integrate signals from a huge array of ligands to modulate innate immunity. These ligand-receptor interactions are conserved across domains of life (40). In many parts of the body they function as "danger" signals alerting the immune system to threat (41). However, gut mucosal immune wiring is not the same as in the periphery. Receptors which generate proinflammatory responses in the periphery can have opposite anti-inflammatory or regulatory effects when activated in the gut. Examples are the actions of Toll-like receptors TLR4 and TLR5 (42, 43). Agents such as lipopolysaccharide (LPS) that are inflammatory when given intravenously can be anti-inflammatory after oral administration (44). Indeed, repeated oral administration of LPS can be protective against subsequent intravenous challenge (37), reminiscent of original observations of Wells and Osborne in 1911 (23).

The action of EDP1867 and EDP1815 in the small intestinal axis is dependent on transfer of immune signals from the intestine to the periphery. The mechanism of this effect has been traced through three steps (Figure 1): first, primary interactions with pattern recognition receptors; second, trafficking through mesenteric lymph nodes, resulting in; third, the generation in the periphery of circulating $CD4^+$ T-cells whose ability to regulate inflammation can been shown by adoptive cell transfer into untreated animals. The study of EDP1867 mentioned above (4), experimentally elucidated this mechanistic chain of events.

TLR2 engagement in the gut was required to initiate a cascade of events leading to the generation of a population of $CD4^+$ cells which have inflammation resolving effects in the periphery. TLR2 is expressed on many immune cell types and has a promiscuous range of ligands and functions (45). As well as stimulating inflammation it has been reported to induce dendritic cell mediated tolerance under some conditions (46) and even to maintain gut barrier function enhancing immune homeostasis (47). In our studies with orally-delivered EDP1867, antibody-mediated blockade of TLR2 prevented the *in vivo* efficacy in a mouse model of inflammation. Although we have not yet characterized the primary target cell in the small intestine, this demonstrates that TLR2 is at least one of the molecular targets required to mediate systemic effects, and that the relevant cells bearing TLR2 are likely to be more abundant in the proximal intestine. Subsequent unpublished studies

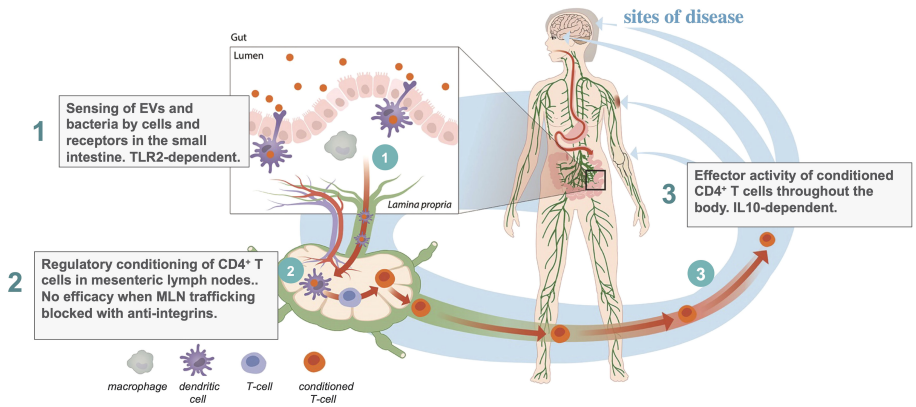

FIGURE 1
Three step model of inflammation resolution in the small intestinal axis.

comparing the pharmacology of a range of microbial strains, extracellular vesicles (EVs), and an orally delivered TLR2 agonist have revealed that TLR2 engagement is necessary but not sufficient for the systemic anti-inflammatory effect.

Mesenteric lymph nodes (MLN) are the critical interface between gut mucosal and peripheral immunity. If lymphocyte trafficking through MLNs is blocked using a cocktail of anti-integrin antibodies against α4β7 (LPAM–1) and CD62L, then there is no signal transfer from the gut to the periphery, and no efficacy of EDP1867. The anti-integrins do not, themselves, block the inflammatory response. This provides key evidence that immunological signals in the gut have a route to the periphery. This requirement for MLN trafficking to regulate peripheral inflammation by signals in the gut is an interesting inverse to the efficacy of vedolizumab in the treatment of inflammatory bowel diseases (48, 49). Vedolizumab is an antibody that prevents lymphocyte trafficking to the gut by blocking human α4β7, leading to a reduction in bowel inflammation, the inverse effect to our observations of signalling from the gut to the periphery.

We next determined the peripheral cellular effect of the primary events in the gut and MLNs. Consistent with earlier reports on tolerance induced by gliadin and PSA (26, 50), treatment with EDP1867 led to the generation of a population of $CD4^+$ T cells that could adoptively transfer the inflammation resolving effect from donor animals treated with EDP1867 to naïve recipient animals which had a subsequent challenge. This is also the case for animals treated with EDP1815 (manuscript in preparation). Whilst the generation of therapeutically effective T cells in donors was TLR2-dependent, their anti-inflammatory effector function in recipients was not. The converse was the case for IL-10, which was not required in the donors but was for effector function in recipients. This T cell dependent mechanism results in long-lasting effects weeks after the cessation of treatment. This was a breakthrough finding, both in understanding the immune mechanism resulting in the peripheral anti-inflammatory effects, and for the therapeutic potential of this approach. By engaging oral tolerance mechanisms, the small intestinal axis can generate circulating T cells with regulatory function which induce generalized inflammation resolution without any apparent adverse effects in either preclinical or clinical studies to date.

Since the publication on EDP1867 we have observed that the anti-inflammatory T cells induced *via* the small intestinal axis are noncanonical $FoxP3^-$ CD4 cells. Although differing from most reports of Tregs, this fits with a growing literature on FoxP3-independent T cells that nevertheless have regulatory functions. Van der Veeken et al. (5) reported a peripheral Treg transcriptional program which is induced by events in the intestine and is independent of FoxP3. Hong et al. (26) described populations of noncanonical helper T cells which appear to mediate non-responsiveness to oral gliadin. Sefik et al. (51) observed a distinct population of $ROR\gamma^+$ T-cells induced by intestinal symbionts. Johnson et al. (50, 52) reported that capsular polysaccharide A from *B. fragilis* induced T cells capable of regulating airway hyperreactivity that are not canonical $FoxP3^+$ Tregs. This latter report is particularly interesting because it showed that a non antigen-specific systemic anti-inflammatory agent acting in the gut could regulate the OVA antigen specificity used to generate airway inflammation.

What mediates microbial function within the small intestinal axis

Microbial and eukaryotic cells shed lipid nanoparticles which comprise part of the cell membrane and cytoplasmic molecular contents, variously known as extracellular vesicles, outer membrane particles, or exosomes. These may be the major mediators of inter-cellular communication (53–55). Unlike the cells themselves, they are small enough for Brownian motion (56) which enables them to diffuse across the local microenvironment to signal to neighboring cells and tissues.

Most preparations of microbes contain EVs. Being sub-microscopic they are not generally tracked. The earliest descriptions of bacterial EVs by electron microscopy were in the 1960s (57, 58). It is now emerging that the EVs may mediate biological effects which have been ascribed to microbial cells. Thus oral delivery of EVs of *Akkermansia muciniphila* was protective in a mouse model of DSS colitis (59) and also able to improve gut permeability with possible benefits in a range of metabolic diseases (60), activities that have been attributed previously to the microbe itself.

EDP1867 contains EVs. When the EVs were separated from the microbial cells and independently tested in an *in vivo* model of DTH, the purified EVs exhibited the full anti-inflammatory effects that were observed in the microbial preparation containing both cells and EVs. All of our mechanistic observations on the signaling path from gut to periphery are common to EV and microbial preparations. Although roughly a thousand-fold smaller in volume than the parent microbial cells, we have been unable to detect them outside of the gut in our studies after oral administration suggesting that their primary site of action is the intestinal mucosa. In a direct comparison, EVs resolved systemic inflammation after oral but not intravenous or intraperitoneal administration.

Taken together, we have observed systemic anti-inflammatory effects that depend on primary interactions in the small intestine with both non-viable microbial cells and EV preparations that cannot colonize the gut microbiome. Since EVs are not living entities, they provide the clearest evidence for a drug-type rather than a microbiological-type mechanism, likely using the potent mechanisms required for normal food

tolerance. The pharmacology cannot be attributed to a probiotic type of action.

A point to note is that the functional activities of bacteria and EVs in host interactions are often attached to taxonomic names, from species up to phylum level. There is not a sufficiently meaningful definition of a bacterial species (61) to allow function to be clearly associated with taxonomy. The variability within what is classed as a species is sufficient for the biology of interactions with the immune system to differ even between strains of the same species. Bacterial function in the gut-immune axis must be defined at the level of the individual clonal strain as much as by taxonomy.

Clinical potential of targeting the small intestinal axis

The broad-spectrum and potent inflammation resolving activity described above in animal models, supported by the CD4 T cellular mechanism of action, has the potential to translate to humans to address the unmet clinical needs of people with immune-mediated inflammatory diseases (IMID). Despite significant recent therapeutic advances, immune homeostasis is not restored in a large majority of IMID patients nor is remission achieved sufficiently often.

Our observations, interpreted in light of the many contributions cited in this article, suggest a path to solving Pabst and Mowat's dilemma of the differential immunological effects of the upper and lower gut by delivering components of microbes to the proximal tolerogenic regions at pharmacological doses. This is supported by a weight of preclinical data, and now clinical trial results which are in preparation for publication. EDP1815 has to date been administered to over 700 people, including patients with psoriasis, atopic dermatitis or COVID-19. Encouraging efficacy has shown that this principle of addressing gut tolerance mechanisms with a mucosal microbial preparation can have effects in humans. A phase 2 study produced clinically meaningful outcomes amongst 250 patients with mild and moderate psoriasis (62). Another phase 2 study in 400 patients with atopic dermatitis is under way. There have been no safety or tolerability issues in studies to date that differ from placebo controls. These agents can be manufactured at large scale and reasonable cost, which establishes the potential of a new class of effective, safe and affordable oral anti-inflammatory medicines.

Concluding remarks

We have described effector functions of killed mucosal microbes and their EV constituents acting on the small intestinal axis to resolve systemic inflammation without systemic exposure. Gut mucosal immunity not only regulates tolerance to food and gut contents, but it is also a significant factor in maintaining systemic non-inflamed homeostasis without compromising parenteral immunity to pathogens. As well as contributing to the understanding of the gut-immune axis, our observations suggest the possibility of a major advance in the treatment of inflammatory diseases.

Data availability statement

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

Author contributions

The first draft of the manuscript was written by MB and revised by AI and IM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MB and AI are employees of Evelo Biosciences Inc. IM is a non-executive member of the Board of Directors of the company.

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A diet enriched in omega-3 PUFA and inulin prevents type 1 diabetes by restoring gut barrier integrity and immune homeostasis in NOD mice

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Introduction: The integrity of the gut barrier (GB) is fundamental to regulate the crosstalk between the microbiota and the immune system and to prevent inflammation and autoimmunity at the intestinal level but also in organs distal from the gut such as the pancreatic islets. In support to this idea, we recently demonstrated that breakage of GB integrity leads to activation of islet-reactive T cells and triggers autoimmune Type 1 Diabetes (T1D). In T1D patients as in the NOD mice, the spontaneous model of autoimmune diabetes, there are alterations of the GB that specifically affect structure and composition of the mucus layer; however, it is yet to be determined whether a causal link between breakage of the GB integrity and occurrence of autoimmune T1D exists.

Methods: Here we restored GB integrity in the NOD mice through administration of an anti-inflammatory diet (AID- enriched in soluble fiber inulin and omega 3-PUFA) and tested the effect on T1D pathogenesis.

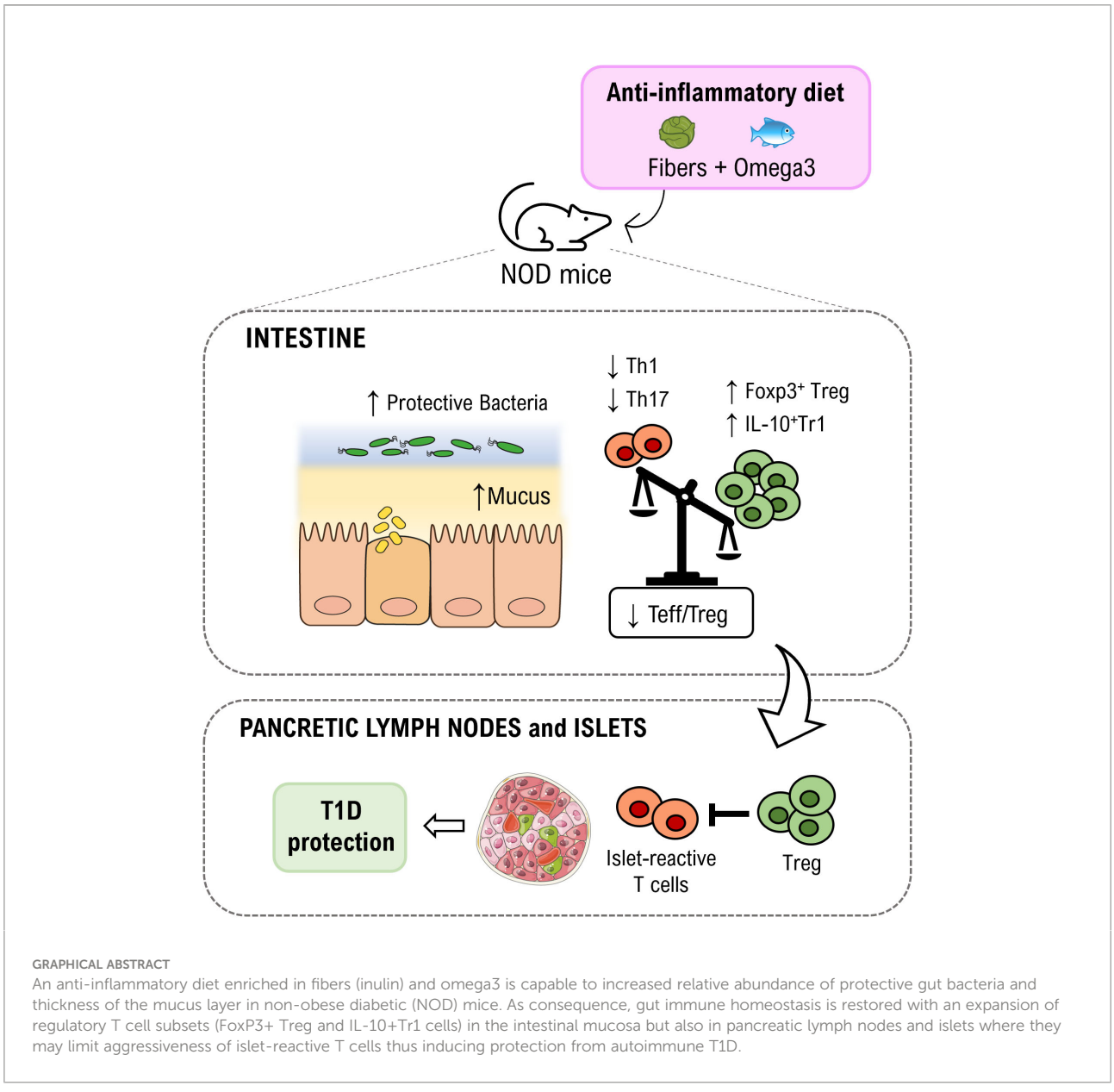
Results: We found that the AID prevented T1D in NOD mice by restoring GB integrity with increased mucus layer thickness and higher mRNA transcripts of structural (Muc2) and immunoregulatory mucins (Muc1 and Muc3) as well as of tight junction proteins (claudin1). Restoration of GB integrity was linked to reduction of intestinal inflammation (i.e., reduced expression of IL-1β, IL-23 and IL-17 transcripts) and expansion of regulatory T cells ($FoxP3^+$ Treg cells and $IL\text{-}10^+$ Tr1 cells) at the expenses of effector Th1/Th17 cells in the intestine, pancreatic lymph nodes (PLN) and intra-islet lymphocytes (IIL) of AID-fed NOD mice. Importantly, the restoration of GB integrity and immune homeostasis were associated with enhanced concentrations of anti-

inflammatory metabolites of the ω3/ω6 polyunsaturated fatty acids (PUFA) and arachidonic pathways and modifications of the microbiome profile with increased relative abundance of mucus-modulating bacterial species such as *Akkermansia muciniphila* and *Akkermansia glycaniphila*.

Discussion: Our data provide evidence that the restoration of GB integrity and intestinal immune homeostasis through administration of a tolerogenic AID that changed the gut microbial and metabolic profiles prevents autoimmune T1D in preclinical models.

KEYWORDS

anti-inflammatory diet (AID), Type 1 diabetes, gut barrier integrity, mucus layer, gut microbiota, regulatory T cells



GRAPHICAL ABSTRACT
An anti-inflammatory diet enriched in fibers (inulin) and omega3 is capable to increased relative abundance of protective gut bacteria and thickness of the mucus layer in non-obese diabetic (NOD) mice. As consequence, gut immune homeostasis is restored with an expansion of regulatory T cell subsets (FoxP3+ Treg and IL-10+Tr1 cells) in the intestinal mucosa but also in pancreatic lymph nodes and islets where they may limit aggressiveness of islet-reactive T cells thus inducing protection from autoimmune T1D.

Introduction

T1D is a multifactorial autoimmune disease characterized by autoimmune destruction of insulin-producing beta cells of pancreatic islets of Langerhans (1). The numerous genetic and environmental factors that regulate T1D pathogenesis are still largely unknown. Recent evidence indicates that the gut environment is fundamental to modulate autoimmunity at sites distal from the intestine including the pancreatic islets in T1D (2). The microbiota composition, intestinal inflammation and breakage of GB integrity are all important players in modulating the effector phenotype and aggressiveness of self (islet)-reactive T cells and for triggering the early events of autoimmune T1D (3–5). In particular, the integrity of the GB composed of the epithelial barrier and the mucus layer is crucial to prevent beta cell autoimmunity and T1D. In support to this view, alterations of the GB integrity and of the mucus layer are found in humans and preclinical models of T1D (e.g., the NOD mice) and precede the clinical signs of disease (5–10). Furthermore, we recently found that low-grade intestinal inflammation and GB damage in TCR transgenic *BDC2.5NOD* mice carrying a large islet-reactive T cell repertoire triggers activation of the diabetogenic T cells and T1D (10). A causal link between GB alterations and T1D pathogenesis and the mechanisms through which GB damage leads to activation of beta cell autoimmunity are yet to be determined. The GB is an important physical barrier that avoids uncontrolled passage of microbial components into the gut mucosa and systemic circulation (11). Changes to the protective mucus layer, the body's first line of defense at the mucosal epithelia, can alter exposure of the underlying epithelium to foreign antigens and favor activation of self(islet)-reactive T cells (10, 12). Moreover, the GB and, specifically, the mucus layer contain important immune regulatory factors such as mucins and anti-microbial peptides (AMP) that are essential to maintain immune homeostasis and induce Treg cell differentiation at the intestinal and systemic level thus suppressing inflammatory/effector T cells (13, 14), possibly including self(islet)-reactive T cells.

The diet composition is fundamental to maintain gut immune homeostasis and GB integrity and to prevent inflammation (15). Dietary factors provoke or protect from intestinal inflammation and breakage of GB integrity both directly and through microbiota modulation (16–19). Some dietary substances such as saturated fatty acids compromise the GB and allow passage of luminal contents (food antigens, microbiota components) into the mucosal and submucosal layers in proximity to immune cells (20). This results in activation of immune cells, inflammation and further damage of GB integrity. Other dietary components such as soluble fibers and PUFA play a beneficial role on GB integrity. For example, dietary administration of omega-3 PUFA maintains immune tolerance in the gut by directly suppressing inflammation and promoting a beneficial anti-inflammatory microbiota profile (21, 22). The direct anti-inflammatory effect of omega-3 PUFA is related to block of release of proinflammatory eicosanoids and modulation of the functional phenotype of macrophages (23) with release of tolerogenic cytokines such as IL-10 and increased differentiation of regulatory T cells at the expenses of effector Th17 cells (24). The omega-3 PUFA effect on the microbiota profile includes a decrease of pro-inflammatory bacteria such as the Enterobacteriales with concomitant expansion of beneficial bifidobacteria and lactobacilli (25).

Nonfermentable fibers are another dietary factor that is fundamental to maintain gut immune tolerance and prevent inflammation. Dietary fibers are neither digested nor absorbed in the intestine but they critically modulate gut microbiota composition by favoring growth of bifidobacteria and lactobacilli that degrade fibers into SCFAs (butyrate, propionate and acetate), tolerogenic metabolites promoting FoxP3 Treg cell differentiation and preventing intestinal inflammation (26), and/or directly secrete anti-inflammatory metabolites such as the lactic acid (27). Importantly, dietary fibers also have direct microbiota-independent beneficial effects. For example, they trigger release of tolerogenic cytokine IL-10 by dendritic cells (28) and directly promote GB integrity acting *via* modulation of epithelial tight junction proteins (29), goblet cell function (30), and glycocalyx maturation (31). Fibers such as inulin-type fructans induce changes of the intestinal mucosa characterized by higher villi, deeper crypts, increased number of Goblet cells (GC), and increased production of mucins resulting in a thicker mucus layer on the colonic epithelium (32–34). Moreover, inulin promotes formation of the epithelial glycocalyx that is crucial to regulate bacterial-epithelial crosstalk and to support GB function (31).

In order to demonstrate that the GB alterations found in the spontaneous preclinical model of T1D, the NOD mice, are mechanistically linked to T1D pathogenesis we exploited a dietary approach, an anti-inflammatory diet (AID) enriched with omega-3 PUFA and inulin, to restore GB integrity and immune homeostasis in NOD mice and test the effect on T1D pathogenesis.

Materials and methods

Mice

Female NOD and BALB/c mice were purchased from Charles River Laboratories. All mice were maintained under specific pathogen-free conditions in the animal facility at San Raffaele Scientific Institute and all experiments were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) according with the rules of the Italian Ministry of Health.

Dietary regimens

Immediately after weaning, 4-weeks old NOD mice were separated in two groups, housed in different cages and fed with either an anti-inflammatory diet (AID) or standard (STD) diet. The AID was designed with the supervision of a nutritionist and purchased from Rieper S.p.A. (Altromin C1000). The AID diet was enriched with fish oil (3,6%), linseed oil (2%) and corn oil (7,7%), for a total of 13,3% of fats (1:3 PUFA ω3:ω6 ratio with specifically 20g/kg ω3 and 60g/kg ω6). The STD diet contained only 5% of fats (100% Corn oil) with 1:190 PUFA ω3:ω6 ratio (0,15g/kg ω3 and 28,5g/kg ω6). AID diet contained 8% of fibers (100% Inulin) while the STD diet with only 5% of fibers (100% cellulose). For more information about composition of the AID see Supplementary Table S1.

Diabetes incidence

Diabetes was monitored by weekly measurements of blood glucose levels with a GB35 Ascensia Breeze 2 glucometer (Bayer). The animals were considered diabetic after two consecutive blood glucose measurements > 250mg/dl according to standard method (35).

In vivo gut permeability assay

Intestinal permeability was determined by FITC-dextran assay as previously described (36). Briefly, 20 mL/kg of body weight of PBS containing 25 mg/mL FITC-conjugated dextran (FITC-dextran; molecular mass, 4.4 kDa; FD4, Sigma-Aldrich) was administered to each mouse by oral gavage. After 4 h, blood was collected and the concentration of fluorescein was determined by spectrophotofluorometry (Wallac Victor; Perkin-Elmer Life Sciences) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm using serially diluted samples of the FITC-dextran marker as standard.

Cell isolation

Intestinal mononuclear cells were isolated from the small and large intestinal lamina propria as previously described (Lefrancois and Lycke, 2001; Weigmann et al., 2007). Briefly, after the removal of Peyer's patches, small and large intestines were flushed with PBS, opened longitudinally, and incubated twice with 5 mM EDTA and 1 mM DTT for 20 min at 37°C to remove epithelial cells and adipose tissue. Then, the intestines were cut into small pieces and digested in HBSS containing 0.5 mg/ml Collagenase D (Roche Diagnostics), 1 mg/ml Dispase II (Roche Diagnostics) and 5 U/ml DNase I (Sigma-Aldrich) for 20 min at 37°C in a shaking incubator. The digested tissues were washed, resuspended in 5 ml of 40% Percoll (Sigma-Aldrich) and overlaid on 2.5 ml of 80% Percoll in a 15-ml Falcon tube. Percoll gradient separation was performed by centrifugation at 1000 g for 20 min at 20°C. The interface cells were collected and used as intestinal lymphocytes for FACS analysis. Splenocytes and pancreatic lymph node (PLN) cells were isolated by meshing of the tissues. For isolation of intra-islet lymphocytes (IIL), freshly collected pancreata were cut into small pieces with a scissor and digested 3 times for 15min at 37°C with agitation in 3 ml/pancreas of Hank's Balanced Salt Solution (HBSS) 1X with $Ca2^{+}$ and $Mg2^{+}$ complemented with 1 mg/ml of Collagenase IV (Gibco). The digestion was stopped by extensive washing with HBSS containing 5% FBS and 5-minutes incubation on ice to let clumps of undigested pancreatic tissue to settle. Intra-islet lymphocytes (IIL) were collected from the supernatants of the three digestion/incubation cycles and extensively washed in HBSS 5% FBS before use in FACS analysis.

Flow cytometry

Single cell suspensions from different organs were resuspended in staining buffer containing PBS, 1% FBS and 0.09% NaN_3 and stained with monoclonal antibodies against surface markers. For intracellular cytokine staining, single cell suspensions isolated from different organs were stimulated for 2.5 hours with the Leukocyte Activation Cocktail (BD Bioscience). Cells were first stained for surface markers, then fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Bioscience), and finally stained for intracellular cytokines. The following antibodies were used: FITC anti-mouse CD3 (17A2, BD Biosciences), PercCP-cy5.5 anti-mouse CD4 (RM4-5, BD Biosciences), APC anti-mouse INF-γ (XMG1.2 BioLegend), APC-cy7 rat anti-mouse IL-17A (TC11-18H10. BD Bioscience), PE anti-mouse IL-10 (JES5-16E3 eBioscience), eFluor 647 anti-mouse Foxp3 (FJK-16s, eBiosciences) and APC anti-mouse CD25 (PC61, BioLegend). Dead cells were stained with Fixable Viability Dye eFluor 506 (eBioscience) and excluded from the analysis. Flow cytometry was performed using FACSCanto III (BD Biosciences) and data were analyzed with FCS Express V4 software (*De Novo* Software). See Supplementary Figure 1 for gating strategy.

RT-qPCR analysis

Immediately after sacrifice, mice's colons were flushed with PBS, opened longitudinally and placed in 500µl of TRIzol reagent (Life Technologies). After homogenization with TissueRuptor (QIAGEN), RNA was extracted by adding 100µl of chloroform, precipitating the acqueous phase with 300µl of 70% ethanol and purifying RNA with RNeasy Mini Kit (QIAGEN). RNA was retrotranscribed with SuperScript III

First-Strand Synthesis System following manufacturer's instructions (Life Technologies). Real time qPCR assay was performed with SYBR Select Master Mix (Life Technologies) using primers specific for different tight junction proteins, mucins and cytokines (Supplementary Table S2) on a ViiA 7 Real-Time PCR System (Life Technologies). Transcript expression was normalized against Rpl32 gene (housekeeping gene). See Supplementary Table 2 for the list of primers used in this study.

Histology

Segments of the distal colon were fixed in Carnoy's fixative (60% dry methanol, 30% chloroform, and 10% acetic acid), washed in absolute methanol, ethanol, xylene, embedded in paraffin wax and sectioned at 6 µm. For mucus layer structure evaluation, colon sections were stained with Alcian Blue staining kit, pH 2.5 (Sigma-Aldrich) and images acquired with Axio Imager M2m Light Microscope (ZEISS). The proportion of GC in colon sections was determined within the total number of cells in randomly selected stained sections. To assess histopathological signs of diabetes, pancreata were fixed in formalin, embedded in paraffin, sectioned into 4-µm slices and then stained with hematoxylin–eosin for insulitis score.

16S rRNA microbiota analysis

Total DNA was isolated from the mucosa and luminal content of the large intestine of NOD mice using PowerFecalTM DNA Isolation kit (MoBio) following the manufacturers' instructions. Microbiome characterization was performed by amplification of three regions of the 16S rRNA (V3, V4, V5) using universal primer pairs. The analysis of metabolically active microbiota was performed by pyrosequencing of rRNA cDNA 16S (GS Junior, Roche Diagnostics GmbH). Sequences with a high-quality score were used for the taxonomic analysis with QIIME (Quantitative Insights into Microbial Ecology version 1.6).

Metabolomic analysis

Fresh snap-frozen feces were collected from mice after 10 weeks of diet and used for metabolomics analysis. Metabolites were extracted three times using the following mixture of solvent, acetonitrile:methanol:water = 4:4:2. The collected supernatants were filtered on 96 well-plate with positive pressure manifold (0.22 µm pore size), dried under gentle stream of nitrogen and recomposed with 100 µl of water containing 0.1% formic acid. A QC sample was prepared by taking 20 µL from each sample. The samples were analysed by LC-MS/MS in 5 technical replicates by injecting 3 µl on the TripleTOF 5600+ mass spectrometer (SCIEX) connected to the UPLC 1290 (Agilent Technologies). The chromatographic separation occurred on a Waters Acquity UPLC HSS T3 column (100x2.1 mm, 1.8 µm) through the following 25 minutes gradient of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) at flow rate of 600 µl/min: 1 min of equilibration at 2% B; from 2% B to 95% B in 14 min; washing step at 95% B for 5 min, re-equilibration at 2% B for 5 min. The mass spectrometry analysis was performed both in positive and negative polarity, in the range of m/z 50-500 (TOF-MS scan with an accumulation time of 0.15 sec), with a SWATH acquisition comprising 10 windows of 45 Da each (accumulation time of 0.07 sec). The MS-DIAL software version 4.7.0 was used to analyze MS and MS/MS data for the identification of metabolites.

Statistical analysis

Cumulative diabetes incidence was calculated using the Kaplan-Meier estimation, whereas statistical significance was evaluated by the log-rank test. Statistical significance of the differences between 2 or more samples for qRT-PCR data and immunological data was calculated by unpaired 2-tailed Student's *t*-test or ANOVA respectively. Metabolic profile data, normalized to feces weight, were submitted to ANOVA test, with FDR correction. We could observe 17630 features in positive polarity and 10535 features in negative polarity with FDR p-value < 0.05. The PCA analysis was performed using an in-house developed R script, while the heatmap was obtained using the MeV software v. 4.9.0 (2). For statistical analysis of the microbiome, microbial reads were discriminated against human reads with BMTagger (ftp.ncbi.nlm.nih.gov/pub/agarwala/bmtagger/) and mapped to the collection of all available genomes (https://www.ncbi.nlm.nih.gov/genome/) with Kraken2 for exact alignment of k-mers and accurate read classification (37). Relative abundance profiling and differential analysis were performed with DESeq2 upon variance-stabilizing transformation (38). Visualization was performed with ggplot2 (39). Species alpha diversity and dominance indices were calculated with vegan (https://cran.r-project.org/web/packages/vegan). *p* values <0.05 were considered statistically significant.

Results

Administration of AID prevent autoimmune diabetes in NOD mice

NOD mice that spontaneously develop autoimmune diabetes have a defect of the GB that primarily affects the mucus layer structure and composition (10), however a causal

relationship between loss of gut barrier integrity and autoimmunity in this preclinical model of T1D is yet to be determined. Here, we restored GB integrity in NOD mice and test the effect on T1D occurrence. We decided to use a dietary approach containing nutrients known to reduce intestinal inflammation and promote GB function. To this aim, we elaborated a specific anti-inflammatory diet (AID) that includes omega-3 PUFA capable to reduce gut inflammation and promote immune tolerance, i.e., FoxP3$^+$ Treg and IL-10$^+$Tr1 cell differentiation (21–24), and inulin, a soluble fiber, that plays a direct beneficial effect on the GB and mucus layer integrity and promotes FoxP3$^+$ Treg cell differentiation (26, 28–31) (Figure 1A and Supplementary Table S1 for diet composition). Female NOD mice were either fed with standard (STD) diet or with the AID starting at 4 weeks of age just after weaning and before onset of beta cell autoimmunity and first signs of GB damage occur (10). In our animal facility female NOD mice normally develop spontaneous autoimmune diabetes with an incidence of 80-90% between 16 and 30 weeks of age. Our data reveal that the administration of the AID protected female NOD mice from

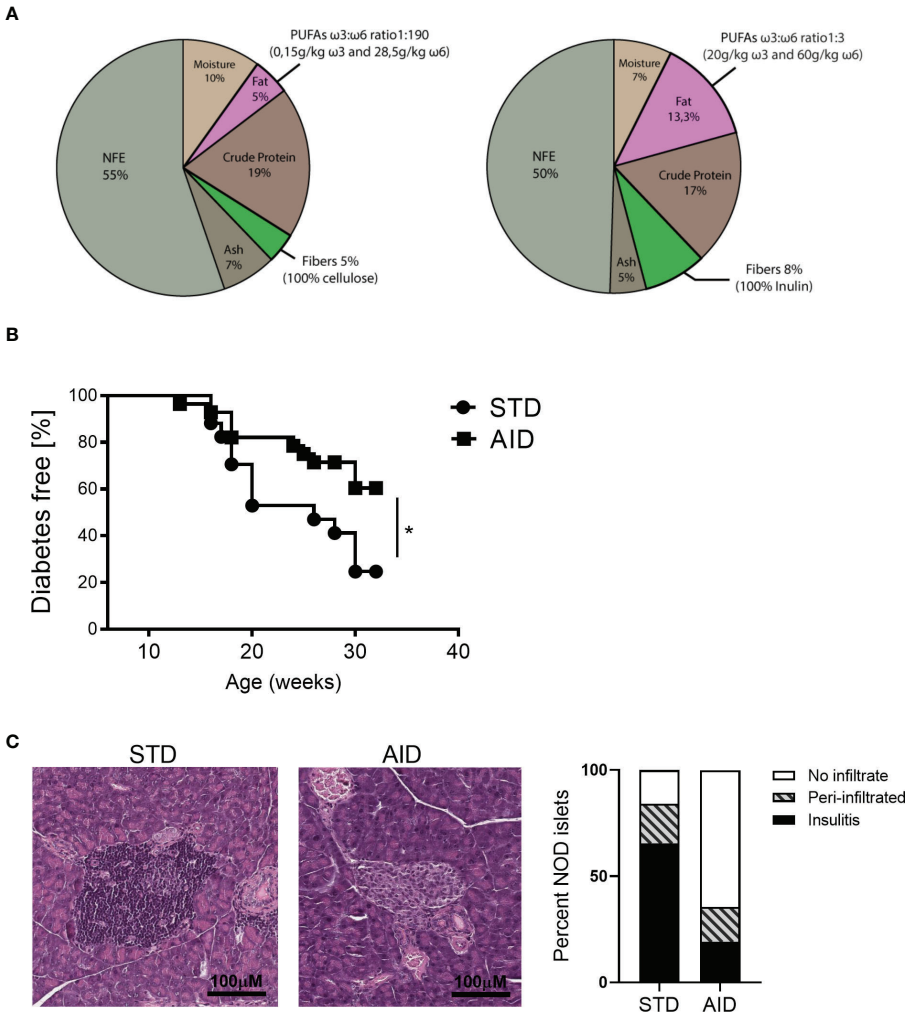


FIGURE 1
NOD mice fed with anti-inflammatory diet were protected from autoimmune diabetes. **(A)** 4-weeks-old female NOD mice were fed with anti-inflammatory diet (AID) or standard (STD) diet up to 32 weeks of age (end of the experiment). In the AID the total amount of fats was 13,3% (3,6% of fish oil, 2% of linseed oil and 7% of corn oil), with PUFA ω3:ω6 ratio of 1:3 (20000mg/kg ω3 and 60000mg/kg ω6), and 8% of fibers (100% Inulin). STD diet contained 5% of fats (100% corn oil) with PUFA ω3:ω6 ratio of 1:190 (150 mg/kg ω3 and 28500 mg/kg ω6) and 5% of fibers (100% Cellulose). NFE, nitrogen-free extract **(B)** Incidence of autoimmune diabetes in NOD mice fed with AID (n=25) and STD diet (n=20). **(C)** Diabetic mice from each group (AID or STD diet-fed) were sacrificed after two consecutive measurements of hyperglycemia (> 250mg/dl), while diabetes resistant mice were sacrificed at the end of the experiment (32 weeks of age). Hematoxylin and eosin staining of pancreatic tissues was performed to detect lymphocyte infiltrates. On the left: one representative image of pancreatic tissue showing islet infiltration (in a diabetic NOD mouse fed with STD diet) or intact islets (in a diabetes-resistant NOD mice fed with AID diet) are shown. On the right: percentages of pancreatic islets infiltrated, peri-infiltrated with lymphocytes or intact (no infiltrates) out of total analyzed islets in Hematoxylin and Eosin-stained pancreata of NOD mice fed with AID or STD diet. Five randomly selected sections were analyzed from each mouse (n=6 mice per group). *$p < 0.05$.

occurrence of T1D (Figure 1B) with 40% of diabetic mice in the AID-fed group compared to 80% in the STD diet-fed counterparts at 32 weeks of age (end of experiment) ($p< 0.05$). The protective effect of the AID was confirmed at the histological level and NOD mice fed with the AID showed reduced degree of insulitis and increased number of intact islets (70%) compared to STD diet-fed mice (15%) (Figure 1C).

AID restored GB integrity by promoting expression of tight junction proteins and mucins

The GB alterations in the NOD mice are detectable at 10-12 weeks of age concomitantly with onset of beta cell autoimmunity and thus suggesting that they may have a triggering effect on T1D pathogenesis (10). In order to assess whether prevention of T1D in AID-fed NOD mice is linked to restoration of GB integrity, we performed a gut permeability test (FITC-dextran) and measured by RT-qPCR the expression of mRNA transcripts of tight junction proteins and analyzed different biomarkers of mucus layer integrity (mucus layer thickness, percentage of GC, mRNA expression levels of mucins) in the intestine of AID vs STD diet-fed NOD mice. Our FITC-dextran test confirmed previous findings of increased gut permeability in NOD mice compared to non-autoimmune mice (Supplementary Figure 2) and a decrease of gut permeability in AID-fed NOD mice compared to STD diet-fed counterparts at 24 weeks of age but not earlier in the pre-diabetic phase (14 weeks of age) (Supplementary Figure 2). Importantly, we observed that AID-fed NOD mice had increased mRNA expression levels of claudin 1 (CLDN1), one structural protein of the intestinal epithelial barrier (IEB) (Figure 2A; $p<0.0001$). Importantly, mRNA transcripts relatives to several mucins including the mucus structural mucin Muc2 ($p<0.0001$) and immune-regulatory mucins

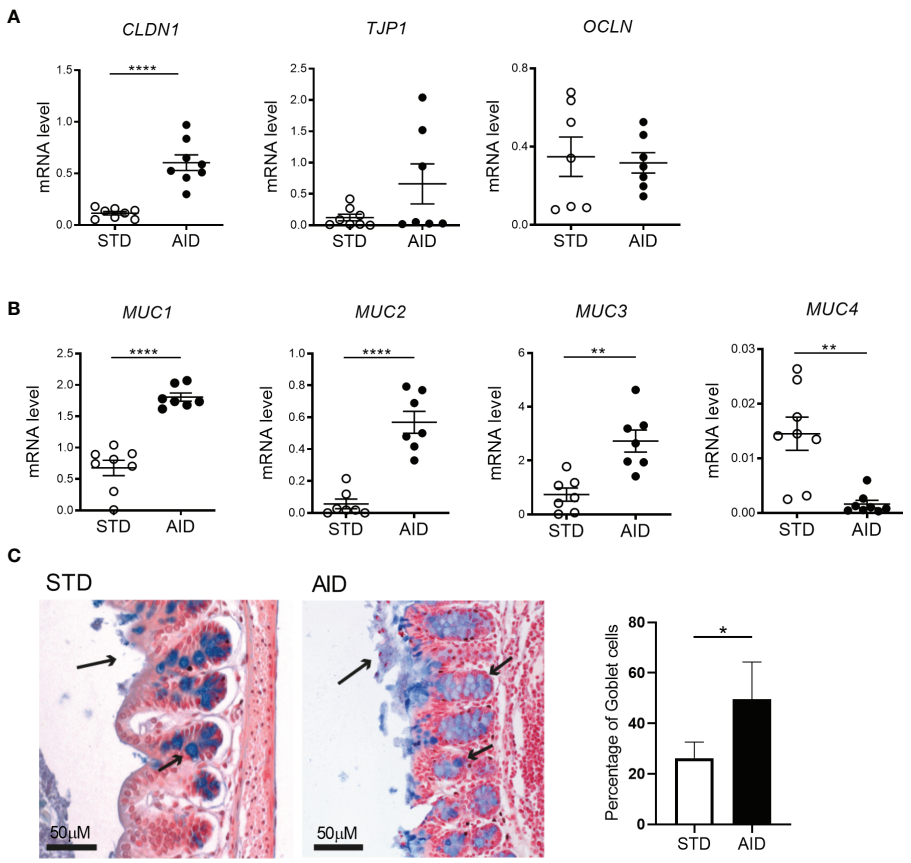


FIGURE 2
AID restores gut barrier integrity in NOD mice. **(A)** RT-qPCR analysis of claudin 1 (Cldn1), tight junction protein 1 (Tjp1) and Occludin (Ocln) on tissue homogenates from the intestine of NOD mice fed with anti-inflammatory (AID) or standard (STD) diet at 14 weeks of age (n=8 per group). **(B)** RT-qPCR analysis of Muc1, Muc2, Muc3, and Muc4 mucin genes in the intestine of NOD mice fed with AID or STD diet (n=8 per group). **(C)** Alcian Blue staining of colon sections (20X) of NOD mice fed with AID or STD diet. The arrows on the apical side of the intestine (long arrow) indicate the thickness of the mucus layer while the smaller arrows along the crypts point to GC (stained in Blue). The percentage of GC in the two groups (right panel) was calculated within the total number of epithelial cells on randomly selected sections (n=5 per group, 6 sections/mouse). All data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

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Muc1 ($p<0.0001$) and Muc3 ($p<0.01$) were also up-regulated in the intestinal tissue of AID-fed NOD mice (Figure 2B), while Muc4, a pro-inflammatory mucin (40), is decreased in AID-fed NOD mice (Figure 2B; $p<0.01$). Restoration of the mucus layer integrity in AID-fed NOD mice was confirmed at the histological level with an almost complete absence of mucus layer in STD-fed NOD mice that was corrected by administration of AID (Figure 2C). In line with this observation, we measured a significantly higher percentage of GC in the large intestine of AID-fed NOD mice compared to their STD diet-fed counterparts (Figure 2C; $p<0.05$).

AID promoted differentiation/expansion of regulatory FoxP3$^+$ Treg and IL-10$^+$Tr1 cells

Next, we asked whether restoration of the GB integrity (IEB and mucus layer) and protection from T1D in AID-fed NOD mice were associated with reduction of inflammation and induction of tolerogenic mechanisms in the gut mucosa. Specifically, since T1D occurrence is associated with acquisition of an effector phenotype by diabetogenic T cells in the gut (10), we asked whether T1D protection in AID-fed NOD mice was linked to a shift of functional phenotype of intestinal T cells from an effector Th17/Th1 type to a regulatory type (FoxP3$^+$ Treg and IL-10$^+$ Tr1 cells). First, we analyzed gut inflammation by measuring mRNA expression levels of inflammatory cytokines IL-1β, IL-23 and IL-17 in the intestinal tissues and found a statistically significant reduction of those inflammatory cytokines in AID-fed NOD mice (Figure 3A; $p<0.001$ for IL-1β and IL-17 and $p<0.0001$ for IL-23). Furthermore, our FACS analysis of the functional phenotype of intestinal T cells revealed that AID feeding in NOD mice significantly reduced the relative percentages of effector Th1 and Th17 particularly in the small intestine (Figure 3B; $p<0.05$ for Th17 and $p<0.01$ for Th1 cells) and to a lesser extent in the large intestine (Supplementary Figure 3) while simultaneously promoting expansion/differentiation of FoxP3$^+$ Treg cells in the small and large intestine (Figure 3C; $p<0.01$ and Supplementary Figure 3) and IL-10$^+$ Tr1 cells in the small intestine (Figure 3C; $p<0.01$). Importantly, improved immune tolerance mechanisms, i.e., increased FoxP3$^+$ Treg and IL-10$^+$ Tr1 cells, and decrease of Teff cell differentiation found in the intestinal mucosa of AID-fed mice were spread to pancreatic lymph nodes (PLN) and islets. In fact, we detected a decrease of effector Th17 cells in the PLN (Figure 4A; $p<0.05$) and, most importantly, a decrease of effector Th17 and Th1 cells within the intra-islet lymphocytes (IIL) of AID-fed NOD mice (Figure 4B; $p=0.05$ for Th17 cells and $p<0.01$ for Th1 cells). Simultaneously, we measured an increase of the relative percentages of both FoxP3$^+$ Treg cells and IL-10$^+$ Tr1 cells in the PLN (Figure 4C; $p<0.01$ for FoxP3$^+$Treg cells and $p<0.05$ for Tr1 cells) and IIL (Figure 4D; $p<0.05$ for FoxP3$^+$Treg cells and $p<0.01$ for Tr1 cells) of AID-fed NOD mice compared to their STD diet-fed counterparts.

AID modified the microbial and metabolomic profile in the intestine of NOD mice

To clarify the mechanisms responsible for restoration of GB integrity and immune homeostasis in AID-fed NOD mice, we analyzed the microbial and the metabolic profile at the intestinal level. Our 16s rRNA analysis of the gut microbiota profiles (from the intestinal mucosa and luminal content) showed important modifications in AID-fed NOD mice compared to STD diet-fed controls. Specifically, we detected an increased alpha-diversity (Shannon index) in the AID-fed NOD mice (Figure 5A), a characteristic of the gut microbiota profile that is normally associated with reduced intestinal inflammation and protection from T1D. We also measured statistically significant differences in the relative abundance of 86 bacterial taxa (Figures 5B, C and Supplementary Table 3). Notably, we found a very high increase in AID-fed NOD mice of the relative abundance of two *Akkermansia* species (log2fold change of 8.7 for *Akkermansia muciniphila* and 2.1 for *Akkermansia glycaniphila*) (Figure 5D and Supplementary Table 3; $p=6.9X10^{-14}$ *and* $p<0.01$ respectively), two bacterial species associated with mucus-regeneration (41–44). We also detected a statistically significant augment of relative abundance of beneficial species previously linked with dietary administration of omega-3 PUFA and soluble fibers such as bifidobacteria (*Bifidobacterium pseudolongum* and *Bifidobacterium animalis*) (21, 22, 26): (Figure 5D and Supplementary Table 3; $p=4X10^{-5}$ and $p<0.05$ respectively). Conversely, some pro-inflammatory bacterial strains such as *Bacteroides intestinalis* and *Streptococcus* sp. KS 6 were decreased in AID-fed NOD mice (Figure 5D and Supplementary Table 3; $p=4X10^{-4}$ and $p<0.01$ respectively). Next, we asked whether the AID modified the metabolic environment in the intestine of NOD mice. Our untargeted metabolic analysis with high performance liquid chromatography tandem-mass spectrometry (LC-MS/MS) revealed a completely different metabolic profile in AID-fed NOD mice compared to NOD mice fed with STD diet (Figure 6A). Importantly, the metabolic profile of AID-fed NOD mice was much closer to that of a control non-autoimmune mouse strain (Balb/c mice) (Figure 6A), even if the Balb/c mice clustered in two groups possibly due to different environmental conditions (Figure 6A). Numerous metabolites were differentially represented in NOD mice fed with AID compared with STD diet (Figure 6B) including several metabolites involved in the ω6/ω3 PUFA metabolic pathway that perform pro-resolving actions in experimental

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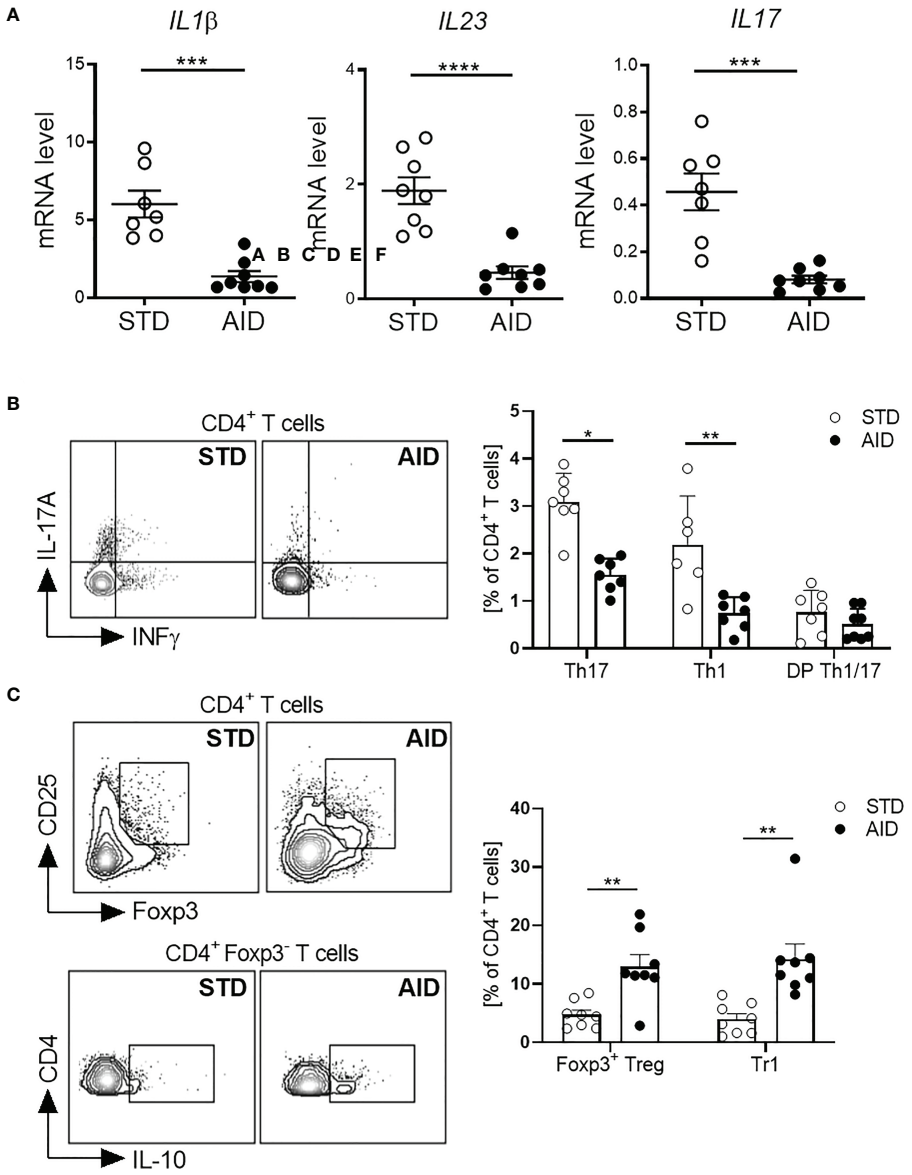

FIGURE 3
AID reduces intestinal inflammation and promotes gut immune homeostasis in NOD mice. **(A)** RT-qPCR analysis of cytokine genes encoding interleukin-1β (Il1b), subunit p19 of IL-23 (Il23), and IL-17A (Il17a) on tissue homogenates from the intestine of NOD mice fed with anti-inflammatory (AID) or standard (STD) diet at 14 weeks of age (n=8 per group). **(B)** Representative flow cytometry plots (*Left*) and percentages (*Right*) of INF-γ^+CD4$^+$ (Th1 cells), IL-17$^+$CD4$^+$ (Th17 cells) and INF-γ^+IL-17$^+$CD4$^+$ (DP Th1/17 cells) cells out of total CD4$^+$ T cells in the small intestinal tissue of 12-week-old NOD mice fed with AID or STD diet (n=7-8 mice/group). **(C)** Representative flow cytometry plots (*Left*) and percentages (*Right*) of FoxP3$^+$CD25$^+$CD4$^+$ (FoxP3$^+$Treg cells) and IL-10$^+$FOXP3$^-$CD4$^+$ (Tr1 cells) cells out of total CD4$^+$ T cells in the small intestinal lamina propria of 12-week-old NOD mice fed with AID or STD diet (n=7-8 mice/group). All data are presented as mean percentages ± SEM. **p < 0.05; **p < 0.01.*

models of colitis such as Docosapentaenoic acid (DPA), Docesahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) (45–47) and in the arachidonic acid metabolic pathway (Eicosanoic acid/Arachidic acid) involved in regulation of mucosal immunity and gut epithelial barrier function (48) (Figures 6B, C and Supplementary Table 4; *p<0.05* for DHA and DPA, *p<0.001* for EPA and *p<0.01* for Eicosanoic/Arachidic acid).

Discussion

Previous reports indicated that specific diet regimens such as an omega-3 PUFA-enriched diet or a diet supplemented with tolerogenic metabolites (acetate, propionate) prevent T1D in humans and preclinical models (49–51). Those dietary regimens counter-regulate T1D possibly by promoting growth of beneficial bacteria and dampening intestinal inflammation (51,

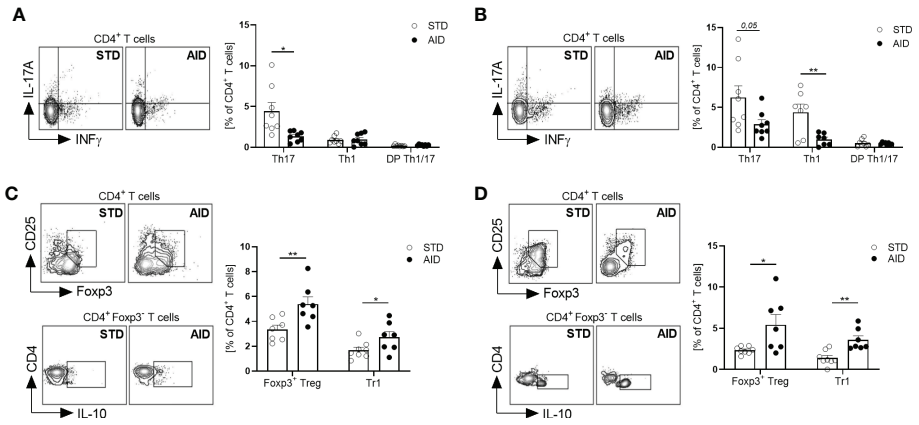

FIGURE 4
AID reduces the Teff/Treg cell ratio in the pancreatic lymph nodes and islets of NOD mice. **(A, B)**: Representative flow cytometry plots (*Left*) and percentage (*Right*) of INFγ+CD4+(Th1), IL-17+CD4+ (Th17) and INF-γ^+IL-$17^+$$CD4^+$ (DP Th1/17 cells) cells out of total CD4+ T cells in pancreatic lymph nodes (PLN) **(A)** and pancreatic intra-islet lymphocytes (IIL) **(B)** of 12-week-old NOD mice fed with AID or STD diet (n=8 per group). **(C, D)** Representative flow cytometry plots (*Left*) and percentage (*Right*) of $FoxP3^+CD25^+CD4^+$ ($FoxP3^+$Treg cells) and IL-$10^+FOXP3^-CD4^+$ (Tr1 cells) cells out of total $CD4^+$ T cells in the PLN **(C)** and pancreatic IIL **(D)** of 12-week-old NOD mice fed with AID or STD diet (n=8 per group). All data are presented as mean percentages ± SEM. *$p < 0.05$; **$p < 0.01$.

52), however an association between dietary protection from T1D and restoration of GB integrity was never demonstrated. Here we specifically designed an anti-inflammatory diet aimed at restoring GB integrity and mucus layer structure in NOD mice. Our data showing protection from T1D in AID-fed NOD mice with restoration of GB integrity provide proof-of-concept that an inflammatory gut environment and GB damage with modifications of mucus layer structure and composition play a causal role in the autoimmune pathogenesis of T1D in the NOD mice.

Recent evidence indicates that damage of the GB plays a pathogenic role in T1D. In line with this idea, several signs of intestinal inflammation with increased intestinal permeability, GB damage associated with lymphocyte infiltration and presence of pro-inflammatory cytokines are present in patients and preclinical models of T1D (5–10). The observation that breakage of GB integrity with subsequent increased antigen trafficking and occurrence of low-grade intestinal inflammation precede the onset of T1D suggests that GB damage is directly related to autoimmune pathogenesis rather than secondary to diabetes-induced metabolic alterations (5, 10). A causal link between loss of GB integrity and occurrence of T1D was demonstrated by the finding that induction of low-grade intestinal inflammation in TCR transgenic *BDC2.5NOD* mice with an expanded self(islet)-reactive T cell repertoire promoted intestinal activation of the diabetogenic T cells thus triggering autoimmune diabetes (10). Damage of the GB integrity with alterations of the mucus structure and composition are found in the NOD mice, the spontaneous preclinical model of T1D (10), however there is yet no evidence that those defects are not an epiphenomenon but rather play a direct pathogenic role in T1D occurrence. Here, we demonstrated that restoring GB integrity in NOD mice through administration of an AID that specifically increased expression of tight junction proteins and mucins thus restoring a normal mucus layer architecture plays a beneficial role and prevented T1D. How does the GB damage provoke activation of beta cell autoimmunity and T1D occurrence? The GB is an important gatekeeper that regulates the interaction between the gut commensal microbiota and the immune system. The GB maintains the physical separation between microbial species and immune cells residing in the gut mucosa but also plays important immune regulatory functions. Specifically, the mucus layer contain immune-regulatory molecules such as Muc1 and Muc3 that are crucial to maintain immune tolerance towards bacterial antigens and prevent inflammation (53). Here, we did not detect an amelioration of the gut permeability in the prediabetic phase in AID-fed mice but only after the onset of diabetes in the STD-fed group, thus indicating that restoration of physical separation between gut mucosa and the intestinal lumen is not crucial to prevent beta cell autoimmunity in AID-fed NOD mice. Conversely, we observed an increase of mucus structural protein and immune regulatory mucins that preceded occurrence of beta cell autoimmunity (14 weeks of age) and thus could play a direct beneficial effect on dampening the autoimmune process in T1D. In fact, in the NOD mice the GB damage mostly relates to defects of mucus layer structure and composition leading to loss of immune homeostasis and inflammation with a predominance of effector Th1/Th17 cells and a defect of regulatory T cells in the gut (10). Our data demonstrate that those mucus layer alterations and defective gut immune homeostasis are directly linked to T1D pathogenesis in NOD mice. In fact, in AID-fed NOD mice, restoration of GB integrity with increased structural mucus layer

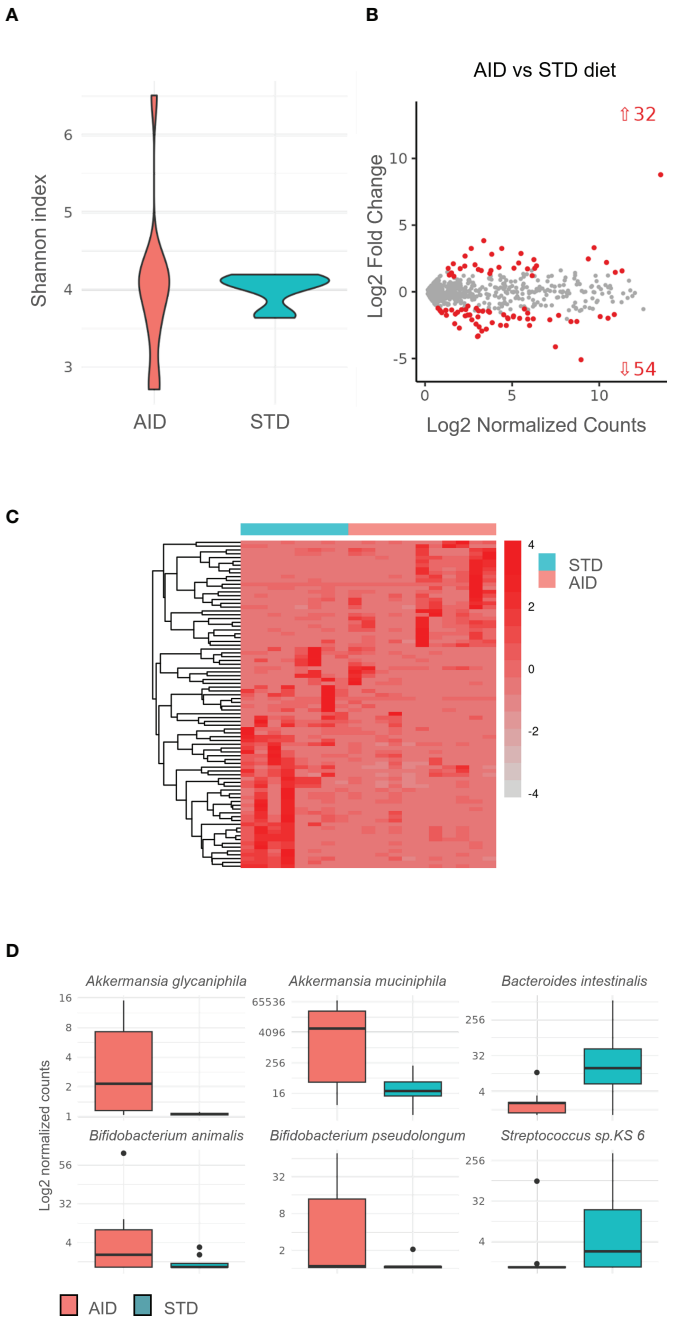

FIGURE 5
Gut microbiota profiles of AID and STD-fed NOD mice. Microbiota isolated from luminal content and brushed intestinal mucosa of AID or STD-fed NOD mice at 12 weeks of age (n=6 per group) was assessed by 16S Amplicon Sequencinq of DNA and analysed with QIIME software. **(A)** alpha-diversity in AID vs STD-diet fed NOD mice based on Shannon index. **(B)** Differentially expressed bacterial species between AID and STD diet fed NOD mice. **(C)** Heatmap of selected differentially represented bacterial species in AID vs STD-diet fed NOD mice. **(D)** Relative abundance of selected differentially represented bacterial species in AID vs STD-fed NOD mice.

component Muc2 and immune-regulatory mucins (Muc1 and Muc3) and subsequent increase of FoxP3$^+$ Treg and IL-10$^+$ Tr1 cells prevented T1D. The increased relative percentages of FoxP3$^+$Treg/Tr1 cells and decrease of effector T cells (Th1/Th17) was not limited to the gut mucosa but a shift of the functional phenotype of T cells from a pro-inflammatory Th1/Th17 type to a protective Treg phenotype (FoxP3$^+$ Treg and IL-10$^+$Tr1) was also detected in the draining PLN and intra-islets lymphocyte (IIL) infiltrates of AID-fed NOD mice. Recent evidence in different preclinical models of T1D indicates that

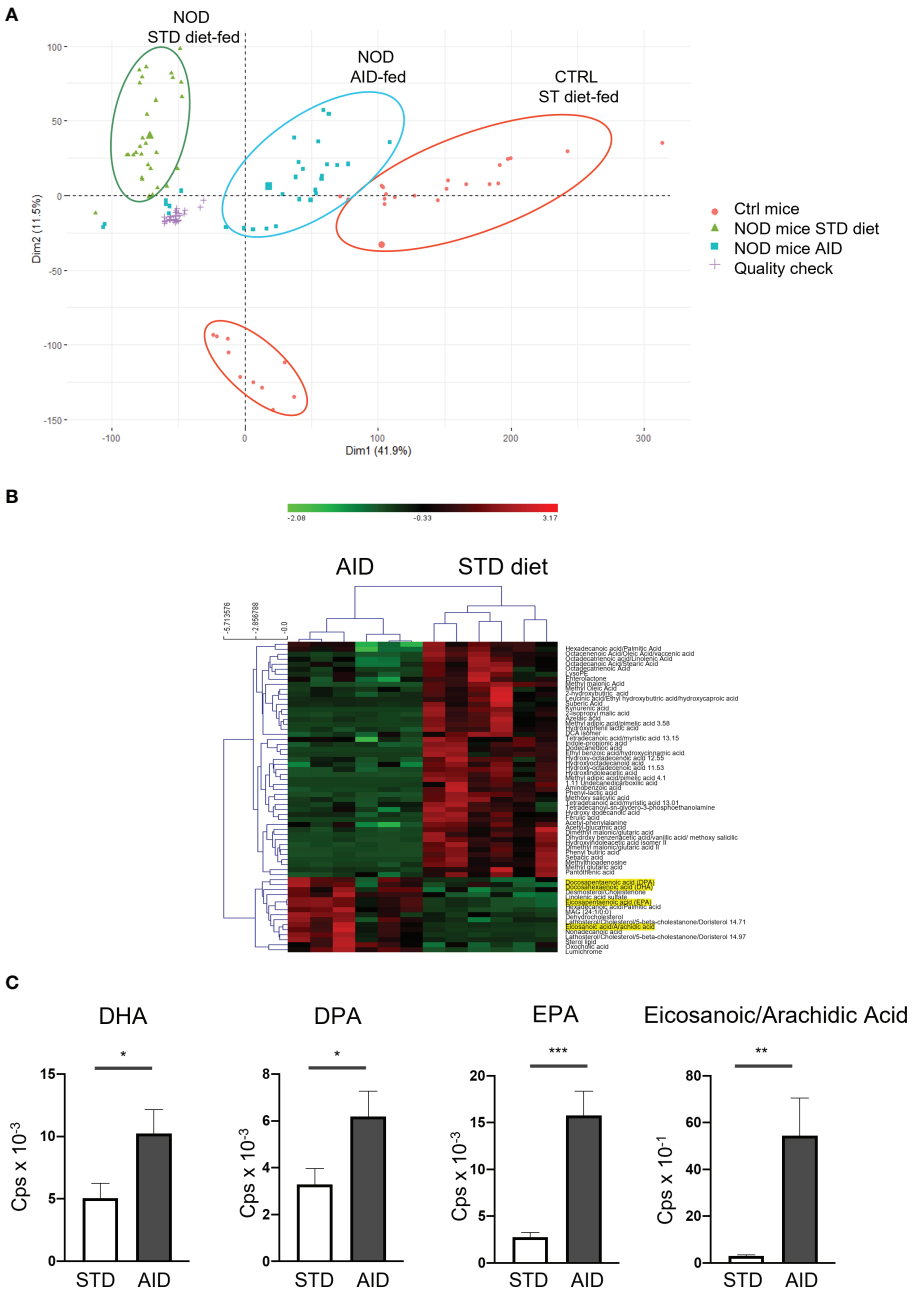

FIGURE 6
Metabolomic profiles of stools from AID or STD diet-fed NOD mice. **(A)** Representative PCA analysis of 17630 features in positive polarity with FDR *p-value* < *0.05* in STD-diet and AID-fed NOD mice and non-autoimmune Balb/c mice (n=6 per group). Technical replicates for the QC sample clustered together, showing a very reproducible LC-MS/MS analysis. The larger symbol per group indicates the mean of all the samples. **(B)** Heatmap of the peak areas for the 62 annotated metabolites which were significantly different between STD-fed vs AID-fed NOD mice. The overrepresented metabolites are in red, while the underrepresented ones are indicated in green. 15 out of 62 metabolites were upregulated in the group of AID-fed NOD mice. **(C)** Selected differentially represented metabolites in AID vs STD-fed NOD mice. Data are expressed as mean percentages ± SEM. **p < 0.05; **p < 0.01; ***p < 0.001.*

self-(islet)-reactive T cells are activated at the intestinal level, possibly through microbiota-induced mechanisms of molecular mimicry (54, 55) and acquire effector phenotype (Th1/Th17) (10). Hence, we speculate that a tolerogenic gut environment induced by AID can shift the functional phenotype of self(islet)-reactive T cells from an effector Th1/Th17 type towards a regulatory FoxP3$^+$ and/or Tr1 cell type at the intestinal level, thus counter-regulating T1D pathogenesis.

How does the AID restore GB integrity in the NOD mice? Alterations of the gut commensal microbiota composition have

been reported in patients and preclinical models of T1D (4, 56–58). Diet is one of the most effective measures to modulate the composition of the commensal gut microbiota (18). Previous reports indicate that diets enriched in omega-3 PUFA or inulin fibers promote a beneficial gut microbiota composition promoting overgrowth of anti-inflammatory bifidobacteria and lactobacilli (21, 22, 26). Here, we found that our AID enriched in omega-3 PUFA and inulin had a strong impact on the gut microbiota composition of NOD mice. First, in T1D-protected AID-fed NOD mice we detected a higher overall diversity (alpha diversity based on Shannon index), a condition previously associated with a decreased risk to develop clinical T1D in humans (3). In addition, we found an increased relative abundance of beneficial species previous associated with omega-3 PUFA and inulin such as bifidobacteria (*Bifidobacterium pseudolongum* and *Bifidobacterium animalis*). However, the most significant modification that we observed in the gut microbiota composition of AID-fed NOD mice was an 8-fold increase in the relative abundance of *Akkermansia muciniphila* and a 2-fold increase in *Akkermansia glycaniphila*. Those are mucus-degrading bacteria belonging to the Verrucomicrobia phylum that produce a wide array of mucin degrading enzymes fundamental to promote mucus regeneration, increase mucus layer thickness and maintain its function of physical and biological barrier (41–44). Importantly, the levels of *A. muciniphila* inversely correlate with intestinal inflammation (59) and previous reports associated the presence of *Akkermansia muciniphila* in the commensal gut microbiota with diabetes protection in NOD mice (60, 61). The absence of this mucus-degrading strain in a specific NOD mouse colony (NOD/Jax mice) was linked with high diabetes incidence, while transferring of *A. muciniphila* to those NOD mice either through oral gavage or co-housing with a diabetes-resistant NOD mouse colony (NOD/MrkTac) prevented T1D. The protective effect of *A. muciniphila* was related to enhanced mucus production, restoration of physical GB integrity and immune homeostasis with increased number of regulatory FoxP3$^+$ Treg cells and transcripts levels of Treg-associated cytokines IL-10 and TGF-β in PLN and islets (61). In accordance with those findings, in our AID-fed NOD mice T1D protection was associated with high increase in *A. muciniphila*, restoration of the mucus layer architecture with augmented mRNA expression of structural (Muc2) and immune-regulatory mucins (Muc1 and Muc3) and enhanced FoxP3$^+$ Treg and IL-10$^+$ Tr1 cells in the PLN and IIL.

Another important mechanism through which the AID could counter-regulate T1D is through modulation of the intestinal metabolic profile. Recent evidence indicates that the metabolites that are present in the gut mucosa are important regulators of adaptive immunity at the intestinal level but also systemically (62). We found that AID-fed NOD mice have an activation of the ω3/ω6 PUFA metabolic pathway and arachidonic pathway that are associated with reduction of intestinal inflammation and restoration of GB integrity in different colitis models (45–48).

Diet composition is one of the strongest environmental factors affecting the composition of the gut microbiota but also the GB integrity and maintenance of immune homeostasis in the gut and systemically. Our data provide evidence that an AID designed to promote GB integrity and function is capable to prevent occurrence of T1D by improving mucus layer architecture and composition and promoting immune tolerance, i.e., FoxP3$^+$ Treg and IL-10$^+$ Tr1 expansion, in the intestine but also in PLN and islets of NOD mice. The intestinal environment is important for modulating T1D in preclinical models but also in humans affected by T1D. Commensal gut microbiota alterations, intestinal inflammation and GB damage are all factors that increase the risk to develop T1D in genetically susceptible individuals. Our results could pave the way to new dietary approaches aimed at restoring GB function and immune homeostasis with the final goal to prevent T1D in genetically "at-risk" children.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI Sequence Read Archive; PRJNA913009.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the IRCCS San Raffaele Scientific Institute according with the rules of the Italian Ministry of Health (IACUC #868).

Author contributions

MLC and MAC performed *in vivo* experiments and FACS analysis. IC and AN designed the AID and analyzed data. RF and LM performed 16s rRNA and statistical analysis on microbiota profiles. MU and AA performed mass spectrometry and analyzed metabolomic data. MLC analyzed data and prepared figures. NM and FU contributed to design the study and to interpret data. MF served as principal investigator, analyzed and interpreted data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

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Gut microbial profile of treatment-naive patients with primary biliary cholangitis

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Background and aims: The pathogenesis of primary biliary cholangitis (PBC) is associated with alterations of gut microbiota. We compared the gut microbiota of PBC patients and healthy controls from Zhejiang Province and assessed the use of these data for the diagnosis of PBC.

Methods: First, 16S rRNA gene sequencing was used to characterize the gut microbiota of treatment-naive PBC patients (n=25) and matched healthy controls (n=25). Then, the value of gut microbiota composition for the diagnosis of PBC and assessment of PBC severity was determined.

Results: The gut microbiota of PBC patients had lower diversity based on three different metrics of alpha-diversity (ace, Chao1, and observed features) and fewer overall genera (all $p<0.01$). PBC patients had significant enrichment of four genera and significant depletion of eight genera. We identified six amplicon sequence variants (*Serratia*, *Oscillospirales*, *Ruminococcaceae*, *Faecalibacterium*, *Sutterellaceae*, and *Coprococcus*) as optimal biomarkers to distinguish PBC patients from controls based on receiver operating characteristic analysis (area under the curve [AUC] = 0.824). PBC patients who were anti-gp210-positive had lower levels of *Oscillospiraceae* than those who were anti-gp210-negative. KEGG functional annotation suggested the major changes in the gut microbiota of PBC patients were related to lipid metabolism and biosynthesis of secondary metabolites.

Conclusion: We characterized the gut microbiota of treatment-naive PBC patients and healthy controls from Zhejiang Province. The PBC patients had significant alterations in their gut microbiota, suggesting that gut microbiota composition could be useful as a non-invasive tool for the diagnosis of PBC.

KEYWORDS

primary biliary cholangitis, fecal microbiome, biomarkers, amplicon sequence variants (ASV), diagnosis

Introduction

Primary biliary cholangitis (PBC) is a chronic auto-immune cholestatic liver disease that is characterized by fibrosis and destruction of the interlobular bile ducts (1). Studies of the pathogenesis of PBC have mainly focused on genetic susceptibility (2), environmental factors (3), and immune factors (4). However, there is increasing emphasis on the effect of gut microbiota in PBC (5, 6) because the liver and intestine are linked by the portal vein, forming a gut-liver axis (7). Previous studies confirmed that many patients with chronic liver diseases, including PBC, have different severities of dysbiosis of gut microbiota (8). Disruption of the tight junctions (TJs), important structures composed of multiple proteins that help to maintain intestinal homeostasis, can lead to a leaky gut (9). Patients with PBC often have many gut microbes and metabolites that can penetrate the intestinal mucosal barrier and are then transported to the liver *via* the gut-liver axis (10), where they can trigger an immune-mediated attack against the small bile duct (11). Therefore, studies of alterations in the gut microbiota of patients with PBC may improve the diagnosis and treatment of this disease and understanding of its pathogenesis.

Few previous studies have examined the characteristics of the gut microbiota of PBC patients, although there were two notable recent studies of this topic. Furukawa et al. (12) studied PBC patients from Japan and reported they had significantly reduced diversity of gut microbiota, with abnormal increases of *Enterococcus*, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*, and a significant decrease of *Clostridiales*. They also found that the use of ursodeoxycholic acid (UDCA) and proton pump inhibitors (PPIs) were important confounding factors because they affected the composition of gut microbiota. Tang et al. (13) studied PBC patients from the outpatient clinic of Shanghai Renji Hospital and found alterations in their fecal flora, with increases in eight bacterial genera and decreases in four bacterial genera relative to controls. In particular, *Klebsiella* was significantly more abundant in PBC patients and positively correlated with the level of serum total bilirubin. However, there have been no studies of the gut microbiota of patients with PBC in Zhejiang Province, and populations from different regions often have significant differences in their gut microbiota (5).

In this study, we compared the characteristics of the gut microbiota in treatment-naive PBC patients and healthy controls from Zhejiang Province to identify specific gut microbiota markers that have potential use for the diagnosis and treatment of PBC. Our focus was to determine the correlation of different gut microbiota with liver functional indexes, and then evaluate the use of gut microbiota for the diagnosis of PBC and determination of PBC severity in an effort to reduce the need for invasive testing.

Methods

Study cohort and sample collection

This study compared 25 treatment-naive PBC patients and 25 healthy matched controls. All PBC patients were from the inpatient department of Hangzhou Xixi Hospital affiliated to Medical School of Zhejiang University, and healthy controls were from the physical examination center. Blood samples were collected for analysis of liver function (including alkaline phosphatase [ALP], gamma-glutamyl transferase [GGT], alanine transaminase [ALT], aspartate transaminase [AST], and total bilirubin [TB]) and immunological tests (including IgG, IgM, anti-mitochondrial antibodies [AMA], anti-mitochondrial M2 antibodies [AMA-M2], anti-sp100 antibodies, and anti-gp210 antibodies). Fecal samples were collected for analysis of gut microbiota. Each PBC patient received an ultrasound examination and a liver biopsy. All fecal samples were freshly collected at the hospital, stored using the Longseegen Stool Storage Kit (No : LS-R-P-007, Guangdong, China), and frozen at −20°C within 3 h after collection. Histological analyses of liver samples were evaluated using the Ludwig staging system (14). All samples were collected from June 2021 to June 2022.

The 2018 PBC practice guidelines of American Association for the Study of Liver Diseases (AASLD) (13) were used for the diagnosis of PBC. Each enrolled PBC patient had all three of the following criteria: (*i*) elevation of ALP; (*ii*) presence of AMAs or other PBC-specific auto-antibodies (including those against sp100 or gp210); and (*iii*) histology results indicating nonsuppurative destructive cholangitis with destruction of the interlobular bile ducts. The two major exclusion criteria for PBC patients were: (*i*) previous standardized treatment for PBC or use of UDCA and (*ii*) use of antibiotics, lactulose, probiotics, PPIs or other drugs that might alter the gut microbiota within the previous 2 months. Patients with PBC-autoimmune hepatitis (PBC) overlap syndrome, as defined by the Paris criteria (15), were also excluded. All healthy controls, who were matched for age, sex, and BMI, had the following characteristics: (*i*) no significant abnormalities in routine blood tests, liver or kidney function tests, fasting glucose, serum lipids, or liver ultrasound results; (*ii*) no infection by the hepatitis B or C virus; and (*iii*) no use of antibiotics, probiotics, or other drugs that might alter gut microbiota within the previous 2 months.

This study was conducted according to the principles of the Declaration of Helsinki and was approved by the Medical Ethics Committee of Hangzhou Xixi Hospital (Ethical Approval No. 2020, Science and Education Section, Medical Ethics Committee No. 41). Each study participant signed a written informed consent document before participation.

DNA extraction and 16S ribosomal RNA gene sequencing

Primers were designed to amplify specific regions of the 16S V3-V4 region, and an amplified fragment of about 420 bp was obtained. The paired-end data of 2×250 bp were sequenced using the Illumina Novaseq 6000 platform, and longer sequences were obtained by splicing for 16S analysis. Standard data cleansing techniques were used to improve the accuracy and reliability of these measurements. In particular, the raw sequencing data were first de-noised and filtered to obtain validated (cleansed) data, and clustering of amplicon sequence variants (ASVs) and species classification were then performed using the validated data. Based on the clustering results of the ASVs, taxon identification and abundance were determined, with annotation for each ASV sequence. ASVs were also analyzed to determine species richness and evenness within samples. Common and unique ASVs among different samples or groups were identified by determining their abundance, and were presented in Venn diagrams, petal diagrams, and calculations of alpha diversity. Data obtained from the National Center for Biotechnology Information (NCBI) 16S rRNA database (BioProject ID : PRJNA892581, https://submit.ncbi.nlm.nih.gov/subs/bioproject/SUB12178067/overview) were used for identification.

Bioinformatic analysis of 16S rRNA sequencing

PICRUSt (https://picrust.github.io/picrust/) is a bioinformatics tool that uses 16S rRNA sequences to determine the functional profiles of microbial communities (16). Determination of the gene functions of sequenced microbial genomes also allows comparisons of different groups. Functional gene abundance enrichment of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways at different levels (1–3) were obtained using PICRUSt functional prediction from the 16S rDNA sequences. Clusters of Orthologous Genes (COG), KEGG Orthology (KO), and KEGG metabolic pathway predictions were also performed.

Statistical analysis

SPSS version 22.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis and calculation of the statistical significance of differences between groups. Linear discriminant analysis effect size (LEfSe) analysis was used to identify differences in taxa and pathways between the two groups. Receiver operating characteristic (ROC) curves were constructed and area under curve (AUC) values were calculated to assess the diagnostic performance of the model using the pROC package in R software. Spearman's rank correlation was calculated to determine correlations between the two groups, and Pearson's correlation was calculated to determine correlations of gut microbiota with clinical indexes. A Wilcoxon rank-sum test was used to determine the significance of differences in continuous variables in the two groups.

Results

Characteristics of participants

We used strict patient selection and exclusion criteria, and collected stool samples from 25 treatment-naive PBC patients and from 25 healthy controls, with matching for age, gender, and BMI (Table 1). Most of the PBC patients were middle-aged women, and the two groups were similar in most baseline measurements except GGT and ALP, which were higher in the PBC patients (both $P < 0.001$). Measurements of autoantibodies showed that 96% of PBC patients were positive for AMA-M2, 60% were positive for anti-sp-100 antibodies, and 84% were positive for anti- gp210 antibodies. The ultrasound examinations of all PBC patients indicated no obvious abnormalities, such as signs of a liver mass or cirrhosis. Liver biopsy testing showed that all PBC patients had Ludwig stage I or II disease.

Fecal microbiomes in PBC patients and controls

Analysis of alpha diversity of the microbiomes of PBC patients and controls showed that the PBC patients had significantly reduced richness and evenness (both $P < 0.05$, Figure 1A). We also compared the two groups in terms of beta-diversity using principal coordinate analysis (PCoA) (Figure 1B) and non-metric multidimensional scaling with 2 axes (NMDS2) (Figure 1C). These results also indicated significant intergroup differences. Therefore, the fecal microbial communities in patients with PBC were distinct from those of the healthy controls. Consistent with these results, a comparison of the overall composition of the fecal microbiomes in the two groups indicated many taxonomical differences (Figure 2).

Phylogenetic characteristics of the fecal microbial communities in PBC patients

LEfSe cladogram analysis of PBC patients (red) and controls (green) indicated clear differences between these groups (Figure 3A). Least discriminant analysis (LDA) of genus scores showed that 12 microbial biomarkers clearly distinguished PBC patients and controls (Figure 3B). In particular, there were 4 predominant genera in PBC patients (*Acidimicrobiia*, *Yersiniaceae, Serratia*, and *ucg_010*; all $P < 0.05$ and LDA > 3), and 8 predominant genera in the controls (*Faecalibacterium*, *Ruminococcaceae*, *Sutterellaceae*, *Oscillospiraceae, Parasutterella, Clostridia, Coprococcus*, and *Christensenellaceae*; all $P < 0.05$ and LDA > 3).

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TABLE 1 Characteristics of treatment-naive PBC patients and matched healthy controls.

Characteristic	PBC patients (n=25)	Controls* (n=25)	*p* value
Age, median years (min–max)	55 (43–77)	57 (24–77)	0.979
Female, n (%)	21 (84%)	22 (88%)	0.946
BMI, median kg/m^2 (min–max)	21.33 (19.12–23.5)	20.77 (20.12–21.56)	0.058
Hepatic function tests, median (min–max)			
ALP, U/L	199 (144–392)	77 (60-100)	0.001#
GGT, U/L	150 (103–292)	32 (21-45)	0.001#
ALT, U/L	25 (15–45)	25 (16-40)	0.345
AST, U/L	33 (17–69)	24 (16-50)	0.161
TB, µmol/L	14.6 (4.79–45.17)	17.1 (13.9-28.8)	0.865
Immunoglobulins, median (min-max)			
IgG, g/L	14.05 (9.37–31.66)		
IgM, g/L	2.62 (0.94–9.13)		
Autoantibody positivity, n (%)			
AMA	20 (80%)		
AMA-M2	24 (96%)		
sp100	15 (60%)		
gp210	21 (84%)		
Histological results			
Ludwig stage I/II/III/IV, n (%)	16/9/0/0 (64%, 36%, 0%, 0%)		

*Data from controls were from routine physical examinations, and therefore did not include immunoglobulins, autoantibodies, and histological results. # with staitistical significance.

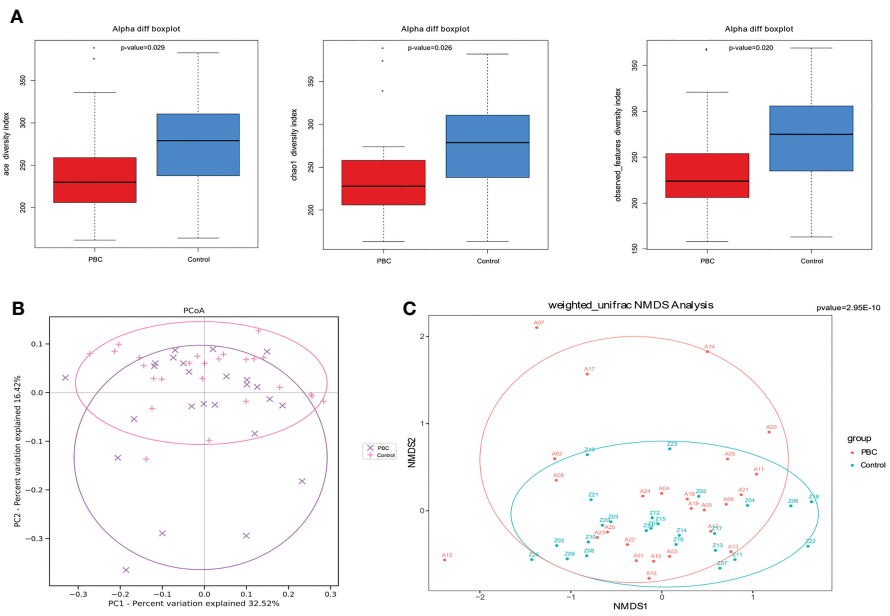

FIGURE 1
Gut microbial diversity in PBC patients and healthy controls. **(A)** Ace diversity index (left), Chao1 diversity index (center), and observed features index (right) were significantly lower in PBC patients. **(B)** PCoA based on weighted UniFrac matrix analysis showed the two groups had differences in overall fecal microbiota composition ($p < 0.01$). **(C)** Beta-diversity based on was NMDS analysis of the weighted UniFrac matrix showed distinct separation of the two groups in the direction of the NMDS2 axis ($p<0.01$).

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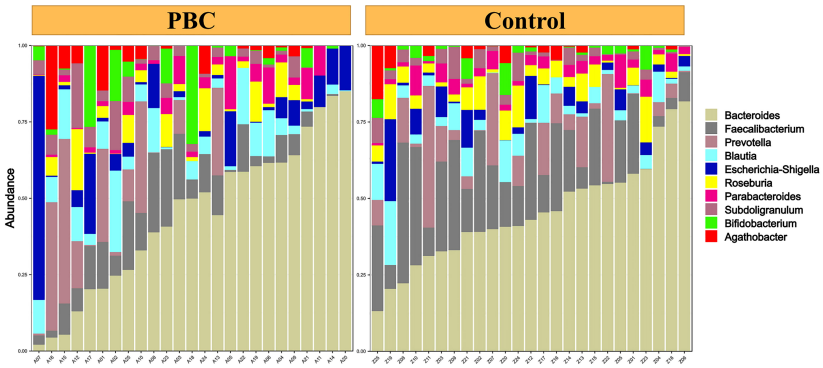

FIGURE 2
Abundance of different taxa in the fecal microbiota of each PBC patient (left) and each healthy control (right).

Gut microbiome signature and diagnosis of PCB

Spearman correlation analysis of the 12 microbial genera identified above showed negative correlations in the abundances of genera enriched in PBC patients with genera enriched in controls (Figure 4A). Multivariable stepwise logistic regression analysis showed that 6 genera (*Serratia*, *Oscillospirales*, *Ruminococcaceae*, *Faecalibacterium*, *Sutterellaceae*, and *Coprococcus*) reliably discriminated PBC patients from controls (data not shown). Subsequent ROC analysis based on these 6 genera led to an AUC of 0.824 (95% CI: 0.71, 0.94, Figure 4B). ROC analysis using 9 genera did not significantly improve the predictive performance (AUC: 0.834, 95% CI: 0.72, 0.95).

Correlation of fecal microbiome characteristics and PBC disease severity

We then used Pearson's rank test to analyze the relationship of PBC-associated genera and clinical indices of disease severity in PBC patients, with control for potential interference by age, gender, and BMI. The results showed that enrichment of *Serratia* and *Yersiniaceae* were positively related to the IgG level, and enrichment of *Oscillospiraceae* was negatively related to anti-gp210 antibody status (Figure 5A). In addition, calculation of the level of *Oscillospiraceae* in patients who were anti-gp210-positive and anti-gp210-negative showed that the anti-gp210- positive group had a lower level of *Oscillospiraceae* ($P < 0.05$, Figure 5B).

We then compared the functional and metabolic profiles of the gut microbial communities in PBC patients and controls using PICRUSt (Figure 6). The results indicated that 42 KEGG categories were significantly different in PBC patients and controls. Notably, the categories of lipid metabolism and biosynthesis of other secondary metabolites were abnormal in the microbiomes of PBC patients.

Discussion

We used 16s rRNA sequencing of gut microbiota to compare 25 treatment-naive PBC patients and 25 matched controls who were from Zhejiang Province. Diversity measures indicated the PBC patients had significantly reduced richness and evenness of gut

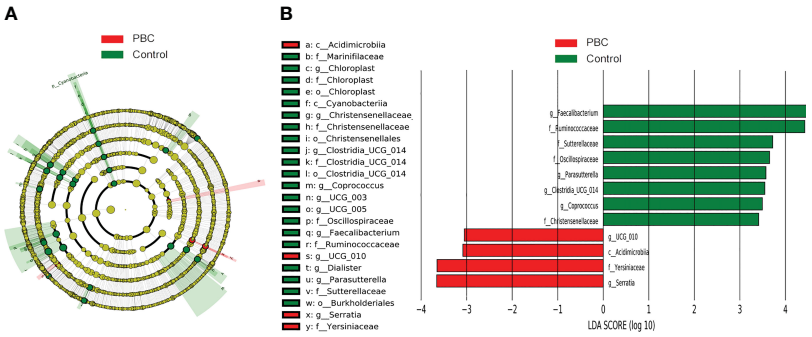

FIGURE 3
LEfSe and LDA analyses based on ASV characterizations of microbiota in PBC patients and matched healthy controls. **(A)** Cladogram. Red: PBC group, Green: control group, Node color: microbial groups that play important roles, yellow node: microbial groups that do not play an important role. Gut taxa indicated by the letters in the figure are shown in the legend on the right. **(B)** Twelve gut taxa with LDA scores greater than 3 or less than −3. Green: enriched in the control group, Red: enriched in the PBC group.

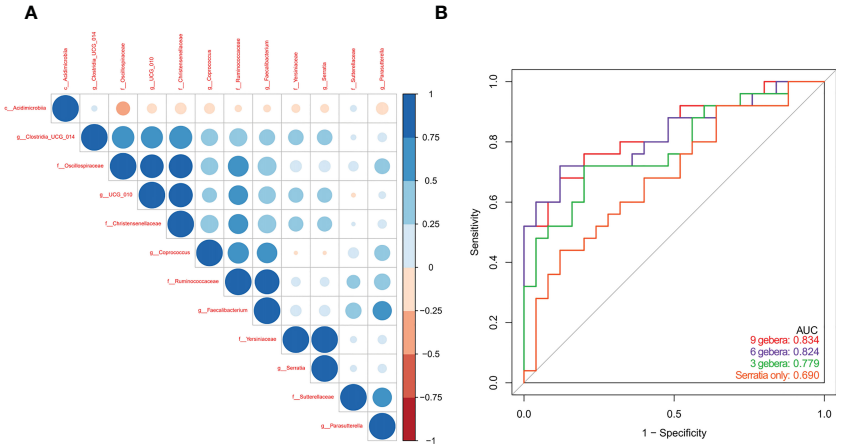

FIGURE 4
(A) Spearman correlations of the 12 PBC-associated genera in treatment-naive PBC patients and healthy matched controls. **(B)** ROC analysis based on a multivariable logistic regression model using 9 genera, 6 genera, 3 genera, and Serratia alone.

microbiota. Our NMDS2 and PCoA results also indicated significant differences in the abundances of different gut microbiota in PBC patients and controls. Our differential gut microbiota analysis of the 2 groups identified significantly greater abundances of 4 taxa and significantly reduced abundances of 8 taxa in PBC patients. Spearman correlation analysis showed a negative correlation in the abundance of *Acidimicrobiia* and *Oscillospiraceae*; enrichment of *Oscillospiraceae* and *Christensenellaceae* in the controls; and enrichment of *Serratia* and *Yersiniaceae* in the PBC patients. Multivariable logistic regression indicated that 6 ASVs (*Serratia*, *Oscillospirales*, *Ruminococcaceae*, *Faecalibacterium*, *Sutterellaceae*, and *Coprococcus*) were optimal biomarkers for distinguishing PBC patients from matched controls. Subsequent ROC analysis indicated these taxa provided an AUC of 0.824, and the AUC value of *Serratia* alone was 0.690.

Correlation analysis of different clinical indices with the 12 taxa that differed between the groups indicated that PBC patients who were anti-gp210-positive had lower levels of *Oscillospiraceae* than patients who were anti-gp210-negative. Previous research found that anti-gp210-positive PBC patients tend to have a poorer prognosis and to develop a range of complications, especially earlier onset of portal hypertension (17). Therefore, we speculate that a severe depletion of *Oscillospiraceae* may be a predictor of poor prognosis. In addition, the serum IgG level in PBC patients was positively correlated with the levels of *Serratia* and *Yersiniaceae*. Although elevated serum IgM levels are more common in PBC patients than in patients with other autoimmune liver diseases (18), some PBC patients with systemic rheumatic diseases (especially Sjögren's syndrome) have elevated serum IgG levels (19). Therefore, we speculate that if a PBC patient presents with abnormally enriched *Serratia* and *Yersiniaceae* in the gut microbiota, clinicians should remain alert to the possibility of other extrahepatic autoimmune diseases.

Other studies of the gut microbiota in PBC patients examined patients from different geographical locations. A study in China

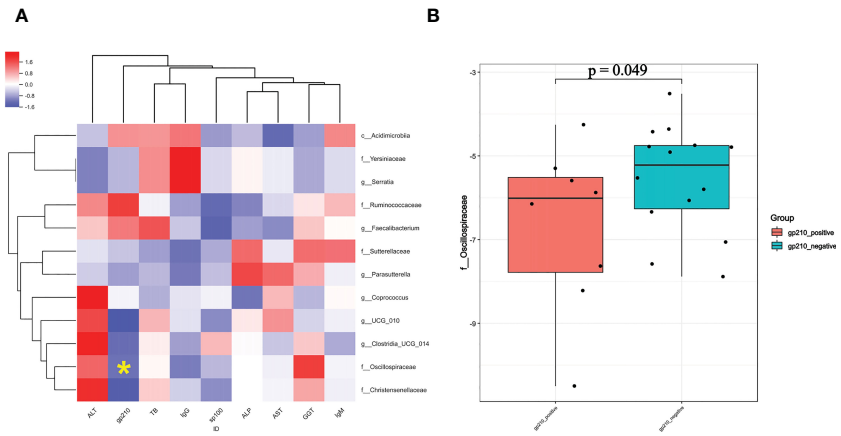

FIGURE 5
(A) Pearson correlation coefficients for the relationships of 12 gut genera (vertical axis) with 9 clinical indices (horizontal axis) in PBC patients (red: positive correlation, blue: negative correlation). **(B)** Level of Oscillospiraceae in PBC patients who were anti-gp-210 positive and anti-gp-210 negative. *enrichment of Oscillospiraceae was negatively related to anti-gp210 antibody status.

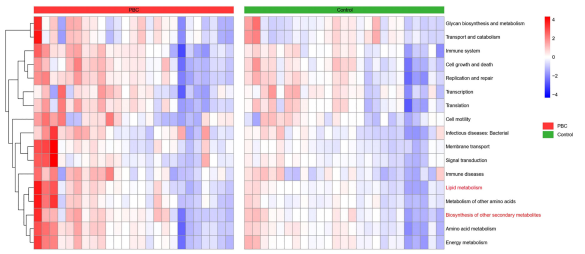

FIGURE 6
Heatmap of Spearman correlations between KEGG categories (vertical axis) and the levels of microbiome taxa (horizontal axis) in the PBC group (left) and the control group (right).

reported sequences from the gut microbiota of 394 healthy subjects from seven different cities and found that ethnicity and especially geographic location were the main factors affecting gut microbiota composition (20). The most enriched genus in our PBC patients was *Serratia*, but a study performed in Shanghai reported the most enriched genus in PBC patients was *Klebsiella (*2), and a study in Japan reported that *Lactobacillales* was the most enriched genus (12), although all of these genera are in the *Enterobacteriaceae*. A recent study in Shenzhen (southern China) showed that the normal bilirubin group of PBC patients had a lower abundance of *Gemmiger*, *Blautia*, *Anaerostipes*, and *Coprococcus* genera than a high bilirubin group of PBC, in which *Holdemania* was absent (21).Therefore, when screening gut flora for the diagnosis of PBC it is important to consider the geographical location of the patient.

PBC patients are diagnosed using a combination of biochemical markers, autoantibodies, and liver histopathology. Although most guidelines do not require liver histopathology for diagnosis, all PBC patients in our study received liver biopsies and assessment using the Ludwig staging system. All of our patients had Ludwig Stage I or II. There were several reasons for our focus on patients with early-stage PBC. First, the main aim of this study was to assess the value of gut microbiota composition as an early diagnostic marker for PBC. Second, patients with advanced-stage PBC are often hospitalized for various complications, especially due to portal hypertension, and often receive medications that can alter the gut microbiota. All of our PBC patients were treatment-naive and had no history of using UDCA. UDCA can reshape the bile acid profile (22) and affect the gut microbiota composition.

Our results indicated that PBC patients had 4 significantly up-regulated genera and 8 significantly down-regulated genera. Three microbiota alterations particularly attracted our attention.

First, *Serratia* and *Yersiniaceae* were significantly enriched in PBC patients. When there is an intestinal microecological imbalance, an immune response against bacterial antigens may also lead to attacks of structurally similar human antigens — an autoimmune-mediated injury (23). There is evidence that antibodies from patients with autoimmune liver diseases react with specific microbial proteins. For example, the AMAs from PBC patients bind to *Escherichia coli* proteins (24), and *Serratia*, *Yersiniaceae*, and *E. coli* are all in the *Enterobacterales*. Therefore, we speculate that a "mosaic effect" occurs (25)when the body creates antibodies against *Serratia*, *Yersiniaceae*, and their metabolites and these antibodies then mistakenly attack bile duct epithelial cells, manifesting as immune-mediated bile duct injury.

A second notable alterations is that the level of *Faecalibacterium* was significantly reduced in PBC patients, a finding apparently unique to PBC patients from Zhejiang Province. *Faecalibacterium* is a major producer of intestinal butyrate and plays a crucial role in maintaining intestinal homeostasis and host health (26). Butyrate is one of the main energy sources of colon cells, and is important for maintaining the integrity of the intestinal mucosal barrier (27). More specifically, butyrate strengthens the intestinal mucosal barrier by activating AMP-activated protein kinase (AMPK) to promote the expression of proteins that function in TJs (28). Butyrate also regulates the gut microbiota by modulating the pH of the intestinal lumen (29), which is beneficial for bacteria that produce short-chain fatty acids (30);it maintains epithelial hypoxia status and limits overgrowth of nitrate-respiratory-dependent bacteria (31); and it stimulates the growth of villi and the production of mucin (32). Therefore, we suggest that the significant deficiency of *Faecalibacterium* in PBC patients contributed to the increased permeability of the intestinal mucosal barrier, and the migration of many bacteria into the liver *via* the gut-liver axis, ultimately manifesting as immune-mediated damage to the small bile ducts.

A third notable alteration is that the level of *Ruminococcaceae* was significantly reduced in PBC patients, also apparently unique to PBC patients from Zhejiang Province. *Ruminococcaceae* plays an important role in the conversion of primary bile acids into secondary bile acids (33), and impaired production of secondary bile acids can cause dysbiosis of gut microbiota and induce intestinal inflammation (34). Sinha et al. (35) compared a control group that had familial adenomatous polyposis (FAP) with a group that had ulcerative colitis, and showed severe depletion of *Ruminococcaceae* in the ulcerative colitis patients. Bajaj et al. (36) reported increased serum levels of IL-6 and lipopolysaccharide-binding protein and a decreased butyrate/isobutyrate ratio in patients with alcoholic liver disease, and that these changes were associated with a depletion of *Ruminococcaceae*. Therefore, we speculate that the depletion of *Ruminococcaceae* in PBC patients decreases the conversion of primary bile acids into secondary bile acids, leading to interruption of enterohepatic circulation and aggravation of cholestasis, and eventually to intestinal inflammation and exacerbation of the dysbiosis of gut microbiota.

The results of our KEGG functional annotation analysis suggested that the intestinal flora of PBC patients had abnormal expression of two pathways: lipid metabolism and biosynthesis of secondary metabolites. Many anaerobic intestinal microbes, such as species in the *Ruminococcaceae*, *Coprococcus*, and *Oscillospirales* (all detected in our study) produce short-chain fatty acids (SCFAs) by fermentation of dietary fiber (37, 38). SCFAs have a beneficial effect on health due to their anti-inflammatory effects (39), consistent with our finding of abnormal expression of this pathway in PBC patients. Secondary metabolite synthesis by the intestinal flora in our PBC group was also abnormal. This indicates insufficient conversion of primary bile acids into secondary bile acids in these patients, a condition that can further aggravate cholestasis (40). Dietary supplementation with *Ruminococcaceae* is a potential method for promoting bile acid metabolism and ameliorating cholestasis (41).

Our study has certain shortcomings, especially the relatively small sample size. However, we were only able to enroll 25 PBC patients because the incidence of this condition is very low, and the number of PBC patients who are treatment-naive is much smaller. Second, UDCA is relatively safe, and many patients with cholestatic liver disease have already taken UDCA before receiving a definitive diagnosis of PBC. However, because UDCA affects the composition of the intestinal flora, we had to exclude these patients. Third, a liver biopsy is not necessary for the diagnosis of PBC, but we excluded PBC patients who were diagnosed without liver biopsy results because of our need to use strict inclusion criteria and to enroll patients with definitive diagnoses.

Our study provided a comprehensive comparison of the gut microbiota of treatment-naive PBC patients and matched healthy controls from Zhejiang Province. The results provide new insights into the pathogenesis of PBC and the possible use of non-invasive biomarkers for the diagnosis or stratification of PBC patients.

Data availability statement

The data presented in the study are deposited in the National Center for Biotechnology Information (NCBI) 16S rRNA database and the BioProject ID is PRJNA892581.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Hangzhou Xixi Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Study concept and design, acquisition of data, analysis and interpretation of data: Y-JZ, G-XY, S-LD. Drafting of the manuscript: Y-JZ, G-XY, BX. Critical revision of the manuscript for important intellectual content and study supervision: Q-FJ. All authors contributed to the article and approved the submitted version.

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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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The role of Nrf2 in the pathogenesis and treatment of ulcerative colitis

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Ulcerative colitis (UC) is a chronic inflammatory bowel disease involving mainly the colorectal mucosa and submucosa, the incidence of which has been on the rise in recent years. Nuclear factor erythroid 2-related factor 2 (Nrf2), known for its key function as a transcription factor, is pivotal in inducing antioxidant stress and regulating inflammatory responses. Numerous investigations have demonstrated the involvement of the Nrf2 pathway in maintaining the development and normal function of the intestine, the development of UC, and UC-related intestinal fibrosis and carcinogenesis; meanwhile, therapeutic agents targeting the Nrf2 pathway have been widely investigated. This paper reviews the research progress of the Nrf2 signaling pathway in UC.

KEYWORDS

Nrf2, ulcerative colitis, oxidative stress, intestinal fibrosis, colorectal cancer

1 Introduction

Ulcerative colitis (UC) is a nonspecific, chronic, relapsing inflammatory disorder mainly involving the mucosa and submucosa of the colorectum. The occurrence and frequency of UC have witnessed a continuous escalation in the progressive timeline of recent years (1). The disease has a long course and a wide range of lesions. Furthermore, it is essential to note that the principal clinical indications linked to this ailment encompass frequent diarrhea, excruciating abdominal pains, viscid mucus discharge, excrement that is conspicuously blood-stained, and even additional debilitating symptoms that cause a tremendous amount of distress and utterly corrode the quality of an individual's life (2). It is also paramount to comprehend that this unfortunate medical predicament might lead to a sequence of menacing and detestable complications, such as damage to the intestinal tract resulting in the development of intestinal fibrosis and ultimately culminating in the malignant and life-threatening ailment commonly known as colorectal cancer (1).

The underlying causes of ulcerative colitis are murky and complicated. Yet the evidence is mounting that oxidative stress and inflammation are also closely related, except in genetics, intestinal flora, host immune system, and environmental factors (2). An accumulating corpus of empirical data has demonstrated the significant contribution of oxidative stress in inciting

the inflammatory response that precipitates the onset of UC. Its multifaceted effects have impinged upon the disorder's progression (3). Dysregulation of the immune system and pronounced inflammatory response events elevate reactive oxygen species (ROS) levels within the organism, thus perturbing redox balance and precipitating oxidative stress. The ensuing upsurge in ROS levels and consequential enhancement of oxidative stress indices result in deleterious cumulative damage to the fundamental biomolecules. Additionally, the imbalance between oxidative and antioxidant systems facilitates the activation of oxidative stress-associated pathways, which mediate cellular senescence, apoptosis, and necrosis (4). Nuclear factor erythroid 2-related factor 2 (Nrf2), a crucial transcriptional regulator involved in redox homeostasis, exerts a pivotal role in facilitating antioxidant responses within the organism (5). The Keap1/Nrf2 signaling pathway is constituted by the principal modulator Nrf2 and its counteractive inhibitor Kelch-like ECH-associated protein 1 (Keap1). This signaling pathway has been confirmed to exert a safeguarding influence on animal models and individuals with ulcerative colitis. In this regard, the Keap1/Nrf2 signaling pathway is vital as an antioxidant defense mechanism (3).

This paper aims to provide a comprehensive overview of Nrf2, including its physiological configuration and function, its involvement in intestinal maintenance and development, and its research progress in addressing ulcerative colitis and related complications. Among multiple aspects of Nrf2, we also focus on the therapeutic applications of modulating the Keap1/Nrf2 pathway in ulcerative colitis.

2 The physiological structure and function of Nrf2

2.1 The physiological structure of Nrf2

Nrf2 was initially cloned from the human leukemia cell line (K562) and identified as a Cap-n-collar (CNC) alkaline leucine zipper transcription factor family member (6). Nrf2 consists of seven Neh domains (Nrf2-ECH homology), each with a different function. The Neh1 domain is characterized by a remarkably conserved basic region-leucine zipper (bZIP) architecture. The Neh2 region mediates interactions with Keap1 through DLG and ETGE motifs. The carboxyl terminus of Neh3 has been found to facilitate the regulation of the antioxidant response element (ARE)-mediated transcription through its association with the chromo ATPase/helicase DNA binding protein (CHD6) (7). Neh4 and Neh5 regions, on the other hand, are instrumental in initiating downstream gene transcription, which is crucial for the transactivation of Nrf2. Notably, the regulatory region of Neh6 is characterized by the prevalence of serine residues and is responsible for the regulation of Nrf2 degradation via a mechanism independent of KEAP1. Furthermore, retinoic acid X receptor alpha (RXRα) has been reported to reduce the cytoprotective effect of Nrf2 by directly binding to the Neh7 domain (8).

Keap1 acts as a substrate adaptor protein for the E3 ubiquitin ligase complex, which comprises Cullin3 (Cul3) and Rbx1 to form a functional E3 ubiquitin ligase complex (Keap1-Cul3-E3). This complex plays a crucial role in regulating the activity of Nrf2 (9). Keap1 contains five domains, namely N-terminal region (NTR), intervention region (IVR), Broad complex, Tramtrack and Bric-à-Brac region (BTB), diglycine repeat region (DGR), and C-terminal domain C (CTR). The DGR region, the Kelch region, is the Neh2 junction region of Keap1 and Nrf2 (10) (Figure 1).

2.2 Triggers for activation of the Nrf2 signaling pathway

2.2.1 Keap1-dependent activation of Nrf2 signaling pathway

Upon Nrf2 entering the nucleus, bZIP cooperates with small Maf proteins to form a heterodimer, enabling Nrf2 to recognize, bind to antioxidant response element (ARE), and initiate downstream related gene transcription (11). Under typical physiological circumstances, the BTB domain within the Keap1 protein interacts with the Cul3

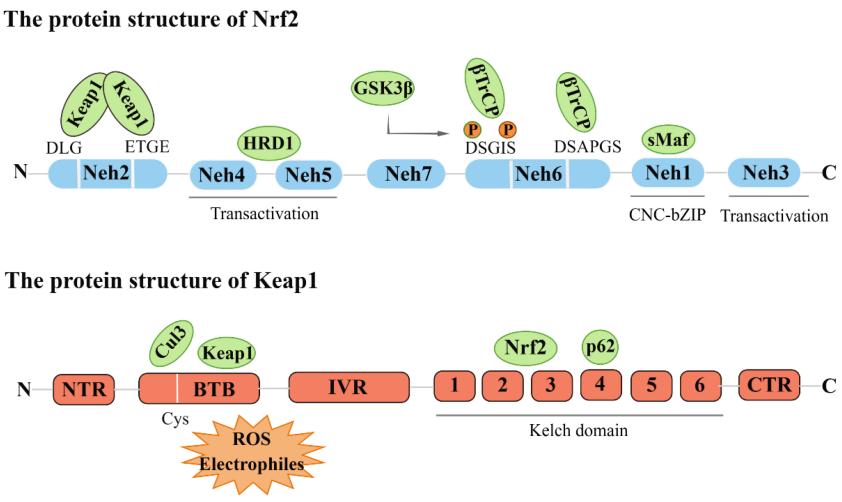

FIGURE 1
The protein structure of Nrf2 and Keap1.

protein. In contrast, the DGR domain targets various lysine residues in the Neh2 domain of Nrf2, thereby facilitating its ubiquitination. Consequently, the ubiquitinated Nrf2 undergoes degradation via the proteasome pathway (12). However, upon exposure to oxidative stress, particular cysteine residues within Keap1 undergo modification, which results in a structural alteration of the Keap1-Cul3-E3 ubiquitin ligase complex. This alteration disrupts the ubiquitination process of Nrf2, enabling its translocation into the nucleus. Subsequently, the nuclear Nrf2 forms a complex with sMaf proteins and binds to the ARE region. This binding event initiates a coordinated activation program that induces the expression of multiple cytoprotective genes, ultimately enhancing cellular defense mechanisms (13).

In addition to the Keap1/Nrf2 pathway, an atypical mechanism of Nrf2 activation, the autophagy-lysosome pathway, is driven by autophagy dysfunction, which also plays a crucial role in mediating oxidative stress (14, 15). The SQSTM1/p62 is a typical receptor for selective autophagy that degrades ubiquitinated substrates and plays a crucial role in regulating various signaling cascades, encompassing the Keap1/Nrf2 pathway (16). Dysregulation of autophagy processes culminates in the build-up of the autophagy-associated adaptor molecule SQSTM1/p62, consequently leading to the entrapment and subsequent functional impairment of various interacting proteins, including Keap1 (17). It has been reported that SQSTM1/p62 competes with Nrf2 for binding to Keap1, and this interaction can sequester Keap1 into autophagosomes, thereby preventing Keap1-mediated Nrf2 degradation and leading to Nrf2 pathway activation (18). Notably, the activation of Nrf2 must be tightly controlled. Over-accumulation or over-expression of Nrf2 has been reported to be detrimental and sometimes fatal (Figure 2).

2.2.2 Keap1-independent activation of Nrf2 signaling pathway

Protein kinases are important regulators of many cellular processes through the phosphorylation of specific proteins. Recent studies have highlighted the crucial role of protein kinases in the modulation of Nrf2 activity. Among them, protein kinase C (PKC) has been shown to play a significant role in the activation and expression of Nrf2. Salvianolic acid B has been demonstrated to induce the expression of Nrf2, HO-1, and GCLC by activating the PI3K and PKC pathways, thus providing protection against APAP-induced liver injury (19). Similarly, protocatechualdehyde has been found to protect the liver from APAP-induced injury through the protein kinase Cε/Nrf2/HO-1 pathway and to alleviate cerebral ischemia-reperfusion-induced oxidative injury (20). Further investigations have revealed that the phosphorylation of Nrf2 at Ser-40 by PKC represents a key signaling event leading to the activation of ARE-mediated cellular antioxidant responses (21). Different isoforms of PKC may also play a role in mediating the phosphorylation of Nrf2 (22). Specifically, Chen et al. identified PKC-δ as the major PKC isoform responsible for the phosphorylation of Nrf2 Ser40, whereas PKC α and β activate Nrf2 at different time points, namely early and late stages, respectively (23).

Recent studies have highlighted the beneficial impact of AMP-activated protein kinase (AMPK) on the activation of Nrf2 and the subsequent induction of antioxidant enzymes in response to oxidative stress (24). AMPK has been found to trigger the expression of SOD and HO-1 via the Nrf2/ARE signaling pathway, leading to enhanced cellular antioxidant capacity and improved cell survival (25). Furthermore, AMPK has been shown to stimulate the nuclear accumulation of Nrf2 through phosphorylation at serine 550 (26).

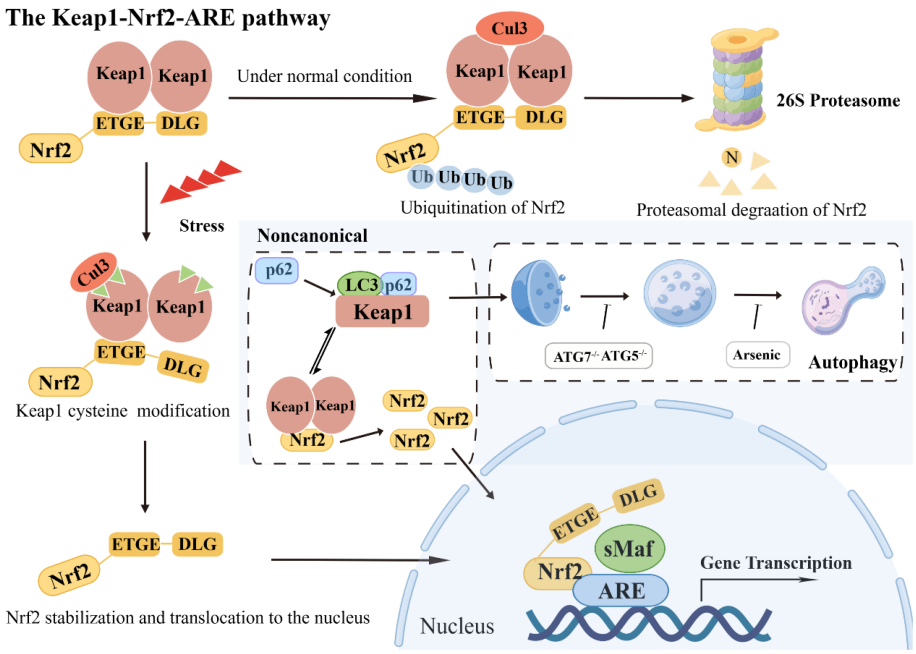

FIGURE 2
The Keap1-Nrf2-ARE pathway: classical and noncanonical pathways.

However, the interplay between AMPK and Nrf2 transcription warrants further investigation to better understand the underlying mechanisms of this signaling pathway.

Moreover, three Mitogen-activated protein kinases (MAPKs) have been identified as playing a role in the modulation of Nrf2 activity. Extracellular-signal-regulated kinases 1 and 2 (ERK1/2) are involved in both cell proliferation and defense mechanisms. Senkyunolide I has been found to protect the rat brain against focal cerebral ischemia-reperfusion injury by upregulating p-Erk1/2, Nrf2/HO-1, and inhibiting caspase 3 (27). Similarly, Gastrodin has been shown to safeguard the midbrain from oxidative stress in mice by blocking the ERK1/2-Nrf2 pathway (28). In addition, c-Jun N-terminal kinases (JNKs) and p38 MAPK are crucial mediators of oxidative stress reduction. Research indicates that Protocatechuic acid induces the expression of antioxidant/detoxification enzymes via JNK-mediated Nrf2 activation in mouse macrophages (29). Moreover, Ma et al. have identified p38 MAPK/Nrf2 signaling as a critical molecular network responsible for the development of temozolomide resistance in gliomas (30). Furthermore, Diallyl trisulfide has been shown to inhibit tumor growth by suppressing Nrf2/Akt activity and activating p38/JNK signaling (31). Meanwhile, Qi et al. have demonstrated that the activation and interplay between PI3K/Akt and Nrf2/HO-1 signaling pathways may play a role in regulating the hormesis of Z-ligustilide in PC12 cells subjected to oxygen and glucose deprivation (32). Similarly, reactive oxygen species and PI3K/Akt signaling have been shown to be key factors driving Nrf2-mediated heme oxygenase-1 expression in sulforaphane-treated human mesothelioma MSTO-211H cells (33). In conclusion, further investigation is necessary to gain a deeper understanding of the Keap1-independent Nrf2 pathway and its activation.

3 The role of Nrf2 in intestine development

The transcriptional activity of Nrf2 is essential for maintaining normal intestinal architecture in mice (34). The mouse intestine begins to establish around 9.5 days after conception. During the 9.5-14.5 days of embryonic development, pure epithelial cells transform into endodermal tubes, and the length and circumference of the intestine gradually increase (35). Subsequently, differentiated cells of absorptive and secretory lineages appeared from day 14.5, and epithelial remodeling and transient villi appeared in the gut on day 15. During 14.5-21 days of embryonic development, intestinal cells proliferate, and crypts and stem cell nests gradually form (36). Most colonic structures and cellular aggregates in adults exist prenatally, with fully developed colonic crypts manifested within 12-15 days following birth (35).

Intestinal development is mainly driven by the coordination between Notch and Wnt signaling pathways, and it is worth noting that Nrf2 can affect the activation of these signaling pathways (37). Studies have validated the significance of intercommunication between the Nrf2 and Notch pathways in regulating gastrointestinal tract maturation (38). Research in this area has shown that the proximal region of the promoter of the Notch downstream effector Math1 gene in mice has a functional ARE sequence, and the activation of Nrf2 signaling in the intestinal epithelium can lead to intestinal elongation and extension through negative transcriptional regulation of the Notch downstream effector Math1 (39). Meanwhile, Nrf2 knockout mouse embryonic fibroblasts decreased the expression of Notch-1 and its related gene signaling (40). There is a delicate balance between the Wnt and Nrf2 signaling pathways. A study confirmed that β-catenin in the Wnt pathway could activate the Nrf2 pathway to a certain extent, and Nrf2 could strongly inhibit β-catenin. Moreover, β-TrCP1 binds to β-catenin to weaken the inhibition effect of Nrf2 on β-catenin (41).

Twenty-five years ago, Chen et al. showed that the Nrf2 gene is highly expressed on the luminal side of the intestine during pregnancy in mice. In addition, they also observed substantial variations in Nrf2 mRNA levels across different organs and gestational days (42). A recent study has shown that Nrf2 levels continue to rise in the hindgut from 14.5 to 18.5 days of embryonic development, while Nrf2 levels decrease in other tissues, such as the lung or heart suggesting that Nrf2 plays a crucial role in intestine development (34). Nrf2 transcriptional deletion resulted in marked elongation of the colon, altered crypt distribution, enlarged goblet cells, and markedly elevated mucin levels (43). Thus, Nrf2 transcriptional activity constitutes an integral regulatory mechanism in the formation of the gastrointestinal tract, modulating the proliferation and differentiation of hindgut cells throughout varied embryonic stages.

4 The role of Nrf2 in ulcerative colitis

4.1 Nrf2 attenuates intestinal inflammation and damage by controlling oxidative stress

Oxidative stress results from dissonance amidst the production and clearance of ROS and reactive nitrogen species (RNS) following the body's exposure to diverse injurious stimuli, which causes the body's oxidation and oxidation antioxidant system imbalance. The antioxidant defense function is weakened, resulting in various pathological changes (44). Under physiological conditions, oxidation and anti-oxidation maintain a dynamic balance in the body. However, when inflammation occurs in the body, this balance is broken. Oxygen free radicals attack their tissues, participate in and generate inflammatory mediators through lipid peroxidation, and activate the inflammatory response (45).

Impairment of the antioxidant defense machinery in the gastrointestinal tract has been implicated in UC etiology. Infiltration of inflammatory cells exacerbates oxidative stress via the upregulation of ROS synthesis in the immune cells (46). Insurmountable ROS release, coupled with the persistent accumulation of oxidative stress, frequently culminates in DNA damage, protein oxidation, and lipid peroxidation, ultimately causing intestinal tissue damage, debilitating the immune system, and precipitating an array of severe pathologies, including UC (47). When UC occurs due to the inflammatory response, the activity of gut-derived vasoconstrictors is enhanced, resulting in intestinal ischemia. At the same time, inflammatory cells in the intestinal mucosa, such as neutrophils and macrophages, enter the intestinal tract from the blood circulation (48). In the damaged part, under the action of cell membrane reduced coenzyme II and NADPH

oxidase, a large number of reactive oxygen radicals such as superoxide anion (H:0), hydrogen peroxide (H2O2), hydroxyl radical (H01), NO free radicals and lipid peroxides (LPO), thereby aggravating intestinal mucosal damage (47, 49).

Cells have evolved a complex protective system to defend against the damage mentioned above, and the Keap1/Nrf2 pathway is the foremost defense mechanism for counteracting oxidative stress (13). The initial research investigating the alleged involvement of Nrf2 in UC was reported first by Arisawa et al. in 2008. They identified that the -686*-684 genotype of the Nrf2 gene was significantly correlated with UC incidence among the Japanese population and closely associated with the chronic persistent phenotype (50). Recent research has established that the expression level of Nrf2 in individuals with UC is lower than in healthy cohorts. Nonetheless, some studies have also revealed a marked increase in the expression level of Nrf2 in the mucosa of the inflammatory intestinal tract among UC patients compared to controls (51, 52). Moreover, Milad's research demonstrated that the phosphorylated form of Nrf2 was expressed at a significantly higher level in individuals diagnosed with moderate and severe UC than in healthy controls. Conversely, the expression level of non-phosphorylated Nrf2 was diminished in moderate to severe UC patients compared to healthy cohorts. These observations suggest that the altered levels of Nrf2 in UC patients may be influenced by the disease's progression or the form of Nrf2 expression (51).

Over the years, related studies on the involvement of the Keap1/Nrf2 axis in the process of UC have found that mice lacking Nrf2 exhibit heightened vulnerability to dextran sodium sulfate (DSS)-induced colitis and an increased susceptibility to colorectal cancer (53–55). Furthermore, compared with wild-type mice, the levels of pro-inflammatory cytokines and lipid peroxidation in the colon of Nrf2 knockout mice treated with DSS were significantly increased, and the expression levels of antioxidant enzymes were decreased (56). Moreover, multiple genetic mutations on Nrf2 have been linked to increased susceptibility and progression of DSS-induced colitis in mice (57, 58). Considering these discoveries, modulation of the Keap1/Nrf2 signaling pathway could represent a promising avenue for managing UC.

In UC, the Keap1/Nrf2 pathway can reduce intestinal inflammation and injury by controlling oxidative stress and play an essential role in protecting intestinal integrity, mainly by regulating inflammatory mediators and inducing the production of antioxidant enzymes (59, 60). Oxidative stress provokes Nrf2 to translocate to the nucleus, where it mediates the transcription of a diverse suite of antioxidant genes, thereby conferring cell protection against the damage induced by oxidative stress (61). In addition, various antioxidant enzymes in the body, such as superoxide dismutase (SOD), glutathione (GSH), and other activations, establish an endogenous defense system against intestinal oxidative stress, protecting the intestinal mucosa from harmful stimuli (62). Currently, most studies focus on regulating inflammatory mediators, inducing the production of antioxidant enzymes, and regulating autophagy by activating Nrf2, thereby reducing oxidative stress damage caused by aggravated ROS and alleviating pathological inflammatory responses (63–66).

In addition, activation of Nrf2 can balance cellular homeostasis, activating a series of signaling pathways targeting inflammation, such as the NF-κB pathway. Numerous studies have shown an interaction between the Keap1/Nrf2 and NF-κB pathways (67, 68). First, sMaf (MafK) can positively regulate NF-κB activity by enhancing the Nrf2 transcriptional coactivator CBP-mediated acetylation of NF-κB p65, suggesting that Nrf2 may indirectly regulate NF-κB activity by inhibiting MafK. Second, Keap1 can inhibit the activation of NF-κB by inhibiting the ubiquitination degradation of IKKβ. Third, the inflammatory response can inhibit NF-κB activity by inducing inflammatory mediators and subsequently reacting with Keap1 to activate the expression of Nrf2 (69, 70). In conclusion, activation of Nrf2 in the gut can inhibit inflammatory pathways or reduce the overreaction of oxidative stress, thereby alleviating intestinal damage and inflammation.

However, we still need to control the expression of Nrf2 strictly. Recently, in a study of transgenic mice constitutively expressing active Nrf2 (caNrf2 mice), Gerstgrer et al. found that symptoms of acute colitis induced by DSS were exacerbated after constitutive Nrf2 expression but not worsened chronic colonic mucosal inflammation (71). The above phenomenon suggests that the redox balance in the body needs to be strictly regulated. Otherwise, the double-edged sword effect is prone to occur. Meanwhile, further extensive studies are therefore imperative for an enhanced understanding of the complex interplay between the Keap1/Nrf2 signaling network and oxidative stress as well as inflammation in the intestine.

4.2 Nrf2 facilitates the maintenance of the intestinal epithelial barrier

The intestine is an important digestive organ of the human body and the largest immune organ of the body. The intestinal epithelium forms a tightly regulated intestinal barrier between the external environment and the body, which can prevent the invasion of harmful substances such as pathogenic bacteria and toxins. It is indispensable in maintaining homeostasis and body health (72, 73).

The intestinal mucosal barrier is a multifaceted structure comprising the surface mucous layer, epithelial cell layer, and mucosal basal layer, alongside the biological barrier facilitated by the resident microflora of the gut. Furthermore, it encompasses a chemical barrier consisting of a range of digestive enzymatic secretions, lysozymes, mucopolysaccharides, glycoproteins, and glycolipids produced by the intestine, an immune barrier that is formed by intestinal associated lymphoid tissue (GALT) and secretory immunoglobulin A (sIgA), and a mechanical barrier consisting of intact intestinal mucosal epithelial cells and intercellular junctions (74–76).

The pivotal role of the intestinal mucosal barrier is attributed to its mechanical barrier, in which epithelial cells and their intercellular junctions are the structural basis for maintaining intestinal epithelial selective permeability and barrier function. It is the key to resisting the invasion of extraintestinal harmful substances or pathogens into the intestinal mucosa (77, 78). The junction between intestinal epithelial cells is the core part of the mechanical barrier. It is controlled by the myosin light chain (MIC), including tight junctions, gap junctions, adhesion junctions, and

desmosome junctions, especially tight junctions, mainly composed of occlusal junctions. The constituents of this barrier include members of the tight junction protein group, such as occludin, claudin, and cadherin, as well as the Zonula Occludens (ZO) family (79, 80). The tight junctions between intact intestinal epithelial cells can prevent intestinal bacteria, toxins, and antigens from entering the lamina propria, prevent the activation of lamina propria immune cells, and induce abnormal intestinal immune responses (81, 82).

A key pathological event in UC is intestinal barrier dysfunction. The destruction of the intestinal barrier will increase intestinal permeability, and intestinal pathogenic bacteria and pathogens will further invade the intestinal mucosa, thereby exacerbating inflammatory cell infiltration and damage, forming a vicious circle, which eventually leads to damage to the intestinal mucosa and ulcer formation (83, 84). Current research confirms the indispensable involvement of Nrf2 in preventing UC. Activation of Nrf2 in animal models of UC exerts a regulatory effect on the expression of tight junction proteins located in the intestinal epithelium, specifically Zonula Occludens-1 (ZO-1) and claudin, thus safeguarding the integrity of the gut barrier (85–87). In DSS-induced mouse colitis models, tight junction protein expression was significantly lower than in the control group, resulting in increased intestinal permeability (88, 89). In the LPS-induced intestinal barrier damage model, the mitochondria-targeted antioxidant MitoQ can prevent intestinal barrier damage by upregulating the expression of Nrf2 downstream regulatory genes. The mechanism may involve activating the Keap1/Nrf2/ARE signaling pathway and inhibiting oxidative stress (90). Concurrently, the study revealed that Nrf2 confers a safeguarding effect in a traumatic brain injury-induced intestinal mucosal injury model. In contrast to wild-type mice, Nrf2-deficient mice demonstrated enhanced susceptibility to traumatic brain injury-induced intestinal inflammation, characterized by elevated intestinal permeability and augmented plasma endotoxin levels, exacerbating the decline in intestinal barrier function (91). Another study in the same model confirmed that ERK/Nrf2/HO-1-mediated stimulation of mitophagy ameliorates intestinal mucosal impairment and barrier dysfunction (92). Furthermore, Nrf2 activation is purported to enhance the protection of tight junction proteins by negating apoptosis of intestinal epithelial cells while concurrently instigating autophagy. In addition, in a study of reflux esophagitis, Nrf2 was found to bind to the promoter of claudin-4 and increase its expression but not to claudin-1. Nrf2 deficiency leads to mitochondrial dysfunction, downregulating claudin-4 expression and ultimately leading to tight junction damage in the esophageal epithelium (93). Further research has demonstrated that the inducement of the Keap1/Nrf2 pathway impacts the regulation of tight junction proteins and governs the regenerative processes of intestinal stem cells, thereby promoting intestinal homeostasis (94, 95).

The intestinal mucus barrier also plays an integral role in maintaining gut health (96). Intestinal mucus is a high-molecular glycoprotein mucus layer secreted by goblet cells, which forms a functional barrier between intestinal microbes and the intestinal epithelium, providing defense by hindering the direct contact of bacteria and harmful substances to the intestinal epithelium (97). Research has documented that diminished levels of intestinal mucus can hinder nutrient absorption through the intestinal mucosa while simultaneously triggering secretion of water and electrolytes within the intestinal lumen. At the same time, plasma-like fluid penetrates further into the lumen, increasing the volume of fluid and the permeation load in the lumen and causing diarrhea (98). Moreover, the study found that Low molecular Seleno-amino polysaccharide (LSA) can protect the intestinal mucosal barrier in rats by activating the Nrf2 pathway and mitigating the anomalous alterations of MUC2 (99). In addition, Singh and his team have found that gut microbial metabolism enhances the integrity of the intestinal barrier through the Nrf2 pathway (59). To conclude, the activation of Nrf2 aids in preserving the integrity of the intestinal epithelial barrier, while further investigations are required to elucidate the underlying mechanism involved.

4.3 Nrf2 is the regulator of intestinal immunity

The abnormal intestinal mucosal immune system is one of the main reasons for the development of UC. Meanwhile, initiating intestinal mucosal immunity and ensuing inflammation hinges upon activating effector T cells. In response to a plethora of stimuli, an array of effector T cell subsets emerge from precursor naive T cells, including Th1, Th2, Th17, and Treg cells (100, 101).

In recent years, in addition to the anti-inflammatory mentioned above and oxidative stress control effects of Nrf2, numerous research endeavors have additionally evidenced that Nrf2 activation holds the potential to modulate the Th1/Th2 equilibrium selectively. In 2012, Rockwell and his team's research found that the induction of Nrf2 can preferentially direct CD4+ T cells toward Th2 differentiation. Specifically, the activation of Nrf2 by tBHQ, a food preservative, has been observed to hinder the production of the Th1 cytokine IFN-γ and simultaneously encourage the generation of Th2 cytokines such as IL-4, IL-5, and IL-13 (102). Three years later, the team found that the Nrf2 activator tBHQ inhibited the production of IL-2 and IFN-γ in activated $CD4^+$ T cells (103). Five years later, the team continued to investigate the effects of Nrf2 activation on the initial events that follow T-cell activation. The results showed that different Nrf2 activators (tBHQ and CDDO-IM) had different effects on early T cell differentiation, and the activation of some cytokines did not depend on the Nrf2 pathway. Although Nrf2 inhibited the expression of early TNF-α and IFN-γ after activation, it was observed to facilitate the generation of IL-2. It displayed no discernible effect on the induction of CD25 and CD69. IL-2 serves as a growth factor for T cells that promotes the development of Th2 cells and is critical for the differentiation and function of Treg cells (104). Other recent studies

on tBHQ also confirmed that tBHQ attenuated 5-fluorouracil-induced intestinal epithelial cell injury by activating Nrf2 (105). Moreover, tBHQ induced an Nrf2-dependent increase in IgM secretion by LPS-stimulated B cells (106). Meanwhile, studies have shown that Nrf2 can regulate the IL-22 response in $CD4^+$ T cells through the AhR pathway (107). This suggests that activation of Nrf2 has a relevant role in regulating both T and B cells.

Another study focused on dimethyl fumarate (DMF). This Nrf2 activator demonstrated that DMF could reduce the inflammatory response in experimental colitis, mainly due to the activation of Nrf2 and its downstream antioxidant genes expression after administration of DMF and simultaneously inhibit the NF-κB signaling (108). A research investigation exploring acute graft-versus-host disease (AGVHD) unveiled that activation of Nrf2 by DMF promoted the development of donor Treg cells and reduced the deleterious response of allogeneic T cells (109). Although the activation of Nrf2 modulates intestinal immunity to varying degrees, the exact mechanism has not been fully elucidated (Figure 3).

5 The role of Nrf2 in intestinal fibrosis in UC

The accumulation of excessive extracellular matrix caused by chronic inflammation is a characteristic feature of intestinal fibrosis, which is a prevalent complication found in patients with Crohn's disease (CD) and has more recently been observed in those with UC (110). During UC, ECM accumulates in the mucosa and submucosa, leading to its thickening, which results in the shortening and stiffening of the colon (111). Furthermore, fibrosis in UC is notably intertwined with inflammation and the disruption of the epithelial layer, often attributed to damage inflicted on the tight junctions (112).

The abnormal accumulation of ECM characterized by fibrosis may be due to overproduction or reduced degradation of ECM. Despite recognizing the TGF-β1/SMADs signaling pathway as the chief driving force behind intestinal fibrosis, numerous pro- and anti-fibrotic endogenous factors, such as ROS and Nrf2, have been identified as interactors of this pathway (113, 114). Several studies have shown that Nrf2 exerts an anti-fibrotic effect in various organs and that this protective effect is closely related to the classical pathway of fibrosis, TGF-β1/SMADs (115–117).

TGF-β1 and its receptor are highly expressed in animal models of fibrous stenosis and intestinal fibrosis. They can signal to the downstream Smads family of proteins, promoting extracellular matrix protein deposition and fibroblast transformation, thereby accelerating fibrosis (118, 119). At the same time, TGF-β1 promotes the generation of ROS while concurrently obstructing the activity of antioxidant enzymes, thereby creating an asymmetry within the redox homeostasis system, while ROS is also an essential mediator in activating the TGF-β1/Smads pathway (120). Therefore, redox disorders caused by dysregulation of the Nrf2 pathway induce fibrosis through massive ROS production and activation of the TGF-β1/Smads pathway (113). Guan et al. showed that tBHQ reduced fibrosis in mice with chronic fibrous colitis and human intestinal fibroblasts by inhibiting the TGF-β1/Smads signaling pathway. At the same time, $Nrf2^{-/-}$ promoted TGF-β1-induced intestinal fibroblast differentiation by pretreating human intestinal fibroblasts with tBHQ or siNrf2. $Nrf2^{-/-}$ could promote TGF-β1-induced intestinal fibroblast differentiation (114). Thus, the inhibition of intestinal fibrosis by Nrf2 is accomplished through

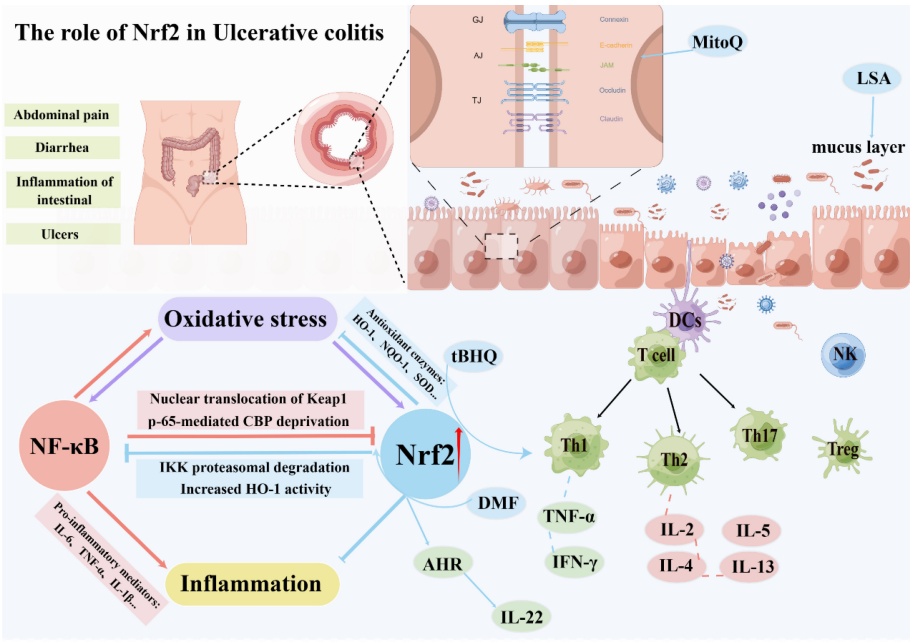

FIGURE 3
The role of Nrf2 in ulcerative colitis.

the ROS/TGF-β1/Smads pathway, which has been demonstrated in both *in vitro* and *in vivo*.

Parallel results were observed in CCD-18Co, a type of normal human colonic fibroblasts, following stimulation with TGF-β1. Suppression of Nrf2 amplified the expression of the TGF-β1/Smad signaling cascade in CCD-18Co fibroblasts (116, 121, 122). These findings highlight the potential of Nrf2 activation in constraining the TGF-β1/Smad signaling axis and its consequent alleviation of intestinal fibrosis. Among colon-derived CCD-18Co fibroblasts, Nrf2 equipped itself to tone down intestinal fibrosis by putting the brakes on the ROS-dependent TGF-β1 signaling pathway, eliciting ROS scavenging (114, 116, 123).

MMPs and TIMPs regulate the degradation of the extracellular matrix. An irregularity in the functioning of these specific enzymes results in the accumulation of extracellular matrix (ECM), thereby contributing to the development of fibrosis (124, 125). Among the myriad matrix metalloproteinases (MMPs), MMP7 is the most critical component within the intestinal context. Research has demonstrated that in human intestinal epithelial cells, the Nrf2/HO-1 axis effectively suppresses MMP7 activity. Reducing fibrosis by specifically inhibiting MMP7 through modulation of the Nrf2 signaling pathway may greatly benefit the treatment of IBD (113, 126). In addition, growing evidence elucidates the role of MMP-3 in IBD.MMP3 concentrations have been observed to increase in response to oxidative stress, and both its expression and activity exhibit augmentation in mice lacking Nrf2. Significantly, patients diagnosed with CD and UC exhibit elevated levels of MMP-3 (127, 128). Furthermore, research has highlighted that MMP3 concentrations are pivotal in determining the responsiveness to infliximab therapy among individuals diagnosed with IBD. Those who did not respond within one year had significantly higher serum MMP3 levels than controls (129). Furthermore, MMP3 possesses the discriminatory capacity for differentiating pediatric patients with UC from their healthy counterparts (130). The implications of MMP3 in regulating intestinal barrier functionality have been comprehensively examined and elucidated by Giuffrida et al. (131). The above findings reveal new therapeutic strategies to modulate Nrf2 signaling to re-establish cellular homeostasis in intestinal fibrosis.

6 Two-sidedness of Nrf2 in UC-associated colorectal cancer

Colorectal cancer (CRC) is a frequently occurring malignancy with a substantial global burden, characterized by elevated incidence and mortality rates. Part of it is caused by chronic colitis, called colitis-related colon cancer (2). Numerous epidemiological, experimental pathological, and clinical studies have shown that the prolonged presence of inflammatory bowel disease, especially chronic ulcerative colitis, can lead to malignant transformation into colon cancer and even promote the progression and early metastasis of colon cancer (132). Studies have reported that UC is a precancerous lesion of colorectal cancer and that the prevalence of CRC in patients with UC is two to six times higher than in the general population, with the incidence increasing with the number of years of diagnosis (132).

As a classical pathway for cellular defense and survival signaling, the role of Nrf2 in tumors has been of great interest. In recent studies, evidence has emerged indicating that Nrf2 plays a role in the carcinogenic transformation of UC. In patients with active ulcerative colitis, a complex ROS-rich microenvironment consisting of cytokines, chemokines, and inflammatory cells in the inflammatory state of the intestine is a major factor in promoting the cancerous transformation of UC (3, 126). It is thought that Nrf2 plays a dual and controversial role in developing and progressing colorectal cancer associated with colitis (Figure 4).

Nrf2 has a protective role in the early stages of CRC development, and ensuring the appropriate basal regulation of Nrf2 is crucial for averting carcinogenesis in colon tissues. In colon cells, activation of Nrf2 protects colon cells from damage by reducing genotoxic damage produced by oxidative stress, thereby inhibiting colon cancer progression (133). Existing research suggests that when colon cells are subjected to dextran sodium sulfate, it leads to the induction of carcinogenesis in the colonic tissue. At the same time, the addition of CPUY192018, a Keap1-Nrf2-PPI signaling pathway inhibitor, activates Nrf2 to reduce the risk of conversion of ulcerative enteritis to colon cancer (134). In addition, digitalis flavonoids can activate Nrf2 via p38 MAPK, promote Nrf2 nuclear translocation, and stimulate the expression of downstream phase II detoxification enzymes. This process safeguards colonic cells from oxidative stress and subsequently decreases the occurrence of AOM-DSS-induced colorectal cancer (135). Conversely, the absence of Nrf2 regulation compromises the cellular capacity to withstand genotoxic and oxidative stress, resulting in a disrupted intestinal microenvironment, and normal colon cells become more susceptible to damage from oxidative stress and various genotoxic compounds. The study showed that Nrf2 knockout mice and mice normally expressing the Nrf2 gene was exposed to both the colitis-inducing agent DSS and the colon cancer-inducing agent AOM to observe the risk of colon cancer in both groups. The results showed that Nrf2 knockout mice exposed to DSS or AOM developed prolapse, rectal bleeding, and inflammation and increased the number of abnormal crypt foci, indicating that Nrf2 knockouts are more susceptible to colitis or colorectal cancer (53, 54, 136). In addition, the presence of single nucleotide polymorphisms (SNPs) within the promoter region of the Nrf2 gene is correlated with an elevated risk of neoplastic development, and Nrf2-silenced mice showed significantly higher levels of neoplastic damage and tumorigenicity than wild-type mice, as well as significantly higher levels of 8-hydroxydeoxyguanosine in the epithelial DNA of mice. This suggests that identifying molecules that maintain a constant state of Nrf2 could help prevent cancer development at an early stage (137).

Nrf2 has been implicated in the processes of cancer cell proliferation, metastasis, and resistance to radiotherapy during advanced tumor stages. Although Nrf2 was initially identified as a tumor suppressor owing to its protective function against

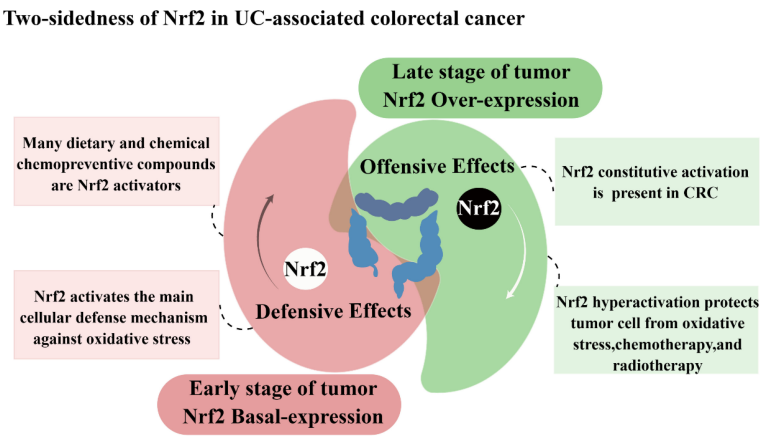

FIGURE 4
Two-sidedness of Nrf2 in UC-associated colorectal cancer.

exogenous and endogenous damage. However, mounting evidence suggests that the over-activation of the Nrf2 pathway facilitates tumor cell survival and protects them from oxidative stress and drug effects. Nrf2 is now thought to accelerate tumor progression, promote metastasis and participate in resistance to radiotherapy in advanced tumor stages.

Nrf2 overexpression can promote the development of colon cancer. One study found that colon cells were subjected to oxidative stress and produced excess ROS, which induced Nrf2 overexpression, leading to inflammation of colon tissue and promoting colon carcinogenesis (138). Arlt and colleagues discovered that heightened Nrf2 expression in colon cancer cells and augmented expression of proteasomal subunit proteins S5a/PSMD4 and α-5/PSMA5 increased proteasomal activity provided anti-apoptotic protection and adequate clearance of abnormal proteins in cancer cells and promoted colon carcinogenesis (139). Sebens et al. found that co-culture of M1-type macrophages with colonic epithelial cells activated Nrf2 expression and proteasome activity in colonic epithelial cells, rendering colon cells resistant to apoptosis, promoting inflammatory carcinogenesis, and increasing the risk of colon carcinogenesis (140, 141).

Nrf2 exhibits a strong association with tumor metastasis. Research has demonstrated that Nrf2 is potentially one of the indicative markers related to metastatic tumor processes. Nrf2 is highly expressed in highly invasive colorectal cancer tissues, and its expression is positively correlated with Duke's stage and clinical prognosis, making it an important marker of colon cancer prognosis (142). Nrf2 regulates colon cancer metastasis by regulating vascular endothelial growth factor (VEGF) and its receptors. Neoangiogenesis is a critical factor contributing to the growth of colorectal cancer tissues. VEGF and its receptor display elevated activity levels during early and advanced (metastatic) stages of colon cancer. At the same time, VEGF family proteins and receptors trigger multiple signaling networks that cause endothelial cell survival, mitosis, and migration (143). One study found that inhibition of Nrf2 blocked the accumulation of HIF-1α in colon cancer cells under hypoxic conditions and inhibited the expression of VEGF and HIF-1α target genes while reducing the growth and angiogenesis of xenograft tumors in mice (144). Currently, anti-VEGF/VEGFR therapy is essential in treating metastatic colon cancer, improving progression-free survival (FPS) and overall survival (OS) in patients with colon cancer (145). Thus, the suppression of Nrf2 may potentially manifest inhibitory effects on the metastasis of colon cancer, thereby highlighting its prospective significance as a target for the treatment of metastatic colon cancer.

Nrf2 plays a vital role in colon cancer chemoresistance. Nrf2, an essential transcriptional regulator of oxidative stress, protects cells from oxidative stress and toxic damage from chemical drugs. However, many studies have reported that persistent overexpression of Nrf2 causes increased resistance of cancer cells to chemotherapeutic drugs, including adriamycin, etoposide, and cisplatin, suggesting that Nrf2 is an important transcription factor for tumor drug resistance (146). 5-Fluorouracil (5-FU) is the most commonly used chemotherapeutic agent in the treatment of colon cancer, but the development of resistance to 5-FU has dramatically reduced its clinical efficacy. The chemotherapeutic agent 5-FU exerts cytotoxic effects on colon cancer cells by inducing the generation of ROS, consequently causing oxidative damage and ensuing cell death. Nonetheless, tumor stem cells (CSCs), a subgroup of colon cancer cells, can counteract 5-FU-induced oxidative damage in colon cancer cells by producing an adaptive cellular response to ROS, closely related to Nrf2 activation, which causes upregulation of antioxidant enzymes and increases cancer cell resistance to 5-FU (147, 148). It was found that FoxO3 overexpression increased the sensitivity of colon cancer cells SW620 and HCT-8 to 5-FU and that the reversal of resistance of human colorectal cancer cells to 5-FU was demonstrated through the involvement of FoxO3 in the inhibition of the Nrf2/TR1 signaling pathway (149). In addition, Kang et al. found that the mechanism of 5-

FU resistance in colon cancer was associated with epigenetic modifications such as DNA demethylation upregulating Nrf2 and HO-1 expression. By comparing the epigenetic changes associated with Nrf2 induction in the 5-FU-resistant colon cancer cell line SNUC5, it was concluded that Nrf2 expression, as well as its nuclear translocation and promoter binding, were markedly elevated in SNUC5/5-FUR cells compared to SNUC5 cells, and further Nrf2 or HO-1 knockdown mediated by siRNA considerably curtailed the proliferation of colon cancer cells both *in vitro* and *in vivo*, leading to heightened sensitivity to 5-FU (150). Cheng et al. demonstrated that cNrf2 exhibited resistance towards 5-FU and oxaliplatin, both *in vitro*, using the HCT116 cell line, and *in vivo*, employing the CRC animal model. This resistance was attributed to the PSMD4-mediated nuclear export of Nrf2, which ultimately activated the NF-κB/AKT/β-catenin cascade, further supporting these findings (151). In addition, cNrf2 and PSMD4-positive CRC patients had a higher rate of chemoresistance.

Nrf2 also plays a key role in promoting resistance to other chemotherapeutic agents in colon cancer. The significant abatement in SW480/Res cell migration and increased induction of apoptosis by oxaliplatin was observed upon inhibition of Nrf2 in colon cancer cells (152). In addition, Nrf2 reduced the sensitivity of NCM460 or Colo320 cells to TRAIL/etoposide by inducing proteasome activity, thereby reducing the apoptosis induced, and tissue immunostaining further confirmed the activation of Nrf2 in the colonic epithelium in the inflammatory region, as well as the increased proteasome expression (140)a. These studies suggest that Nrf2 has an important influence on the development of chemoresistance in colon cancer.

Nrf2 co-regulates CRC progression through interactions with other signaling pathways. In CRC, Nrf2 enhances NF-κB transcriptional activity, which is strongly associated with CRC cell invasion; positive and negative regulation of NF-κB and Nrf2 signaling pathways coexist, which may be closely linked to cell type and tissue microenvironment (153). At the same time, it has been found that Keap1 mutations lead to impairment of the Nrf2-Keap1-ARE signaling pathway, affecting its binding to Nrf2, causing a large accumulation of Nrf2, and increasing the resistance of tumor cells. In malignant tumors, the incidence of Keap1 loss of function is high. Keap1 mutations affect the inhibitory activity of Keap1 on Nrf2, and Keap1 loss of function enhances the survival of tumor cells (154). DeNicola et al. found that Nrf2 transcription was significantly increased in primary mouse cells following the expression of Kras, BRaf, and myc endogenous oncogenic alleles. The upregulation of Nrf2 target genes and the augmented stability of Nrf2 engendered by somatic mutations in both Nrf2 and Keap1 might serve as a mechanism for the increased expression of Nrf2 during tumorigenesis and progression (155). In addition, overexpression of Nrf2 in colon cancer cells could promote colon cancer progression through ERK and AKT signaling pathways (156).

7 The therapeutic potential of modulation of the Keap1-Nrf2 pathway in UC

Nrf2, a crucial transcription factor responsible for regulating cellular defense mechanisms, is intricately associated with the progression of UC, intestinal fibrosis, and CRC. Activation of Nrf2 activity is an effective therapeutic modality against oxidative stress-related diseases (8, 157). Recent studies have demonstrated both sides of Nrf2 in treating malignant tumors. It has been demonstrated through previous studies that the excessive activation of Nrf2 contributes significantly to malignant tumor transformation, treatment resistance, and unfavorable clinical outcomes. Inhibition of over-activated Nrf2 activity in tumor cells can exert anti-tumor effects by disrupting redox homeostasis, antagonizing tumor metabolism, and reversing drug resistance in various ways (15, 158, 159). Therefore, the study of the role of Nrf2 and the molecular mechanism of its activity is becoming a new hot topic.

The literature shows that many medicinal plants and phytochemicals, synthetic chemicals or inducers, and others, such as metformin and short-chain fatty acids, modulate the effects of Nrf2 on UC and UC-associated colorectal cancer by activating Nrf2-mediated antioxidant expression and attenuating NF-κB-associated inflammation (Table 1). Studies have shown that Nrf2 inhibits NF-κB, the most important mediator of UC inflammation, through several cellular and biochemical mechanisms (68, 160, 161). At its peak expression, Nrf2 attenuates the activity of NF-κB primarily by hampering the generation of ROS. The activation of Nrf2 reduces ROS levels, consequently inhibiting the production of NF-κB-dependent pro-inflammatory factors activation mediated by ROS (68). The maintenance of stable Nrf2 activation levels during the initial stages of UC is likely to enhance the intestinal environment, bolster the mucosal barrier, prevent the disruption of the colon, reduce ulceration and microbial metastasis, ultimately suppressing the disease activity index (DAI), restraining the progression of UC, and mitigating the likelihood of subsequent complications (162–164). Moreover, Nrf2 signaling modulates a multitude of genes implicated in redox regulation, protein degradation, DNA repair, xenobiotic metabolism, and apoptosis, which collectively impede the development of colorectal cancer associated with ulcerative colitis (55, 165, 166). Research indicates that in the early stages of various inflammatory disorders, maintaining constant levels of Nrf2 activators can inhibit progression, thereby preventing complications like fibrosis and cancer. However, in advanced cancers wherein Nrf2 expression is elevated, Nrf2 inhibitors may serve as efficacious therapeutic adjuvants that can significantly reduce radiotherapy resistance (3, 126). Nevertheless, further investigations are essential to illuminate the intricate role of Nrf2, along with developing novel drugs capable of modulating the Nrf2 pathway and potentiating its defensive effects.

8 Conclusions and perspective

The Keap1/Nrf2 axis significantly influences the healthy development and maintenance of the gastrointestinal tract's normal function. Given its significant association with UC and its dire complications, the Keap1/Nrf2 axis is a potential therapeutic target for preventing such ailments. In this review, we describe the structure and function of Nrf2 and its role in intestinal development while we elucidate the critical role of Nrf2 in UC and its complications. First, Nrf2 can alleviate intestinal injury and

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TABLE 1 Studies the therapeutic impact of controlling the Nrf2 signaling pathway using medication on UC.

Compound	Optimal Doses (/kg Body Weight)	Model	Potential Mechanism	PMID
Ulcerative colitis				
Natural products				
			Activating the Keap1/Nrf2/HO-1 pathway	
Luteolin	50mg	DSS-induced acute colitis in C57BL/6 mice	Mitigation of colitis in murine models by stimulating the Nrf2 signaling pathway	27569028
Hyperoside	120mg	DSS-induced acute colitis in C57BL/6 mice	The Nrf2 signaling pathway activation mitigates colonic inflammation and diminishes apoptosis.	29162986
Gallic Acid	10mg	DSS-induced acute colitis in BALB/c mice	Stimulates or enhances the expression of Nrf2 and its downstream targets.	26251571
Procyanidin B2	30mg	DSS-induced acute colitis in C57BL/6 mice	Repress oxidative stress *via* Nrf2/ARE signaling	32940048
Caffeic Acid	251mg	DSS-induced acute colitis in ICR mice	Activating the Nrf-2/HO-1 pathway	34867926
Dieckol	15mg	DSS-induced acute colitis in C57BL/6 mice	The suppression of inflammatory signaling and activation of the Nrf2/HO-1 signaling pathway results in the mitigation of colitis.	33331035
Thymoquinone	40mg	DSS-induced acute colitis in C57BL/6 mice	Reducing inflammation through the Nrf2/Keap1 system	33051921
Sinomenine	100mg	DSS-induced acute colitis in C57BL/6 mice	The Nrf2/NQO 1 signaling pathway-mediated alleviation of colitis.	30106158
Matrine	1g	DSS-induced acute colitis in C57BL/6 mice	Activates/upregulates the expression of Nrf2 and its downstream targets	30284461
Berberine	40mg	DSS-induced acute colitis in Sprague-Dawley rats	Alleviation of colitis in rats through the Nrf2-dependent mechanisms	29891588
Rutaecarpine	80mg	DSS-induced acute colitis in C57BL/6 mice	Inhibition of KEAP1-NRF2 interaction and activation of NRF2	31874248
Imperatorin	60mg	TNBS-induced colitis in Sprague–Dawley rats	The modulation of the Nrf-2/ARE/HO-1 pathway in rats.	33098052
Astragalus polysaccharides	300mg	DSS-induced acute colitis in C57BL/6 mice	Activates the NRF2/HO-1 pathway	34562468
6-Shogaol	15mg	DSS-induced acute colitis in FVB/NJ mice	Induce Nrf2 and activate Nrf2 target genes in an Nrf2-dependent manner	28961808
Oligonol	50mg	DSS-induced colitis in C57BL/6 mice	Prevented the relapse of colitis through enhancing NRF2-mediated antioxidative defense mechanism	30149369
Alpinetin	100mg	DSS-induced acute colitis in C57BL/6 mice	Nrf2/HO-1 signaling pathways were found to be activated	29661352
Sulforaphane	15mg	Acid acetic solution induced colitis in Sprague–Dawley rats	Increase the expression of Nrf2 and HO-1	35754320
Crocin	20mg	Acid acetic solution induced colitis in Sprague–Dawley rats	Enhancement of Nrf2 and HO-1 signaling and down-regulation of caspase-3 activity	30530041
Quercetin	20mg	DSS-induced acute colitis in Wistar rats	Reduces the Nrf2 and HO-1 gene expression	35884960
PMID	22mg	DSS-induced acute colitis in ICR mice	Induces the activation of Nrf2/ARE pathway	25874026
Ruscogenins	2mg	TNBS-induced colitis in C57BL/6 mice	Activation of the Nrf2/HO1 signaling pathway	35308175

(Continued)

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TABLE 1 Continued

Compound	Optimal Doses (/kg Body Weight)	Model	Potential Mechanism	PMID
Apocynin	400mg	DSS-induced acute colitis in BALB/c mice	Anti-inflammatory mediators Nrf2 and HO-1 were activated	31141554
Hesperidin	40mg	DSS-induced acute colitis in C57BL/6 mice	Protect against intestinal inflammation via enhanced Nrf2 antioxidant pathway, increases the Treg population	30817082
Carnosic Acid	100mg	DSS-induced acute colitis in BALB/c mice	Modulation of the Keap1/Nrf2 pathway plays a preventive role in colitis.	28887507
Protocatechuic Acid	60mg	TNBS-induced colitis in C57BL/6 mice	Elevated expression of Nrf2 and antioxidant enzymes, coupled with reduced expression of proinflammatory cytokines	28300788
Ficus pandurata Hance	48g	DSS-induced acute colitis in C57BL/6 mice	Facilitation of colonic antioxidative stress attributes, accomplished through the elevation of T-SOD and GSH-Px levels and the augmentation of NRF2 and HO-1 expressions.	34966476
Moringa oleifera Lam	150mg	DSS-induced acute colitis in C57BL/6 mice	Upregulated GSTP1, an Nrf2-mediated phase II detoxifying enzyme	28922365
Atractylenolide III	20mg	TNBS-induced colitis in C57BL/6 mice	Regulating oxidative stress via the FPR1 and Nrf2 pathways	34156157
Pisum sativum L	600mg	DSS-induced acute colitis in C57BL/6 mice	The amelioration of DSS-induced colitis is accomplished by modulating the Keap1/Nrf2 pathway and gut microbiota.	34829046
Rhus chinensis Mill	600mg	DSS-induced acute colitis in C57BL/6 mice	Upregulation of the expression of Nrf2, NQO1 and HO-1	34494061
Crocus sativus	25mg	DSS-induced acute colitis in C57BL/6 and BALB/c mice	Activation of AhR-Nrf2-dependent pathways	34275090
Prunus mahaleb	1300mg	DSS-induced acute colitis in BALB/c mice	The activation of the Nrf2 pathway serves to potentiate mitochondrial oxidative metabolism.	31410984
Bruguiera gymnorrhiza	100mg	DSS-induced acute colitis in BALB/c mice	Mitigating inflammatory and oxidative states is achieved by activating the Keap1/Nrf2 signaling pathway.	32116661
Honokiol	40mg	DSS-induced acute colitis in C57BL/6 mice	Reduction of colitis through the inhibition of oxidative stress and inflammatory signaling.	36578522
Dendrobium officinale	200mg	DSS-induced acute colitis in BALB/c mice	Inhibition of TLR4 and the activation of Nrf2 signaling pathway	34363819
			Regulating Nrf2/NF-kB signaling pathway	
Puerarin	50mg	DSS-induced acute colitis in BALB/c mice	The alleviation of colitis in murine models via the activation of the Nrf2 pathway, coupled with the reduction of NF-κB	31981944
Pectolinarigenin	10mg	DSS-induced acute colitis in C57BL/6 mice	The regulation of the NF-κB/Nrf2 pathway diminishes colitis in murine models.	35931839
Wogonin	50mg	DSS-induced acute colitis in BALB/c mice	The modulation of the Nrf2 signaling pathway curtails TLR-4/NF-κB activation.	24901054
Sericic acid	50mg	DSS-induced acute colitis in C57BL/6 mice	The mitigation of colitis through the modulation of NF-κB and Nrf2 pathways.	36173058
Triptolide	0.02mg	DSS-induced acute colitis in C57BL/6 mice	The stimulation of the NRF2/HO-1 signaling pathway, coupled with the inhibition of the PDE4B/AKT/NF-κB pathway.	33240279
Asperuloside	500ug	DSS-induced chronic colitis in KM mice	Mitigating oxidative stress and inflammation in colitis through the modulation of the Nrf2/HO-1 and NF-κB pathways.	33974900
Dehydrocostus Lactone	15mg	DSS-induced acute colitis in ICR mice	The mitigation of colitis via the modulation of the Keap1-Nrf2 and IKKα/β-NF-κB signaling pathways.	35321327
Geniposide	40mg	DSS-induced acute colitis in ICR mice	The Nrf2/HO-1/NF-κB pathway-mediated abatement of colitis in murine models.	32787366

(Continued)

TABLE 1 Continued

Compound	Optimal Doses (/kg Body Weight)	Model	Potential Mechanism	PMID
Epoxymicheliolide	5mg	DSS-induced acute colitis in ICR mice	The prevention of colitis is accomplished through the inhibition of the TAK1-NF-κB pathway and activation of the Keap1-NRF2 pathway.	36461599
Myristicin	150mg	Acid acetic solution induced colitis in Sprague–Dawley rats	The targeting of endoplasmic reticulum stress, Nrf-2/HO-1, and NF-κB signaling pathways.	36403646
Licochalcone A	80mg	DSS-induced colitis in C57BL/6 mice	Downregulate NF-κB pathway and upregulate Nrf2 pathway	29710547
Leonurine	30mg	DSS-induced acute colitis in C57BL/6 mice	Inhibited TLR4/NF-κB pathway and activated of Nrf2/HO-1 pathway	35253649
GB1a	100mg	DSS-induced acute colitis in C57BL/6 mice	Repression of NF-κB and activation of Nrf2 signaling pathway.	34557497
Diosmetin	50mg	DSS-induced acute colitis in C57BL/6 mice	Elevate the levels of Nrf2 and HO-1 while simultaneously diminishing the acetylated NF-κB and NF-κB ratio by activating the circ-Sirt1/Sirt1 axis.	34262136
Ulva pertusa	100mg	DNBS-induced colitis in CD1 mice	Mitigation of DNBS-induced colitis damage is achieved through the modulation of the NF-κB/Nrf2/SIRT1 signaling pathways.	35893393
Panax ginseng	0.3g	DSS-induced acute colitis in C57BL/6 mice	Concomitant inhibition of the MAPK/NF-κB signaling pathway and activation of autophagy and p62-Nrf2-Keap1 signaling pathways, ameliorating inflammation.	34648903
Rose odorata sweet var. gigantean	500mg	DSS-induced acute colitis in C57BL/6 mice	Regulating the Nrf2/NF-κB signaling pathways	35692958
Perilla frutescens	100mg	DSS-induced acute colitis in ICR mice	Inhibited the activation of both NF-κB and STAT3 and elevated the accumulation of Nrf2	28848431
D-pinitol	40mg	DSS-induced acute colitis in BALB/c mice	Activating Nrf2/ARE and PPAR-γ/NF-κB signaling pathways	33625409
Mesua assamica (King&Prain) Kosterm	200mg	DSS-induced colitis in C57BL/6 mice	Exacerbation of colitis is achieved through the inhibition of NF-κB/STAT3 signaling, and the activation of HO-1/Nrf2/SIRT1 pathways.	36195303
			Activating the Nrf2/HO-1 pathway and suppresses NLRP3 inflammasome	
Cardamonin	60 mg	DSS-induced acute colitis in C57BL/6 mice; TNBS-induced colitis in BALB/c mice	The AhR/Nrf2/NQO1 pathway mediated suppression of NLRP3 inflammasome activation.	30071202
Norisoboldine	40mg	TNBS-induced colitis in BALB/c mice	The modulation of AhR/Nrf2/ROS signaling pathway effectively suppresses NLRP3 inflammasome activation.	29576052
8-Oxypalmatine	50mg	DSS-induced acute colitis in BALB/c mice	The regulatory modulation of Nrf2 and NLRP3 inflammasome signaling leads to a superior anti-colitis effect.	35779424
Rosmarinic Acid	20mg	DSS-induced acute colitis in C57BL/6 mice	Modulation of NLRP3 inflammasome and reestablishment of the Nrf2/HO-1 signaling pathway	33530569
Toosendanin	1mg	DSS-induced acute colitis in C57BL/6 mice	Regulating NLRP3 inflammasome and Nrf2/HO-1 signaling	31520988
			Other functional mechanisms	
Gingerenone A	20mg	DSS-induced acute colitis in C57BL/6 mice	Amelioration of colitis via activating Nrf2-Gpx4 signaling pathway	36135333
Schisandrin B	10mg	DSS-induced acute colitis in C57BL/6 mice	Activation of AMPK/Nrf2 dependent signaling-ROS-induced mitochondrial damage	34628369
Sesamin	100mg	DSS-induced acute colitis in C57BL/6 mice	Through the activation of AKT and ERK signaling pathways, the activation of Nrf2-mediated protective responses against oxidative stress and inflammation is potentiated in colitis.	31534619

(Continued)

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TABLE 1 Continued

Compound	Optimal Doses (/kg Body Weight)	Model	Potential Mechanism	PMID
Aucklandia lappa Decne	1.82g	DSS-induced acute colitis in C57BL/6 mice	Colitis attenuation is attained by co-modulating MAPK and Nrf2/Hmox-1 signaling pathways.	35623504
Ziziphus spina-christi	400mg	Acid acetic solution induced colitis in Wistar rats	The induction of Nrf2 and HO-1 expression suppresses oxidative stress and p38 MAPK expression, resulting in the mitigation of colitis.	29518435
Chemical drugs				
			Activating the Nrf2/HO-1 pathway	
5-ASA	30mg	TNBS-induced colitis in Sprague-Dawley rats	Activates Nrf2-HO-1 pathway by covalently binding to Keap1	28473247
Telmisartan	7mg	DSS-induced colitis in Sprague-Dawley rats	Upregulated the gene expression of Nrf-2 and HO-1	31326516
Coenzyme Q10	100mg	Acid acetic solution induced colitis in Sprague-Dawley rats	Modulation of Nrf2/HO-1 and caspase-3 pathways	28050757
Miconazole	20mg	Acid acetic solution induced colitis in Sprague-Dawley rats	Activation of Nrf2-regulated cytoprotective expression	35205169
			Regulating Nrf2/NF-kB signaling pathway	
Metformin	500mg	LPS-induced colitis in C57BL/6 mice	Alleviated NF-κB phosphorylation, promoted Nrf2 nuclear translocation, and increased the expression of the antioxidative genes	29772687
Olmesartan	10mg	Acid acetic solution induced colitis in Wistar rats	Ameliorates colitis via modulating NFκB and Nrf-2/HO-1 signaling	30594690
Carbocisteine	500mg	Acid acetic solution induced colitis in Sprague-Dawley rats	A Modulator of Nrf2/HO-1 and NF-κB Interplay	35754464
Corynoline		DSS-induced colitis in BALB/c mice	Modulating the Nrf2/NF-κB pathway	35980837
			Activating the Nrf2 pathway and suppresses NLRP3 inflammasome	
Mycophenolate mofetil	50mg	Acid acetic solution induced colitis in Sprague-Dawley rats	Targeting Nrf-2 and NLRP3 inflammasome	33539910
Other				
			Activating the Nrf2 pathway	
Dimethyl fumarate	25mg	DSS-induced chronic colitis in C57BL/6 mice	Activation of Nrf2-mediated antioxidant and anti-inflammatory pathways	32344663
Maggot	1g	DSS-induced acute colitis in C57BL/6 mice	Alleviate inflammation and oxidative stress in colitis via the activation of Nrf2	31827675
FSGHF3	200mg	DSS-induced acute colitis in C57BL/6 mice	Exert a protective effect on colitis via the Nrf2 pathway	31825438
Gloeostereum incarnatum	4g	DSS-induced chronic colitis in C57BL/6 mice	Enhanced the expression levels of Nrf2	32945507
			Regulating Nrf2/NF-kB signaling pathway	
Sodium Butyrate	1g	DSS-induced acute colitis in C57BL/6 mice	Inhibiting oxidative stress and NF-κB/NLRP3 activation via COX-2/Nrf2/HO-1 activation and mitophagy	36867295
Maresin 1	0.3ug	DSS-induced colitis in Sprague-Dawley rats	Alleviates colitis by regulating NRF2 and TLR4/NF-kB signaling pathway	31780371
			An inhibitor of the Keap1-Nrf2 protein-protein interaction	
CPUY192018	10mg	DSS-induced acute colitis in C57BL/6 mice	An inhibitor of the Keap1-Nrf2 protein-protein interaction	27215610

(Continued)

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TABLE 1 Continued

Compound	Optimal Doses (/kg Body Weight)	Model	Potential Mechanism	PMID
Colon cancer				
			Regulating the Nrf2 pathway	
Procyanidin B2	30mg	AOM/DSS-induced colitis-associated colorectal cancer in C57BL/6 mice	The capacity to reduce Nrf2 degradation and increase nuclear translocation of Nrf2 is observed.	32940048
Resveratrol	200mg	AOM/DSS-induced colitis-associated colorectal cancer in C57BL/6 mice	The activation of Nrf2 signaling for preventing colorectal cancer development, facilitated by resveratrol, is contingent on the interplay between Nrf2 and Mpk-1.	30844440
Digitoflavone	5mg	AOM/DSS-induced colitis-associated colorectal cancer in C57BL/6 mice	Elevation of Nrf2 expression, accompanied by its nuclear translocation and downstream expression of Phase II antioxidant enzymes.	24602443
Theobroma cacao	100g	AOM/DSS-induced colitis-associated colorectal cancer in BALB/c mice	Activation of the Nrf2 system	25545372
Glucosinolates	100mg	AOM/DSS-induced colitis-associated colorectal cancer in BALB/c mice	The expression of various Nrf2 target genes is upregulated.	24714741
Crocin	200mg	AOM/DSS-induced colitis-associated colorectal cancer in ICR mice	The suppression of NF-κB and subsequent downregulation of TNF-α, IL-1β, IL-6 expression, alongside the elevation of Nrf2 expression.	23326291
Pterostilbene	250mg	AOM/DSS-induced colitis-associated colorectal cancer in BALB/c mice	Activation of Nrf2 serves to block inflammation and oxidative stress by inducing the expression of HO-1 and GR, hence inhibiting colon carcinogenesis induced by AOM.	21355597
Nobiletin	–	AOM/DSS-induced colitis-associated colorectal cancer in CD-1 mice	The downregulation of iNOS, upregulation of Nrf2-dependent enzymes, and profound modulation of crucial signaling proteins reduce cell cycle progression.	28107678
Wogonin	5mg	AOM/DSS-induced colitis-associated colorectal cancer in C57BL/6 mice	The modulation of Nrf2 activation and diminution of nuclear translocation of NF-κB is observed.	24901054
Cinnamaldehyde	–	AOM/DSS-induced colitis-associated colorectal cancer in C57BL/6 mice	Ubiquitination blockage is observed, concomitant with the upregulation of Nrf2 cytoprotective target genes and rise in cellular glutathione levels.	25712056
			Regulating the Nrf2 pathway and amplifying the HO-1 expression	
Tussilagone	5mg	AOM/DSS-induced colitis-associated colorectal cancer in BALB/c mice	The manifestation of antioxidant effects in suppressing tumor formation is characterized by amplified HO-1 expression.	32290483
AcEGCG	–	AOM/DSS-induced colitis-associated colorectal cancer in ICR mice	The amplification of HO-1 expression is facilitated through ERK1/2 signaling and Nrf2 acetylation.	22409325

- means that the drug dose is not mentioned.
Bold text means grouped activators according to their functional mechanisms.

inflammation by controlling oxidative stress. Secondly, Nrf2 activation modulates the expression of tight junction proteins, consequently supporting and fortifying the integrity of the intestinal epithelial barrier. Thirdly, Nrf2 activation regulates intestinal immunity to varying degrees and influences the development of UC. Finally, Nrf2 is closely associated with intestinal fibrosis and colorectal cancer that occur in UC. In summary, comprehending the intricacies underlying the structure and functionality of Nrf2, developing drugs that target the molecule effectively, and meticulously regulating its activity at different stages of the disease progression could offer new and innovative hypotheses to tackle ulcerative colitis management.

Author contributions

SP and LS wrote the draft of the manuscript. SP drew the figures. XY, LZ, KX, YX, LLZ, and JW contributed to the literature search and discussion. LS and HL designed and revised this manuscript. All authors contributed to the article and approved the submitted version.

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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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Gut microbiota and Sjögren's syndrome: a two-sample Mendelian randomization study

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Background: The link between the gut microbiota (GM) and Sjögren's Syndrome (SS) is well-established and apparent. Whether GM is causally associated with SS is uncertain.

Methods: The MiBioGen consortium's biggest available genome-wide association study (GWAS) meta-analysis (n=13,266) was used as the basis for a two-sample Mendelian randomization study (TSMR). The causal relationship between GM and SS was investigated using the inverse variance weighted, MR-Egger, weighted median, weighted model, MR-PRESSO, and simple model methods. In order to measure the heterogeneity of instrumental variables (IVs), Cochran's Q statistics were utilized.

Results: The results showed that genus Fusicatenibacter (odds ratio (OR) = 1.418, 95% confidence interval (CI), 1.072–1.874, P = 0.0143) and genus Ruminiclostridium9 (OR = 1.677, 95% CI, 1.050–2.678, P = 0.0306) were positively correlated with the risk of SS and family Porphyromonadaceae (OR = 0.651, 95% CI, 0.427–0.994, P = 0.0466), genus Subdoligranulum (OR = 0.685, 95% CI, 0.497–0.945, P = 0.0211), genus Butyricicoccus (OR = 0.674, 95% CI, 0.470–0.967, P = 0.0319) and genus Lachnospiraceae (OR = 0.750, 95% CI, 0.585–0.961, P = 0.0229) were negatively correlated with SS risk using the inverse variance weighted (IVW) technique. Furthermore, four GM related genes: ARAP3, NMUR1, TEC and SIRPD were significant causally with SS after FDR correction (FDR<0.05).

Conclusions: This study provides evidence for either positive or negative causal effects of GM composition and its related genes on SS risk. We want to provide novel approaches for continued GM and SS-related research and therapy by elucidating the genetic relationship between GM and SS.

KEYWORDS

Sjoegren's syndrome, gut microbiota, Mendelian randomization, causal effect, autoimmune disease

Introduction

The peak incidence of Sjögren's Syndrome (SS), a chronic autoimmune illness, occurs around the age of 50 (1). Inflammation of the exocrine glands, particularly the salivary and lacrimal glands, is the main side effect of SS and a contributing reason in excessively dry mouth and eyes. Clinical signs of SS might range from those associated with sicca to systemic illness and cancer (2). Formal criteria for the diagnosis are based on the severe immunologic abnormalities of SS, which include the detection of serum anti-Roantibodies antibodies and localized lymphocytic sialadenitis on labial salivary gland biopsy (3). This illness has a heavy impact since there are few viable treatment choices. Thus, it is crucial to investigate the causes of SS in order to aid in the creation of treatment plans that cause little harm or even no adverse effects.

The biggest known symbiotic microbiological communities in the human body is the gut microbiome (GM), which is made up of bacteria, fungi, viruses, and protozoa (4), and comprises 4 trillion microorganisms (5) and 150 000 microbial genomes (6). The development of the human immune system is crucially influenced by the gut microbiota, which also protects against pathogen overgrowth (7). According to a study, the dynamics of human immune cells were connected to the gut microbiome, indicating that the gut microbiome was responsible for the immune system's regulation (8). Autoimmune illnesses were made more likely by the dysbiosis of the gut microbiome, which impacted immune responses (9). One theory was that autoimmune reactions to nuclear antigens were affected by the presence of commensal GM (10). Previous research has linked the IL-23/IL-17 major cytokine pathway to the development of GM (11) as well as spondyloarthritis (SpA), ankylosing spondylitis (AS), reactive arthritis (ReA) (12), and reactive arthritis induced by bacterial infections. In addition to maintaining intestinal permeability, IL-17 encourages T cell priming and boosts the production of pro-inflammatory cytokines and chemokines by immune cells (13), fibroblasts, endothelium and epithelial cells, and endothelial and epithelial cells (14). The primary source of IL-17 is Th17 cells. Thl7 cell growth results from T cell activation, which is greatly aided by IL-23. Th17 cells contribute significantly to the emergence of SpA by the induction of pro-inflammatory cytokines such IL-17 and TNF-α (15). Moreover, prior research has found that the microbiota diversity of SS is much lower than that of healthy controls (16–22), suggesting that microbial dysbiosis may contribute to the pathogenesis. Nevertheless, whether there is a causal connection between the gut microbiota and SS is yet unknown.

Mendelian randomization (MR) is a unique method to investigate the relationship between GM and SS in this context. To evaluate the causative relationship between exposure and illness outcome, MR constructs IVs of exposure using genetic variations (23). The distribution of genotypes from parent to child is random, therefore common confounding variables have no effect on the connection between genetic variations and outcome, and a causal sequence is justified (24). In this work, two-sample Mendelian randomization (TSMR) study was carried out to assess the causal link between GM and SS using GWAS summary statistics from the MiBioGen and FinnGen consortiums.

Materials and methods

The MR study's assumptions and design

To assess the causal connections between GM taxa and SS, TSMR analysis was performed. Figure 1 shows the flowchart of the MR study between GM taxa and SS. For GM and SS, summary-level data from the genome-wide association studies (GWASs) was acquired. The MR study also met the following 3 assumptions (25) in order to get trustworthy findings (Figure 2): (1) The GM taxa must be closely connected to the instrumental variables (IVs) that are ultimately included for usage; (2) There was no interdependence between the included IVs and confounders (affecting GM taxa and SS); (3) There was no horizontal pleiotropy since SS was only impacted by IVs *via* GM taxa. In the meanwhile, our results were published in accordance with the MR-STROBE recommendations (26).

Data sets

The MiBioGen research is the largest ethnically diverse genome-wide meta-analysis of the GM to date (27). In the GWAS meta-analysis, there were 18,340 people from 24 cohorts. Targeting the V4, V3-V4, and V1-V2 sections of the 16S rRNA gene allowed researchers to characterize the microbial makeup of several cohorts, and 10,000 readings per cohort were selected from all microbiome datasets. An effective sample size of at least 3,000 people and participation in at least three cohorts were the study-wide cutoffs. Through the implementation of a standardized pipeline, microbiome trait loci (mbTL) were mapped to identify genetic loci that affect the relative abundance (mbQTL) or presence

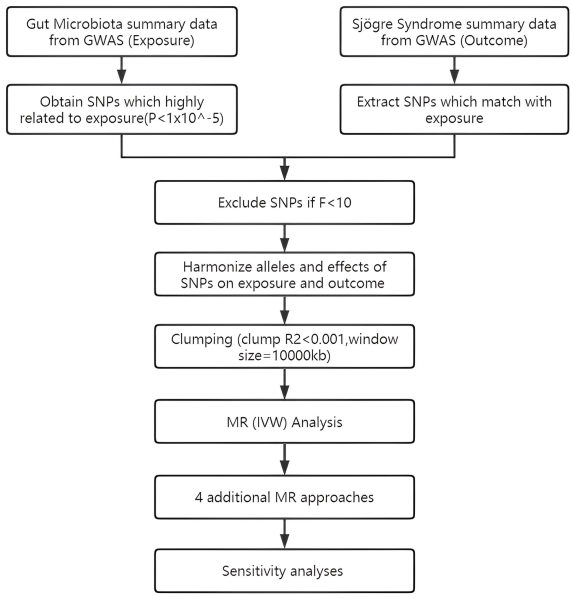

FIGURE 1
Flowchart of TSMR design.

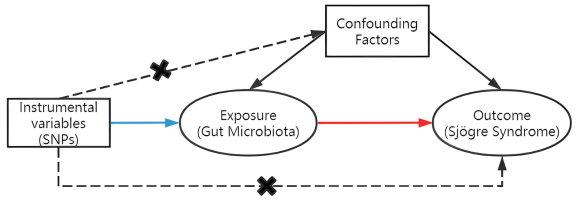

FIGURE 2
Three assumptions of MR.

(microbiome binary trait loci, or mbBTL) of microbial taxa. 211 bacterial taxa's worth of accessible GWAS summary statistics were subsequently used into the MR analysis. SS GWAS data containing 2247 cases and 332115 controls is obtained from FinnGen Release 8, published on Dec 1, 2022(https://www.finngen.fi/en) (28). SS patients are defined according to ICD-10 code M35.0, ICD-9 code 7102, or ICD-8 code 73490 (mostly according to ICD-10 code).

The IVs were chosen using the following selection criteria: First, possible IVs were chosen from single nucleotide polymorphisms (SNPs) connected to each species at a significance level of (P = 1x10-5) (29); Second, the linkage disequilibrium (LD) between the SNPs was calculated using data from the 1000 Genomes Project's European samples as the reference panel, and only SNPs with the lowest P-values were kept among those SNPs with R2 < 0.001 (clumping window size = 10,000 kb); Third, SNPs having a minor allele frequency (MAF) of less than 0.01 were eliminated; Fourth, F-statistic measures the strength of the relationship between genetic variants and exposure, where a higher F-statistic indicates a stronger instrument. The threshold of 10 is widely accepted as it corresponds to an instrument that explains at least 10% of the variance in the exposure variable and has a low probability of weak instrument bias (30). F-statistic for each IV is calculated (F=(beta/se)^2), and only those IVs with an F-statistic greater than 10 were retained.

Statistical analysis

In this investigation, a variety of techniques were utilized to determine if there was a causal relationship between GM and SS, including inverse variance weighted (IVW), MR-Egger, weighted median, weighted model, MR-PRESSO, and simple model approaches. To evaluate the overall impact of GM on SS, the IVW technique coupled a meta-analysis strategy with the Wald estimates for each SNP. The IVW results would be unbiased if there was no horizontal pleiotropy (31). Based on the premise that instrument strength is independent of direct effect (InSIDE), MR-Egger regression enables the evaluation of pleiotropy using the intercept term. The outcome of the MR-Egger regression is consistent with IVW if the intercept term equals zero, demonstrating the absence of horizontal pleiotropy (32). When up to 50% of IVs are invalid, the weighted median technique enables accurate causal connection assessment (33). The weighted mode estimate has been found to have more power to identify a causal impact, less bias, and lower type I error rates than MR-Egger regression in the event that the InSIDE hypothesis is falsified (33). By eliminating large outliers, the MR-PRESSO analysis finds horizontal pleiotropy and makes an effort to decrease it. Nevertheless, the MR-PRESSO outlier test is dependent on InSIDE assumptions and necessitates that at least 50% of the genetic variations be valid instruments (34). The simple mode approach is also less biased than other methods while being less precise since it can reduce bias (33).

In order to assess the stability of significant results, we carried out additional tests for heterogeneity and horizontal pleiotropy using meta-analytic methods. These tests included the modified Cochran Q statistic and the MR Egger intercept test of deviation from the norm (35). To avoid horizontal pleiotropy brought on by a single SNP, a leave-one-out analysis was carried out, which systematically drops one SNP at a time. The packages "TwoSampleMR" (36) and "MRPRESSO" in R version 4.2.1 were used for every analysis.

Mapping SNPs to genes

To further understand the mechanism of the influence of gut microbiota on Sjogren syndrome, we entered the SNPs of each Taxa that were significant in the MR analysis as lead SNPs into FUMA GWAS (37) (a platform that can be used to annotate, prioritize, visualize and interpret GWAS results). These SNPs were mapped to genes using the SNP2GENE tool in FUMA. To understand gene interactions at the protein level, PPI networks were constructed for mapped genes using STRING (38) with 0.4 as the recommended minimum interaction index and default values for all other variables. Analysis and display of PPI data using Cytoscape (V3.9.1).

Deeper MR analysis of transcriptomic

To further verify the causal relationship between mapped genes and SS, we performed a transcriptome Mendelian randomization analysis on them. We obtained cis-eQTLs (cis expression quantitative trait loci, cis-eQTLs) of SNP mapped genes from the eQTLGen consortium (https://eqtlgen.org/). Complete descriptions of the data are accessible in the original publications (39). In a nutshell, the eQTLGen data comprised cis-eQTLs for 16,987 genes and 31,684 blood samples, the majority of which were from healthy people of European ancestry. The whole set of significant cis-eQTL findings (false discovery rate, FDR < 0.05) and allele frequency data was downloaded at 2023/03/10. Using a very low correlation criterion in the setting of a cis-region MR may lead to the loss of causative variants; hence, these eQTLs were clumped using a pairwise linkage disequilibrium (LD) threshold of r2 < 0. 1 (40).The final IVs were obtained for 237 genes from a total of 324 genes. The process of Mendelian randomization was the same as before, but considering the multiple testing problem, we performed an FDR correction, and the result of FDR < 0.05 was considered significant.

Results

Causal effects of gut microbiome on SS

211 Taxa's analysis results are shown in the lollipop plot in Figure 3. Class Methanobacteria, family Methanobacteriaceae and order Methanobacteriales were excluded from the analysis results because MR-Egger showed a different effect direction than the other four methods (), and the final significant six Taxa forest plots are shown in Figure 4. The results of IVW analyses demonstrated that genus Fusicatenibacter (odds ratio (OR) = 1.418, 95% confidence interval (CI), 1.072–1.874, P = 0.0143) and genus Ruminiclostridium9 (OR = 1.677, 95% CI, 1.050–2.678, P = 0.0306) were positively correlated with the risk of SS. Family Porphyromonadaceae (OR = 0.651, 95% CI, 0.427–0.994, P = 0.0466), genus Subdoligranulum (OR = 0.685, 95% CI, 0.497–0.945, P = 0.0211), genus Butyricicoccus (OR = 0.674, 95% CI, 0.470–0.967, P = 0.0319) and genus Lachnospiraceae (OR = 0.750, 95% CI, 0.585–0.961, P = 0.0229) were negatively correlated with SS risk (Figure 4). The MR estimates of weighted median indicated that genus Butyricicoccus (OR = 0.611, 95% CI, 0.384–0.972, P = 0.0377) and family Porphyromonadaceae (OR = 0.521, 95% CI, 0.298–0.909, P = 0.0217) served as protective factors for SS (Supplementary Table S1). However, none of the results were significant after Bonferroni multiple tests correction (P<0.05/211 = 0.000237). We show significant taxa's SNPs in Supplementary Table S2 and Supplementary Figure S1. The heterogeneity test revealed no heterogeneity among the individual SNPs. It seemed unlikely that horizontal pleiotropy would distort the impact of the gut microbiota on SS, according to the findings of the MR-Egger regression and MR-PRESSO global test (Table 1). Leave-one-out analysis revealed that no one SNP was responsible for the causative estimates of GM and SS, which were displayed in Supplementary Figure S2.

Mapping SNPs to genes

To obtain more insight into the biological significance of prior findings, we evaluated the functional annotations of the genetic variants used as IVs in MR through FUMA GWAS tool (37). The SNP hit genes are shown in Supplementary Table S3. STRING was used to generate PPI networks from 324 significant GM's SNPs mapped genes, which were then displayed in Cytoscape to predict the interactions and adhesion pathways of common significant GM's SNPs mapped genes. As shown in the diagram, the PPI network of genes hit by SNPs contains 115 nodes and 202 edges (Figure 5).

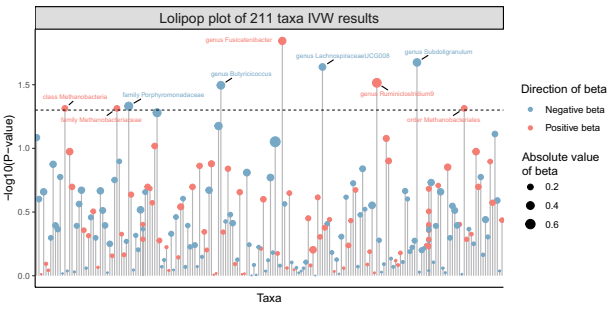

FIGURE 3
Lollipop plot of 211 Taxa's analysis results of GM on SS.

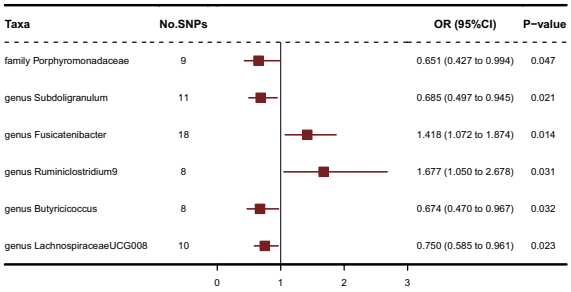

FIGURE 4
Forest plot of IVW analysis results of the effect of GM on SS.

Deeper MR analysis of transcriptomic

We obtained gene expression-related SNPs (eQTLs) from the eQTLGen consortium (https://eqtlgen.org/). As illustrated in Figure 6, the genetically proxied expression of 4 genes was significantly associated with the risk of schizophrenia at FDR < 0.05. Among these genes, ARAP3, NMUR1, TEC were associated with genus Subdoligranulum. SIRPD was associated with genus Ruminiclostridium9. Other genes were not significantly associated with SS. As illustrated in Supplementary Table S4, the effect direction of all five MR approaches was consistent. The heterogeneity test showed no heterogeneity among individual eQTLs. According to the MR-Egger regression results, it seemed implausible that horizontal pleiotropy would bias the effect of gut microbiota on SS (Supplementary Table S5).

Discussion

This was the first study to evaluate the two-way causal link between the GM and SS using a number of complimentary MR methods. This TSMR investigation provided evidence that the presence of family Porphyromonadaceae, genus Subdoligranulum, genus Butyricicoccus, and genus Lachnospiraceae was associated with a lower chance of developing SS, and that genus Fusicatenibacter and genus Ruminiclostridium9 may be factors that increase the likelihood of SS. Further, we performed MR analysis of these genes using these GM-associated SNPs paired on top of the genes, suggesting a causal relationship between these genes and SS, which may indicate that the GM affects SS through these genes.

The immune system and GM interact physiologically during the developing process, and GM has been implicated in the

TABLE 1 Heterogeneity test, pleiotropy test and MR-PRESSO results of genetic variants.

Taxa	Heterogeneity				Pleiotropy		MR-PRESSO	
	MR Egger		IVW		MR Egger		Global Test	
	Cochran's Q	P-value	Cochran's Q	P-value	Egger intercept	P-value	RSSobs	P-value
family Porphyromonadaceae	4.819	0.682	5.311	0.724	0.039	0.506	6.756	0.732
genus Subdoligranulum	5.299	0.808	5.816	0.830	-0.022	0.490	7.116	0.860
genus Fusicatenibacter	14.609	0.553	14.621	0.623	-0.004	0.914	16.421	0.620
genus Ruminiclostridium9	8.863	0.181	8.864	0.263	0.002	0.985	11.699	0.298
genus Butyricicoccus	2.434	0.876	3.132	0.873	0.026	0.436	4.048	0.886
genus Lachnospiraceae UCG008	10.026	0.263	11.081	0.270	0.062	0.386	13.674	0.294

development of chronic inflammatory illnesses and metabolic disorders in humans (7, 41). Recent research suggests that gut dysbiosis affects the immune system in a way that causes autoimmune disorders such rheumatoid arthritis, SLE, systemic sclerosis, ankylosing spondylitis, and Sjögren's syndrome to develop or worsen (42).

Earlier epidemiological investigations discovered a link between GM and SS. Previous research has shown that when SS patients are compared to healthy persons, the GM's -diversity is considerably reduced and its -diversity is changed (18, 43, 44). The severity of dry eye symptoms and GM diversity were shown to be correlated (4). The Firmicutes/Bacteroidetes (F/B) ratio is frequently seen to be lowered in individuals with autoimmune diseases (45, 46), which may be a sign of GM dysbiosis (47, 48). Nevertheless, these studies have considerable drawbacks because of variations in ethnicity, gender, or diet between cohorts, limited sample numbers, and various sequencing techniques, which lead to significant variations in the findings among research. As a result, it is difficult to infer the etiology of GM and SS based solely on prior studies.

Molecular mimicry, metabolite alterations, and the collapse of epithelial tolerance are the primary explanations for how the gut microbiota may affect Sjogren's syndrome (49). One of the most important points is the change of metabolites. Changes in metabolites short-chain fatty acids (SCFAs), which mostly consist of acetic acid, propionic acid, and butyric acid, are one of the significant metabolites produced by gut microbes. SCFAs are essential signaling molecules that control the immune system, cell growth, and metabolism of the host (50, 51). Many SCFA-producing bacteria, including Lachnoclostridium, Lachnospira, and Sutterella, were decreased in systemic autoimmune disorders. It should be noted that these bacteria have a strong pro-regulatory and tolerogenic effect on immunological processes (52). The most frequently cited bacterial product in SS is butyrate. Numerous investigations found that butyrate-producing bacteria were substantially reduced in SS (16, 53, 54), including Faecalibacterium prausnitzii, Bacteroides fragilis, Lachnoclostridium, Roseburia, Lachnospira, and Ruminococcus. This is consistent with our findings. Butyrate, a crucial metabolite produced from the microbiome, supports gut barrier processes by supplying colonic epithelial cells with energy. Furthermore, new studies found that the Clostridia clusters XIVa and IV, as well as butyrate-producing bacteria Bacteroides spp., were crucial for preserving the Treg/Th17 equilibrium. It should be emphasized

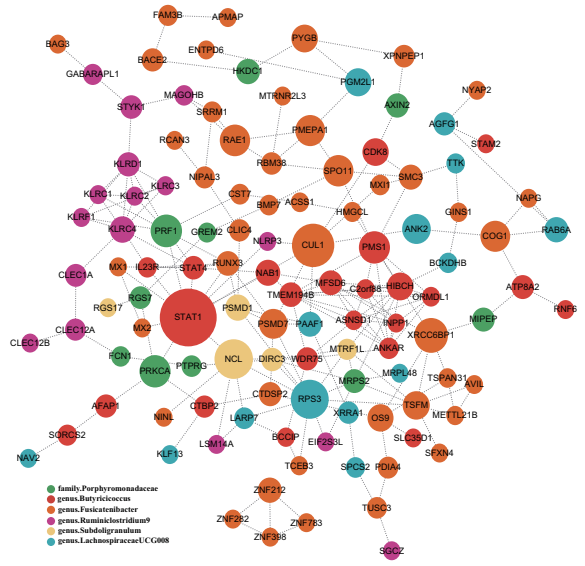

FIGURE 5
PPI network of six significant GM's SNPs mapped genes. Genes are represented by circular nodes, and their interactions are represented by edges. The size of the dots represents the Betweenness Centrality score, with high Betweenness Centrality scores being large and low scoring dots being small, and each of the six bacteria-related genes is represented by six different colors.

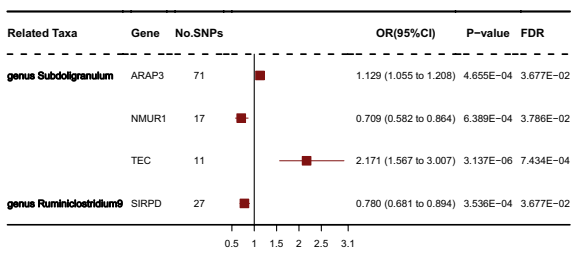

FIGURE 6
Forest plot of IVW analysis results of the effect of mapped genes on SS.

that the immunomodulatory molecule polysaccharide works in concert with butyrate to support the development of Treg cells. a Bacteroides fragilis descendant (55–57). The mucosa barrier's capacity to prevent the colonization of harmful microorganisms will be compromised by disruption of the Treg/Th17 balance (16). Basically, butyrate regulates genes associated with the circadian clock to carry out the anti-inflammation function, which has the ability to alter T cell balances and control the frequency of B cells that produce IL-10 and/or IL-17 (58). These various cues point to a possible role for diminished SCFAs or butyrate-producing bacteria in the pathogenesis of SS through altering immune cell frequency or function, mucosal barrier permeability, and possibly salivary gland secretion.

In addition, there is evidence that a bacterial-derived peptide causes clonal expansion of CD8+ T cells, which is associated with autoimmune diseases such as AS and reactive arthritis (ReA) caused by bacterial infections (59), and that GM may act in the same way in SS. HLA-B27 is a member of the HLA Class I family of MHC genes whose role is to present peptide antigens to CD8 T cells. In the feces of individuals with AS, peptide elution experiments have revealed an abundance of bacterial peptides that are identical to known HLA-B27-presented epitopes, indicating a failure in the clearance of these bacteria, and several peptides provided by APCs-B*27+, but not by B27-negative donors, elicited CD8 responses, which is consistent with these peptides activating the adaptive immune system in AS (60). Within the setting of innate immune activation, proliferation of self-reactive Th17 cells, or microbial mimicry, the initial infectious stage is followed by hyperactivation and disturbed self-tolerance (61). Therefore, CD8 T cells are kept in a state of high activation and do not experience senescence, which results in less effective responses to foreign antigens, possibly as a result of ongoing exposure to bacterial adjuvant (62, 63). This also affects immunological priming and increases the generation of proinflammatory cytokines (TNF, IL-23) that contribute to the clinical signs of joint and gastrointestinal inflammation that are frequently present in AS (60, 64–66). The above studies on GM and AS further demonstrate the causal relationship we found between GM and SS, which is also an autoimmune disease, suggesting that GM may contribute to autoimmune disease through a common pathway. To further explore the immune mechanisms behind the causal relationship between GM and SS, we also built a PPI network using six significant GM's SNPs mapped genes, based on an in-depth understanding of protein biology and prediction of drug targets.

Among the GM-related genes that were causally associated with SS, TEC gene encodes a non-receptor protein tyrosine kinase with a pleckstrin homology domain that is involved in the intracellular signaling mechanisms of cytokine receptors, lymphocyte surface antigens, heterotrimeric G protein-coupled receptors, and integrin molecules, key players in the regulation of immune functions, an integral component of T cell signaling, and plays a distinct role in T cell activation (67, 68). TEC may be associated with possible pathogenic variants of autoimmune diseases such as SS by regulating T cell activation and T cell receptor signaling pathways (69). This may suggest that GM influences the occurrence of SS through TEC. Other genes in this study have not been previously reported to be related to SS, which may be a new finding and provide clues for future studies on the mechanism of action between GM and SS

This study has a number of advantages. By removing confounding variables and reversing the causal inference process, MR analysis was used to establish the causal relationship between gut microbiota and SS. The most comprehensive GWAS meta-analysis was used to acquire genetic variations of the gut microbiome, guaranteeing the reliability of the analytical tools. The MR-PRESSO and MR-Egger regression intercept term analyses were used to identify and rule out horizontal pleiotropy. In addition, a network-based approach is developed to investigate the gene expression patterns from 6 significant GM's SNPs mapped genes and identified molecular targets that may help as potential biomarkers of GM's SNPs mapped genes in this study. It could also provide crucial information about their effects on SS.

This research does have some limitations, though. First, the study data refer only to people of European ancestry and do not include people from other regions. It is still unclear, therefore, whether the results can be regarded as representative of the total community. Second, although it is challenging to determine the extent of sample overlap, there was probably some overlap between the exposure and result research participants. Fortunately, the robust methods employed in this investigation (F statistic substantially greater than 10) should minimize any potential bias brought on by sample overlap (70). Third, the variability of the MiBioGen meta-analysis was extremely high, with only 9 taxa identified in> 95% of the samples, which may have affected the accuracy of the results of this study. Fourth, with only 2247 SS cases in the FinnGen GWAS data, this small sample size may not produce a sufficiently valid beta value, resulting in less statistical power. However, the FinnGen SS GWAS still provides valuable insights into genetic architecture and can serve as a useful starting point for further investigations with larger sample sizes or complementary approaches. Fifth, we did not consider gender factors in the analysis of data, and whether gender differences have an impact on the results needs further study due to data unavailability. Sixth, we did not find GWAS data on "dry eyes" and "dry mouth" for further differentiation studies. Finally, the results were not significant after multiple corrections were performed. But adopting a strict multiple testing correction would have likely been unduly cautious given the biological plausibility and the multi-stage statistical approach, which may have overlooked possible strains that are causally associated to SS. In addition, it is essential to interpret the results of Mendelian randomization analyses in concert with additional measures such as instrument strength and sensitivity analyses as we have done in this study. As a result, we remain optimistic that our research has academic implications. Future studies should aim to validate our current findings with larger sample sizes and diverse populations to establish the robustness of the results obtained. In addition, it would be worthwhile to carry out replication analyses using different MR methods to confirm the identified genetic associations. Meanwhile,

the search for mediating variables in the causal chain of GM and SS is also important for the prevention and treatment of SS.

Conclusions

In summary, we thoroughly evaluated the probable causal relationship between the gut microbiota and SS. Four other bacterial traits had a negative causative direction with SS, whereas two further bacterial features displayed a positive causal direction. Many intestinal bacterial species discovered in this study that may have decreased the incidence of SS may hold promise for SS prevention and therapy.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: Gut microbiota GWAS data is from MiBioGen (https://mibiogen.gcc.rug.nl/). Sjögren's Syndrome GWAS data is from FinnGen R8 release(https://www.finngen.fi/en).

Author contributions

YC and MZ conceived the presented idea. HL and YC performed the manuscript writing. YC and WX was involved in acquisition and processing of data. HL was involved in interpretation of data. YC and HL have contributed equally to this work and share first authorship. All authors contributed to the article and approved the submitted version.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1187906/full#supplementary-material

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The immune role of the intestinal microbiome in knee osteoarthritis: a review of the possible mechanisms and therapies

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Osteoarthritis (OA) is a chronic degenerative joint disease characterized by cartilage damage and synovial inflammation and carries an enormous public health and economic burden. It is crucial to uncover the potential mechanisms of OA pathogenesis to develop new targets for OA treatment. In recent years, the pathogenic role of the gut microbiota in OA has been well recognized. Gut microbiota dysbiosis can break host-gut microbe equilibrium, trigger host immune responses and activate the "gut-joint axis", which aggravates OA. However, although the role of the gut microbiota in OA is well known, the mechanisms modulating the interactions between the gut microbiota and host immunity remain unclear. This review summarizes research on the gut microbiota and the involved immune cells in OA and interprets the potential mechanisms for the interactions between the gut microbiota and host immune responses from four aspects: gut barrier, innate immunity, adaptive immunity and gut microbiota modulation. Future research should focus on the specific pathogen or the specific changes in the gut microbiota composition to identify the related signaling pathways involved in the pathogenesis of OA. In addition, future studies should include more novel interventions on immune cell modifications and gene regulation of specific gut microbiota related to OA to validate the application of gut microbiota modulation in the onset of OA.

KEYWORDS

gut microbiota, osteoarthritis, gut barrier, adaptive immunity, innate immunity, gut microbiota modulation

1 Introduction

Osteoarthritis (OA) is characterized by degenerative joint damage and synovial inflammation and is a leading cause of disability in elderly individuals. It has been reported that over 22.7 million individuals suffer from arthritis-induced activity limitations and 300 million people worldwide are diagnosed with OA (1, 2). OA can be

classified as primary and secondary (such as trauma, surgery on the joint structures and abnormal joints at birth) and usually affects one or several diarthrodial joints, including small joints (e.g., interphalangeal joints) and large joints (e.g., hip joint and knee joint) (3). Previously, biomechanical disorders of the joint and overloading of joints were identified as the causes of OA (4); however, with a growing understanding of OA, it is now widely believed that OA is a much more complex disease with metabolic and inflammatory factor involvement (5). Systemic inflammation and local inflammation of the synovium play crucial roles in the pathogenesis of OA. It has been hypothesized that degraded cartilage can lead to the generation of inflammatory cytokines and metalloproteases (6). Some studies have found a range of immune cells from the synovium in OA patients, including B cells, T cells, lymphoid follicles, granulocytes and plasma cells (7, 8). Moreover, Nedunchezhiyan U et al. proposed a central effect of the innate/adaptive immune response in the pathogenesis of OA (9). To date, researchers have identified several risk factors for OA, such as obesity, estrogen, age, sex, diet, metabolic syndrome, genetics, inflammation, mechanical loading, the oral microbiome and the gut microbiome (10, 11). Most OA patients receive pain-relieving management or joint replacement for end-stage OA with no disease-modifying anti-osteoarthritic drug for OA. Hence, identifying a specific disease-modifying treatment target is imperative.

The gut microbiota comprises multiple microorganisms, including bacteria, yeast, viruses, phages, parasites, and archaea, and due to its important endocrine and immune functions in pathophysiological modulation, the intestinal microbiome has been considered to be a "vital organ" (12). The gut microbiota is involved in many biological functions, including the digestion of food, energetic metabolism, regulation of the immune system, influence of the mucosal barrier and generation of bioactive agents (short-chain fatty acids (SCFAs), estrogen, and serotonin) (13). In clinical studies, Boer et al. reported that the alpha diversity of the gut microbiota was significantly reduced in individuals with OA; however, after adjustment for BMI, the significance was lost (14). The dominant species in the gut microbiome were Bacteroidetes (12.5%) and Firmicutes (77.8%), and plenty of streptococci were linked to worsening levels of pain and local inflammatory responses in the knee joint of OA patients, indicating that the gut microbiota can be a possible starting point for managing knee pain in OA. Bacteroidetes are believed to be associated with anti-inflammatory effects while Firmicutes are supposed to be linked to proinflammatory effects, and the Bacteroidetes/Firmicutes ratio was significantly decreased in OA patients (15). Dysbiosis of the gut microbiome can cause a reduction in the abundance of Bacteroidetes and an increase in that of Firmicutes in OA subjects, which shifts the Bacteroidetes/Firmicutes ratio (15, 16). In addition to the microbiome niches in the traditional gut, Zhao and his colleagues detected bacterial nucleic acids present in the synovium and synovial fluids from subjects with knee OA, which revealed potential correlations with the degree of OA (17). Huang et al. showed that the severity of knee joint damage and synovial inflammation in subjects with OA was positively linked to high levels of lipopolysaccharide (LPS) and the production of intestinal bacteria, and LPS could induce inflammation and damage in joints through macrophage activation and damage-associated molecular patterns (DAMPs), which is named the "two-hit" theory (18, 19). Moreover, Huang et al. showed that mice administered fecal samples from subjects with metabolic syndrome and knee OA exhibited worse OA severity, higher mean circulatory concentrations of inflammatory factors (interleukin-6 (IL-6), IL-1β and macrophage inflammatory protein-1α) and increased intestinal permeability (20), which indicated dysbiosis of the intestinal microbiome played a crucial role in the pathogenesis of OA.

As mentioned above, more studies have focused on the role of the gut microbiota in OA. Many possible mechanisms of the gut microbiota and OA, including low-grade inflammation, metabolic changes and immune modulation, have been proposed. The gut microbiota interacts with risk factors for OA, including obesity, estrogen, age, sex, diet, metabolic syndrome, genetics, inflammation, mechanical loading and exercise (10, 21). Liu et al. reviewed that the gut microbiota, gut microbiota-related components and corresponding metabolites interacted with OA by activating local and systemic innate immune responses (22). Dysbiosis of the intestinal flora can modulate the differentiation of primitive CD4+ T cells into effector T cells or Treg cells, which is crucial for immune homeostasis and joint inflammation (23). Collectively, the gut microbiota may affect the pathogenesis of OA through the immune system. However, the potential mechanisms of the relationship between innate/adaptive immunity and the gut microbiota in knee OA are uncertain. Hence, this paper will summarize the effect of the intestinal microbiota on the intestinal barrier, the immunomodulation of the intestinal microbiota on OA and intestinal microbiota modulation therapy for OA. We hope that the immunomodulation of the gut microbiota in OA patients will be a novel target for OA management in the future.

2 The gut microbiota influences the gut barrier

The gut barrier has selective permeability for nutrients, water and electrolytes, while preventing harmful substances from transiting

Abbreviations: AhR, Aryl hydrocarbon receptor; AMPK, AMP-activated protein kinase; BAs, Bile acids; CNS1, conserved non-coding sequence 1; CPT1A, Carnitine palmitoyl transferase 1A; DAB2, Disabled homolog 2; DCs, Dendritic cells; FcεRI, IgE receptor; FXR, Farnesoid X receptor; GLP-2, Glucagon-like peptide-2; GPBAR1, G protein-coupled bile acid receptor 1; HDAC, Histone deacetylases; HFD, High fat diet; IDO1, Indoleamine 2,3-dioxygenase 1; IL-#, Interleukins-#; LPS, Lipopolysaccharide; LPS, lipopolysaccharide; MCP-1, Monocyte chemotactic protein 1; MD-2 complex, myeloid differentiation protein-2; MUC, Mucin; NLRP, Nod–like receptor protein; OA, Osteoarthritis; OMVs, Outer membrane vesicles; PGN, Peptidoglycan; SAA, serum amyloid A; SCFAs, Short-chain fatty acids; SFB, segmented filamentous bacteria; Slc5a8, Na (+)-coupled monocarboxylate transporter; STAT3, signal transducer and activator of transcription 3; TLR-#, Toll like receptor-#; TNF, Tumor necrosis factor; ZO-1, Zonula occludens 1.

through the gut mucus membrane into other tissues, related organs and the circulatory system. The chemical barrier, biological barrier, mechanical barrier and immune barrier form the gut barrier. Bacterial membranes, tight junctions and intestinal epithelial cells form the mechanical barrier, so deleterious entities, such as microbes, intestinal antigens, and intestinal proinflammatory cytokines, are prevented from entering the blood circulation (24). The chemical barrier is the mucus layer adherent to the intestinal epithelium, and mucins, a kind of high-molecular-weight glycoprotein, are the main components of the mucus layer, which is essential for gut permeability by preventing immediate contact of large particles and microorganisms with the intestinal epithelium (25). The immune barrier consists of gut-associated lymphoid tissues, such as intraepithelial lymphocytes and mesenteric lymph nodes, which prime innate immunity and adaptive immunity in response to the gut microbiota (25–27). The biological barrier refers to the microspatial construction between the gut microbiota and the gut epithelium (27). Gut permeability refers to the nonmediated gut pathway for medium-sized hydrophilic compounds, which acts via a concentration gradient without the participation of the carrier system (28). Solutes pass through the gut barrier through the paracellular pathway or transcellular pathway. The paracellular pathway is defined as the pathway between cells via interstitial spaces and tight junctions, and medium-sized hydrophilic compounds instead of protein-sized molecules are allowed to pass, thus modulating the transportation of gut microbiota products and other polymers (29, 30). The transcellular pathway represents the passive transportation of energy-dependent uptake and lipid-soluble, small hydrophilic molecules via the intestinal epithelium, while harmful substances, such as intestinal microflora products and inflammatory factors, cannot pass through the gut epithelium (31, 32). Various factors, including cytokines, nutritional factors, and local immune tissues, modulate gut permeability, and the gut microbiota is associated with gut permeability via the disruption of tight junction competency and the inhibition of mucin (MUC) gene expression (33). Morbific and symbiotic gram-negative bacteria can produce outer membrane vesicles (OMVs), and OMVs can disrupt tight junctions, interact with intestinal epithelial cells and enhance the transportation of OMVs as well as their contents of bacterial virulence factors into the submucosa. As a result, OMVs can directly interact with macrophages, neutrophils and dendritic cells (DCs) in the submucosa (34). The composition of the intestinal microbiome and intestinal permeability can be modulated by prebiotics in a glucagon-like peptide-2 (GLP-2) manner, which could improve tight-junction integrity (35). In addition, the intestinal microflora also works with the host immune system to participate in the pathogenesis and progression of OA (36). Related studies have observed crosstalk between the imbalance in the intestinal flora and OA by a gut-joint axis; as a result, dysbiosis can induce abnormal intestinal mucosal barrier function and increase the permeability of the intestinal barrier, thereby allowing inflammatory factors and microflora products into the circulatory system and inducing the onset and development of OA (37, 38). Therefore, a well-functioning intestinal barrier is essential to slow the progression of OA. The corresponding mechanisms of the effects of the intestinal microbiota on the gut barrier are summarized in Figure 1.

Gut microbiota dysbiosis can cause changes in bacterial metabolism products and abnormal nutrient absorption, thus affecting gut permeability to allow bacterial products and inflammatory factors into the systemic circulation. Currently, the most studied categories of metabolism in association with host-microbiota interactions are bile acids (BAs), SCFAs, and metabolites of tryptophan. Dietary fibers are fermented by the anaerobic microflora in the gut with the generation of SCFAs as the end products, which indicates a healthy microbial community (45). When gut microbiota dysbiosis occurs, the categories and components of SCFAs in the intestine will be changed. Butyrate, propionate and acetate are the most common representative SCFAs and are considered to be involved in bone metabolism (46, 47). Fermentation of dietary fiber by the gut microbiota in the colon can generate butyrate, and butyrate provides the primary energy for colonic intestinal bacteria. In addition, butyrate can weaken intestinal permeability to enhance the gut barrier (48, 49). Intestinal epithelial goblet cells deprived of glucose could express MUC genes, which could be specifically modulated by butyrate (42). The diverse effects of butyrate a manner dependent on MUC gene expression could affect the properties and composition of the mucus gel; as a consequence, the protective effects of mucus in the gut barrier can be altered (42). In addition, butyrate can facilitate tight junction assembly by activating AMP-activated protein kinase (AMPK) in Caco-2 cell monolayers, which ultimately enhances the gut barrier (43). A high-fat/high-sucrose diet can induce Lactobacillus spp. and Methanobrevibacter spp. abundance in the intestine and can increase serum LPS levels and inflammatory factor levels in the blood and synovial fluid, thus accelerating OA in rat OA models (50). LPS is mainly produced by gram-negative bacteria, and can cause inflammatory responses. An excess of transportation of LPS into the circulatory system is associated with OA. High-fat diet (HFD) consumption markedly enhances gut permeability and reduces zonula occludens 1 (ZO-1) expression, a gene encoding proteins of tight junctions, which ultimately allows a large amount of LPS into the systemic circulation (39, 40). The concentrations of serum LPS, synovial fluid LPS and serum LPS binding protein (LBP) were positively linked to the severity level of knee osteophytes, and synovial fluid LPS was significantly linked to the reduced gap in the knee joint space, higher total Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) scores and self-reported pain scores of the knee (18). Interestingly, serum LPS, serum LBP and synovial fluid LPS were positively associated with the enrichment of activated macrophages (18). In an injury-induced mouse OA model, germ-free mice showed reduced LBP and improved progression of OA (48). Taken together, gut microbiota dysbiosis can accelerate the progression of OA in injury-induced animal models via increased LPS release into the circulatory system, whereas SCFAs can protect the gut barrier from enteric dysbacteriosis.

For many microflora, tryptophan is an important biosynthetic precursor (51). Tryptophan metabolism occurs in the gastrointestinal tract following three pathways: (1) the kynurenine pathway via indoleamine 2,3-dioxygenase 1 (IDO1) in both epithelial and immune cells; (2) gut microorganisms directly transforming tryptophan into compounds, such as indole and its derivatives, which are ligands of the aryl hydrocarbon receptor (AhR); and (3) enterochromaffin cells synthesizing 5-

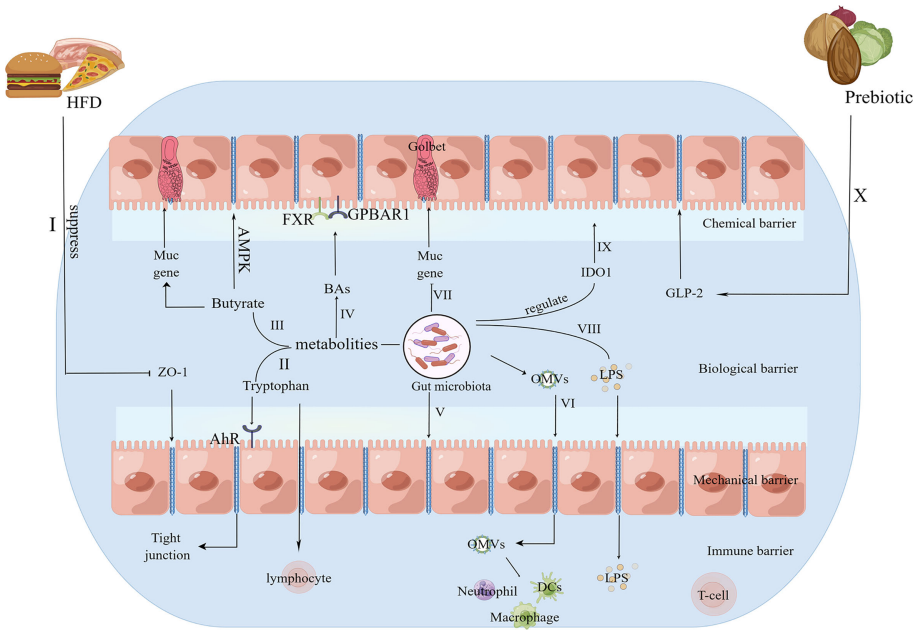

FIGURE 1
The potential mechanisms for the role of the gut microbiota in the gut barrier. I.HFD consumption suppresses the expression of the tight junction protein ZO-1 to increase intestinal permeability (39, 40). II. AhR, a ligand for tryptophan metabolism, acts on epithelial renewal, barrier integrity, and intraepithelial lymphocytes (41). III. Butyrate specifically modulates MUC gene expression in intestinal epithelial goblet cells to affect the mucus layer (42) and enhances the intestinal barrier by facilitating tight junction assembly via AMPK (43). IV. BAs could protect the gut barrier via the interactions of FXR, GPBAR1 and the gut microbiota (44). V. The gut microbiota can disrupt tight junctions (33). VI. OMVs can disrupt tight junctions, interact with intestinal epithelial cells and enhance the transportation of OMVs as well as their contents of bacterial virulence factors into the submucosa. As a result, OMVs can directly interact with macrophages, neutrophils and DCs in the submucosa (34). VII. The gut microbiota inhibits the expression of the MUC gene (33). VIII. LPS produced by the gut microbiota can enter the systemic circulation via tight junctions (39, 40). IX. The gut microbiota regulates IDO1 activity to exhibit a mucosal protective effect (41). X. Prebiotics improve tight-junction integrity via GLP-2 (35). HFD, high fat diet; ZO-1, zonula occludens 1; AhR, aryl hydrocarbon receptor; MUC, mucin; AMPK, AMP-activated protein kinase; FXR, farnesoid X receptor; GPBAR1, G protein-coupled bile acid receptor 1; OMVs, outer membrane vesicles; LPS, lipopolysaccharide; IDO1, indoleamine 2,3-dioxygenase 1; GLP-2, glucagon-like peptide-2.

hydroxytryptamine (5-HT) via tryptophan hydroxylase 1 (TpH1) (52). The AhR signalling pathway works on various types of immune cells, epithelial cell renewal and barrier integrity, thus maintaining the gut barrier and the homeostasis of intraepithelial lymphocytes (53). The gut microbiota can regulate IDO1 activity, and kynurenine-related products, such as Kna, exhibit mucosal protective and immunoregulatory effects (41). 5-HT acts as a pivotal regulator of gastrointestinal tract secretion and motility. Interestingly, the origin of 5-HT determines the impact of 5-HT in controlling bone formation and bone absorption. Ninety-five percent of 5-HT is produced by duodenum chromaffin cells, and gut-derived 5-HT can inhibit bone formation by attenuating the proliferation of osteoblasts through the activation of preosteoblast receptors (54, 55). The gut microbiota can modulate gut 5-HT production through SCFAs, which increase the synthesis of gut-derived 5-HT (56, 57). In contrast, 5-HT produced in the brain can facilitate bone formation via various mechanisms (55). In summary, tryptophan plays a vital role in maintaining the balance between intestinal microflora homeostasis and intestinal immune tolerance.

Cholesterol in the liver can synthesize BAs via two mechanisms. The main mechanism is catalysed via cholesterol-7 α-hydroxylase (CYP7A1), finally generating cholic acid and chenodeoxycholic acid (58). The alternative pathway is accomplished by producing 27-hydroxycholesterol via sterol-27-hydroxylase (CYP27A1), finally generating chenodeoxycholic acid. The gut microbiota can modulate the expression of CYP7A1, CYP7B1, and CYP27A1, thereby influencing BA metabolites (59, 60). On the other hand, BAs can regulate bacterial growth and protect the gut barrier via the interactions of G protein-coupled bile acid receptor 1 (GPBAR1), farnesoid X receptor (FXR) and the gut flora (44). Senescence of chondrocytes is believed to be conducive to the onset of OA. Huang et al. revealed that the bile acid receptor GPBAR1 can play a crucial role in protecting chondrocytes against IL-1β-induced chondrocyte senescence (61). Simon et al. reviewed that BAs can act as determinants of intestinal homeostasis and functional dyspepsia (62). In addition, BAs also influence the components of the intestinal microflora by altering the host's intestinal immunity and the natural antimicrobial defense of the host in liver diseases (63). Due to the effects of BAs in the intestinal immune system, BAs can influence the gut barrier. In summary, gut microbiota dysbiosis can disrupt the gut barrier, increase intestinal permeability and allow harmful bacterial metabolites into the systemic circulation, thus affecting the onset and development of OA. Therefore, more detailed research on the relationships between the intestinal microflora and OA is essential for subsequent targeted interventions for OA.

3 The gut microbiota modulates innate immunity

Innate immunity is a primitive mechanism using germline-encoded proteins to recognize pathogens, thereby inducing immune responses. When a pathogen is encountered, the innate immune cell either kills the pathogen or stimulates the adaptive immune response to deal with the pathogen. The activation of the innate immune system plays a crucial role in the pathogenesis and progression of OA by recognizing DAMPs through interactions with pattern-recognition receptors (PRRs) (64, 65). PRRs, which exist on the cell surface, are cytosolic and endosomal receptors consisting of NOD-like receptors, Toll-like receptors (TLRs) and so on (65). PRRs, which widely exist on the outer membrane of macrophages and other immune cells, can identify a large amount of danger signals, similar to gut microbiota metabolites in the innate immune system; as a consequence, the downstream inflammatory signalling pathway is activated (66). Dunn CM et al. detected microbial DNA and the intestinal microbiome at the same time in the knees of OA patients, and they assumed that enteric dysbacteriosis can stimulate the innate immune system to expedite OA progression (19, 67). In addition, Liu et al. showed that the gut microbiota, gut microbiota-related components and corresponding metabolites interacted with OA by activating local and systemic innate immune responses (22). The process by which the innate immune system affects OA includes the following: 1) synovial joint immune cells are activated and generate DAMPs by interactions with invariable PRRs; 2) host responses to DAMPs activate the innate immune response; and 3) rapid-onset inflammatory responses are initiated (68). The innate immune cells consist of macrophages, neutrophils, DCs, natural killer (NK) cells, and mast cells, among others. Next, we will show the regulation of different innate immune cells in the gut microbiota and OA separately (Figure 2).

3.1 Macrophages in the gut microbiota and OA

In the knee synovium of OA patients, macrophages are the most widespread cell types (82), and are vital components of innate immune cells. Macrophages are a heterogeneous population, and the prominent features of macrophages are the functions of their

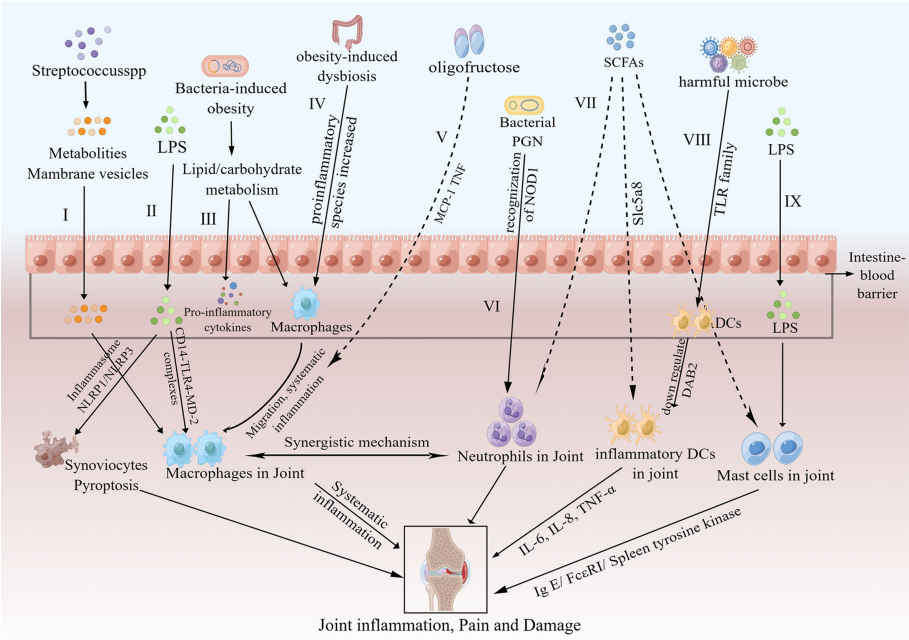

FIGURE 2
Pathways of the effects of the gut microbiota on OA via innate immunity. I. Metabolites and membrane vesicles produced by Streptococcus species could pass through the intestinal barrier and activate macrophages in the synovial lining, thus causing low-level systemic inflammation, joint inflammation and joint damage (69). II. LPS passes through the intestinal barrier and activates joint macrophages via CD14–TLR4–MD-2 complexes; moreover, LPS induces synoviocyte pyroptosis via either NLRP1 or NLRP3 inflammasomes (19, 70). III. Bacteria-induced obesity could increase lipid/carbohydrate metabolism-related gene expression, which activates macrophages and increases proinflammatory cytokine expression (71). IV. Obesity increases the abundance of key proinflammatory species, affects the migration of macrophages to joints and results in downstream systemic inflammation (72). V. Oligofructose could decrease systematic inflammation by inhibiting MCP-1 and TNF (72). VI. Bacterial PGN enhances neutrophil function via the recognition of NOD1 (73). VII. SCFAs reverse the recruitment of neutrophils (74), inhibit the development of DCs through Slc5a8 by inhibition of HDAC (75) and suppress mast cell degranulation (76, 77). VIII. After exposure to harmful microbes, DCs are switched to inflammatory DCs by downregulation of DAB2 (78) and were stimulated by TLRs to produce more inflammatory cytokines, such as IL-6, IL-8 and TNF-α (79). IX. LPS could activate mast cells (80), and mast cell activation expediates the progression of OA via the IgE/FcεRI/spleen tyrosine kinase signalling axis (81). LPS, lipopolysaccharide; CD14–TLR4–MD-2 complex, CD14-toll like receptor 4-myeloid differentiation protein-2 complex; NLRP, nod–like receptor protein; MCP-1, monocyte chemotactic protein 1; TNF, tumor necrosis factor; PGN, peptidoglycan; SCFAs, short-chain fatty acids; DCs, dendritic cells; HDAC, histone deacetylases; Slc5a8, Na (+)-coupled monocarboxylate transporter; DAB2, disabled homolog 2; FcεRI, IgE receptor.

surface markers and cytokine secretion. Based on the surface marker phenotype, macrophages are divided into "classically activated macrophages (M1)" and "alternatively activated macrophages (M2)" (83). Proinflammatory factors, such as LPS and IFN-γ, can activate M1 macrophages, and once activated, they can generate proinflammatory cytokines including inducible NOS (iNOS), IL-6, IL-1β and tumor necrosis factor-α (TNF-α), so M1 macrophages are thought to be proinflammatory. M2 macrophages, triggered by IL-13 and IL-4, are considered to be inflammatory inhibitors and can generate a large number of anti-inflammatory cytokines consisting of arginase, IL-1 decoy receptor and IL-10, which block the activity of IL-1β and TNF-α (83).

CD14 exists in various immune cells, such as monocytes and macrophages, and interacts with complexes of LPS and LBP (84, 85). LPS initiates proinflammatory innate immune responses via the CD14-LPS-LBP complex and the cd14-TLR4-myeloid differentiation protein-2 (MD-2) complex (86). As a result, the secretion of proinflammatory cytokines, including transforming growth factor-beta (TGF-β), matrix metalloproteinases (MMPs), TNF-α, IL-15, IL-10, IL-8, IL-6, IL-1β and free radicals, is induced (87, 88), which contribute to the secondary inflammatory responses in the joint tissue and exacerbate OA. To date, many researchers have conducted a large number of studies on macrophages and macrophage-produced mediators, and they have found that macrophages and their mediators were significantly associated with inflammatory changes and devastating responses in the synovial membrane and cartilage of OA (82). Huang et al. strongly proposed the role of LPS in the pathogenesis and severity of knee osteophytes, joint space size and pain degree in knee OA patients (18) and concluded that a two-hit theory can account for the potential mechanisms of LPS in OA progression (19). The first hit occurs when LPS activates joint macrophages via CD14–TLR4–MD-2 complexes and the second hit occurs when LPS induces the overall inflammatory response and the destruction of the joint structure due to the coexistence and complementarity mechanism, such as the inflammasome pathway or DAMPs in the form of broken cartilage-matrix molecules (19). In addition, LPS can induce fibroblast-like synoviocyte pyroptosis via either nod –like receptor protein (NLRP) 1 or NLRP3 inflammasomes, which contributes to the progression of OA (70). Taken together, LPS plays a vital role in innate immunity and macrophage-associated inflammatory responses, which are important to the pathogenesis of osteoarthritis.

According to the research by Kraus et al., the number of activated macrophages was significantly associated with pain symptoms in the knee as well as the radiological severity of knee OA. In addition, they reported that macrophages are the cause of OA-related pain at the affected joint site (89). Analogously, Daghestani et al. reported that in the synovium and joint capsule from subjects with knee osteoarthritis, many activated macrophages were detected; furthermore, they demonstrated that activated macrophages were positively associated with the degree of knee pain, decreased joint space and joint destruction in knee OA patients (90). Hsueh et al. found that the main immune cell types in the knee joints of OA patients were macrophages and neutrophils, which contribute to the pathogenesis and deterioration of OA in a synergistic way (91). Among the various risk factors for OA, obesity is widely accepted and can affect OA through the dysbiosis of the gut microbiota, which can increase systemic inflammation (92). According to the study by Schott et al., obesity decreases the amount of beneficial Bifidobacteria and increases the number of key proinflammatory species in the murine gut; as a consequence, the migration of macrophages to knee synovial tissues, the activation of downstream circulatory inflammatory responses and more destruction in the knee cartilage are observed (72). Oligofructose, a kind of nondigestible prebiotic fiber, can repair the imbalance in enteric dysbacteriosis, particularly the abundance of Bifidobacterium pseudolongum, and decrease systemic inflammation by downregulating the expression of TNF and monocyte chemotactic protein 1 (MCP-1) (72). In the Rotterdam Study and a large population-based cohort study, Boer et al. revealed that changes in the gut microbiota, especially a large number of streptococci, are significantly linked to enhanced pain symptoms in the knee. The possible mechanism is that metabolites and membrane vesicles produced by Streptococcus species can pass through the gut barrier and activate synovial macrophages to trigger low-grade systemic inflammation and aggravate joint inflammation and injury (14). Bacteria-induced obesity is known to increase the expression of genes involved in carbohydrate and lipid metabolism (69), thus activating macrophages and the migration of related inflammatory cells to fatty tissues, which release proinflammatory mediators into the blood circulation and aggravate systemic inflammation and the development of osteoarthritis (71). According to the research by Huang et al., fecal microbiota transplantation from subjects with OA and metabolic syndrome accelerates OA in mice, which activates TGF-β signalling pathways to regulate multiple immune cells, such as macrophages, NK cells, DCs, T cells and B cells (20). In summary, macrophages play a vital role in the immune responses between the intestinal microbiome and OA, and more studies need to be conducted to uncover the precise mechanism between macrophages, the intestinal microflora and OA, which may provide a novel target for macrophage immunomodulation in OA treatment.

3.2 Neutrophils in the gut microbiota and OA

As the body's first line of defense against microorganisms, neutrophils play a crucial role in innate immunity (93). Brotfain et al. reported that neutrophils from obese subjects were primed with enhanced chemotactic activity, increased superoxide generation and normal adherence and phagocytosis; therefore, neutrophils may participate in the progression of osteoarthritis with obesity as a crucial risk factor (94). Hsueh et al. showed that the number of neutrophils was the highest in knee synovial fluid with elevated TGF-β and elastase, which are significantly associated with the severity of radiographic knee OA (91). Furthermore, neutrophils and macrophages in OA knee joints act in a synergistic manner during the development and deterioration of OA (91). Kyburz et al. revealed that bacterial peptidoglycan (PGN) can activate synovial fibroblasts via TLR2 and induce MMP and

proinflammatory cytokine secretion, thus resulting in joint inflammation and destruction (95). In addition, van der Heijden et al. detected bacterial PGN in knee synovial tissues of individuals suffering from OA (96), where PGN might enhance synovial inflammation. Clarke et al. revealed that PGN, derived from the gut microbiome, systemically primes the innate immune response via the recognition of NOD1, enhancing neutrophil function (73). Interestingly, in mice with no-fiber supplementation for 2 weeks, significant alterations in the intestinal microbiome and significantly enhanced neutrophil-endothelial interactions in the colonic microvasculature were observed by Shen et al. Moreover, supplementation with acetate reversed the recruitment of neutrophils, which indicates the crucial participation of SCFAs in the modulation of neutrophils (74). Taken together, productions and metabolites by the gut microbiota may influence OA progression by interacting with neutrophils. To date, there have been no such studies, and more studies are warranted to uncover the potential relationship between neutrophils, OA and the gut microbiome in the future.

3.3 Dendritic cells in the gut microbiota and OA

DCs are antigen-presenting cells derived from monocytes and can recognize and react to pathogen-associated and danger-associated signals, therefore bridging the innate and adaptive immune systems. Mature DCs mainly activate T cells to prime the adaptive immune system. Unlike macrophages, DCs do not have typical surface markers and are divided into plasmacytoid DCs (pDCs) and myeloid DCs (cDCs), and myeloid DCs are composed of myeloid cDC1 and myeloid cDC2 on the basis of cell lineage and correlate with the differential expression of essential transcription factors, such as interferon regulatory factors 8 and 4 (97). Now, a unified classification of mammalian DCs has been introduced, and another type of DC has been defined as monocyte-derived inflammatory DCs, which are distributed in the inflammatory site (98). In healthy subjects, different DC subsets in the intestinal mucosa are present in the tolerogenic form. Once exposed to microbes, DCs are switched to an inflammatory phenotype by downregulation of disabled homolog 2 (DAB2), which is inhibited by the interactions of the TLR ligands TRIF and MyD88 (78). Butyrate and propionate, bacterial fermentation products, can inhibit the development of DCs through the Na (+)-coupled monocarboxylate transporter (Slc5a8) by inhibiting histone deacetylases (HDACs) (75). In addition, Trompette et al. found that propionate can protect against allergic inflammation by modulating DCs, which are dependent on G protein-coupled receptor 41 (99). Engevik et al. showed that Lactobacillus reuteri surface components and metabolites can promote immature DC maturation and enhance anti-inflammatory IL-10 production by DCs (100). At the OA site, DCs are mainly derived from blood mononuclear cells and manifest a proinflammatory phenotype (101). The TLR family, especially TLR4, plays a vital role in the initiation of DCs in osteoarthritis, which expedites the progression of osteoarthritis (78, 79, 102). Nie et al. showed that TLR 1-8 expression was significantly elevated in DCs of OA mice, and TLRs can stimulate DCs to produce more proinflammatory factors, such as TNF-α, IL-6 and IL-8, while inhibition of TLR in DCs reversed the inflammatory response (79). Therefore, the gut microbiota may affect inflammatory DC function, which interacts with TLR family members to promote the pathogenesis of OA. However, more research is urgently needed to verify the interactive mechanism between the gut microbiota, DCs and OA.

3.4 NK cells in the gut microbiota and OA

NK cells are CD56+CD3- lymphocytes comprising approximately 15% of all circulating lymphocytes, and can kill microorganisms through death-inducing receptors or the release of soluble molecules, including perforin and granulysin, so NK cells are vital components of the innate immune system. Based on the density of CD56 on the cell surface, NK cells are distributed into two cell subsets: CD56(bright) and CD56(dim) (103). CD56(dim) NK cells, with elevated levels of FC gamma receptor III (CD16) and Ig-like NK receptors, exhibit more natural cytotoxicity (103). In contrast, CD56(bright) NK cells produce more cytokines, show lower natural cytotoxicity and are CD16(dim) or CD16(-) (103). In addition, NK cells can interact with other immune cells to exhibit indirect antibacterial ability. For example, IFN-γ, produced by NK cells, can stimulate macrophages/monocytes and neutrophils to migrate and adhere; as a consequence, the related phagocytosis and oxidative killing effects are activated (104). In addition, IFN-γ can help DCs mature and initiate the generation of cytokines, such as IL-12 and TNF-α (104). NK cells can secrete chemotactic and antimicrobial peptides, such as α-defensins and cathelicidin (LL-37), and LL-37 has been proven to be chemotactic for CD4+ T cells and polymorphonuclear leukocytes, which may participate in the antimicrobial progress of NK cells (105).

Huss et al. found that NK cells contained almost 30% of the CD45+ lymphocytes in the synovium of OA patients and expressed the chemoattractant receptors CCR5 and CXCR3. Compared with blood NK cells, NK cells in the synovium of OA patients show a silent phenotype consistent with post-activated exhaustion, which is impaired by cytokine-stimulated IFN-γ production (106). Jaime et al. found that, in comparison with peripheral blood lymphocytes, most NK cells in the synovial fluid were CD56+CD16(-) NK lymphocytes, which expressed a lower number of cytotoxic mediators (perforin and granzyme B) (107). NKG2D (NK group 2, member D) is a danger sensor and a valid activator of immune responses, and NK cells express NKG2D to recognize and clear infected and transformed cells expressing cognate ligands (108). In addition, NKG2D enhances Th1 and proinflammatory Th17 cell effector functions with high production of proinflammatory cytokines during antigen-induced arthritis (108). In summary, unlike NK cells in the blood, NK cells in OA knee synovial fluid present a quiescent phenotype; however, IFN-γ produced by NK cells can activate neutrophils, macrophages, and DCs. In addition, NKG2D expressed by NK cells can activate T cells, so further investigations are needed to interpret the precise mechanism of NK cells in the gut microbiota and OA.

3.5 Mast cells in the gut microbiota and OA

Mast cells act as sentinels in the innate immune system and respond to endogenous danger signals as well as exogenous pathogens rapidly (109). Various factors, such as the IgE receptor FcεRI, IL-33 and complement receptor C5aR, can induce mast cell degranulation to release preformed mediators, such as proinflammatory lipids, tryptases, histamine, chemokines and cytokines (81). Zhang et al. reported that, in mast cells derived from murine bone marrow, butyrate suppresses FcεRI-dependent cytokine production, such as IL-6 and TNF-α via HDAC inhibition (76). Similarly, Folkerts et al. documented that propionate and butyrate could suppress the degranulation of human or mouse mast cells with or without IgE mediation, which was associated with HDAC inhibition (77). Further investigation showed that butyrate downregulated the tyrosine kinases BTK, SYK, and LAT by reducing acetylation at the related promoter regions, which are critical transducers of FcεRI-mediated signals (77). In addition, LPS can activate mature mast cells to produce tryptase, chymase and carboxypeptidase (80). Similarly, Gupta et al. observed that mast cells were activated by LPS once exposed to bacterial infection (110).

It is widely known that mast cells and their mediators are distributed in the synovial fluids and synovial tissues of OA patients (81). In addition, Kulkarni et al. reported that mast cells were differentially distributed in osteophytes and knee synovial fluid, which may further accentuate the inflammatory pathology of OA (111). De Lange-Brokaar et al. revealed that the numbers and degranulation status of mast cells were positively linked to worsening cartilage injury and aggravated synovitis in people with OA, indicating that mast cells contribute to the progression of OA (112). According to Wang et al., mast cell deficiency improved osteoarthritis in mast cell-knockdown mice, and tryptase, a specific product of mast cells, induced inflammation, chondrocyte apoptosis, and cartilage breakdown (81). The possible mechanism of mast cells in the progression of OA is via the IgE/FcεRI/spleen tyrosine kinase signalling axis (81). Interestingly, Zhao et al. proposed a new synovial tissue pathotype (mast cell-low, mast cell-medium, and mast cell-high) for OA patient joints on the basis of the differential expression of prototypical and distinct mast cell markers, and pharmacologic blockade of histamine activity can reduce the severity and OA-related mediators in a mouse OA model (113). Hence, further investigation into the relationship between mast cells in the gut microbiota and OA may provide a novel direction for OA treatment.

4 The gut microbiota modulates adaptive immunity

As described above, most studies have focused on the effect of innate immunity on the gut microbiota and osteoarthritis, especially on macrophages. Recently, some studies have focused on the effect of adaptive immunity on the intestinal microbiome and OA. Lymphocytes account for 10% of nonadipocyte cells in human adipose tissue and include B cells, T cells, innate lymphoid cell group 2 cells, NK cells and NK T cells (114). De Lange-Brokaar et al. reviewed immune cells, their cytokines and synovial inflammation in OA and concluded that most of immune cells found in OA synovial tissues were mast cells, macrophages and T cells while B cells, NK cells and plasma cells were detected in lower amounts; in addition, cytokines related to T cells or macrophages were detected abundantly in OA synovial tissues, indicating that T cells and macrophages were activated in OA synovial tissues (115). To further investigate the immune cells and related inflammation in OA, Klein-Wieringa et al. found that the main immune cells in the synovial tissues of OA patients were T cells and macrophages, followed by mast cells, and most of proinflammatory cytokines were produced by T cells and macrophages even without additional stimulation, among which CD4+ and CD69+ T cells were highly present (116). In addition, the amount of CD4+ T cells in synovial tissues was significantly associated with poor pain feelings classified by the visual analog scale (VAS) (116). Taken together, adaptive immune cells, particularly T cells, play a vital role in the modulation of the intestinal microbiome and osteoarthritis, and the related mechanisms are summarized in Figure 3.

4.1 T cells in the gut microbiota and OA

Among adaptive immune cells, the predominant immune cells are T cells, which are crucial in the progression of OA. To date, it has been widely accepted that significant alterations in Th17 cells, Th9 cells, T memory cells, cytotoxic T cells, regulatory T (Treg) cells and Th1 cells are found in the synovial fluid, synovial tissues and peripheral blood of people with OA (124). However, the role of follicular helper T (TFH) cells, Th22 cells and Th2 cells in the pathogenesis of OA is still uncertain.

The gut microbiota, as a risk factor for OA, has been proposed as a regulator of the T-cell response, especially for Th17 cells (125). Th17 cells, the most primitive subset of CD4+ T cells, are characterized by the production of proinflammatory cytokines, such as IL-22, IL-21 and IL-17, and proinflammatory cytokines, including IL-21, IL-6 and TGF-β, can initiate the induction of Th17 cells (118). TGF-β has a dual role in immune modulation, and low amounts of TGF-β induce the differentiation of Th17 cells whereas high amounts of TGF-β induce Treg cells to inhibit inflammation (118). Imbalances in the intestinal flora can modulate the differentiation of CD4+ T cells into Treg cells or effector T cells, which is crucial for immune homeostasis and joint inflammation (23). Segmented filamentous bacteria (SFB), which accumulate in the synovial fluids and synovium of subjects with OA, can induce Th17 cell accumulation by increasing local serum amyloid A (SAA) production, and increased SAA can stimulate DCs in the lamina propria to induce Th17 cells (23). Clostridia is a predominant type of commensal microorganism in the colon and can induce local Treg cells in the colon to inhibit inflammatory and allergic responses, which presumably are induced by DCs (126). Moradi et al. showed that abundant Treg cells accumulated in synovial fluid and the synovial membrane from individuals with OA and that they existed as activated effector memory cells ($CD62L^{-}CD69^{+}$), while fewer Treg cells were detected in peripheral blood concurrently with

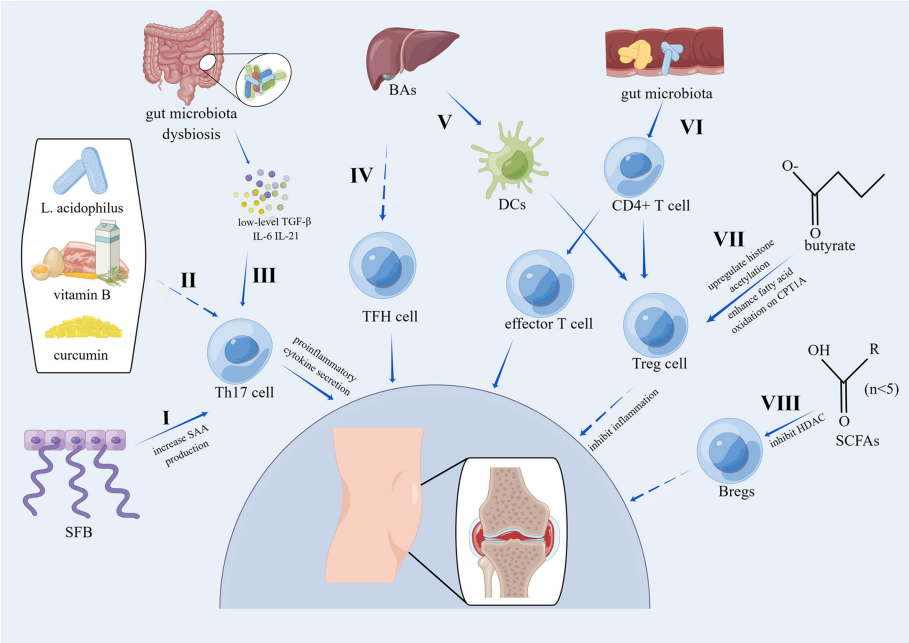

FIGURE 3
Pathways of the effects of the gut microbiota on OA via adaptive immunity. I. SFB induces the accumulation of Th17 cells by increasing local SAA production, which can act on lamina propria DCs to stimulate the induction of Th17 cells (23). II. The combination of L. acidophilus, vitamin B, and curcumin suppresses Th17 cell differentiation by phosphorylating STAT3 (117). III. Activated Th17 cells can secrete proinflammatory cytokines, and Th17 cells are induced by low levels of TGF-β, IL-6, and IL-21 (118). IV. BAs can inhibit the activation of TFH cells (119). V. 3β-Hydroxydeoxycholic acid, a kind of secondary BA, can induce colonic Treg cells by acting on DCs in a CNS1-dependent manner (120). VI. Gut microbiota dysbiosis modulates the differentiation of CD4+ T cells into Treg cells or effector T cells (23). VII. Treg cells have been proven to inhibit inflammation (121) and are induced by butyrate by upregulating histone acetylation or enhancing fatty acid oxidation in a CPT1A-dependent manner (122). VIII. SCFAs can upregulate Breg cells in a manner dependent on HDAC inhibitory activity (123). SFB, segmented filamentous bacteria; SAA, serum amyloid A; DCs, dendritic cells; BAs, bile acids; CPT1A, carnitine palmitoyl transferase 1A; HDAC, histone deacetylases; STAT3, signal transducer and activator of transcription 3; CNS1, conserved non-coding sequence 1.

resting central memory cells ($CD62L^{+}CD69^{-}$) (127). So et al. showed that coadministration of Lactobacillus casei with type II collagen/glucosamine reduced arthritic alterations and suppressed cartilage destruction in an OA model; furthermore, cotreatment of Lactobacillus casei with type II collagen/glucosamine reduced proinflammatory cytokines, such as TNF-α, IFN-γ, IL-6, IL-2 and IL-1β, in CD4+ T cells (128). Jhun and colleagues found that the combination of L. acidophilus, vitamin B, and curcumin suppressed Th17 cell differentiation and maintained the Treg cell population by phosphorylated signal transducer and activator of transcription 3 (STAT3); at the same time, the combination suppressed the proinflammatory Th17-related cytokine IL-17 and increased the anti-inflammatory Treg-related cytokine IL-10 in human peripheral blood mononuclear cells (117). In a rat OA model, the combination of L. acidophilus, vitamin B, and curcumin alleviated pain, protected cartilage, regulated the anabolic/catabolic balance and reduced proinflammatory cytokines, including MCP-1, TNF-α, IL-17 and IL-1β. TFH cells can regulate the activation of B cells to generate immunoglobulins via the secretion of IL-21, and chemokine (C-X-C motif) receptor 5 (CXCR5)+CD4+ T cells are thought to be TFH cells with surface markers, such as the transcription factor, CD40 ligand, programmed death-1, inducible costimulator and CXCR5 (129). Shan et al. showed that, in comparison with healthy individuals, IL-21+ TFH cells were significantly higher in people with stage IV OA in comparison to those with stage II and stage III, and the levels of serum IFN-γ, IL-17A and IL-21 were increased concurrently (130). Furthermore, IL-21+ TFH cells were positively correlated with the level of serum CRP and WOMAC scores of OA patients. Recently, Cheng et al. found that primary bile acid, a metabolite of the gut microbiota, can inhibit the activation of CXCR5+CD4+ TFH cells (119). Butyrate, which is critical for the maintenance of intestinal microbiome homeostasis, can promote the generation of inducible Treg (iTreg) cells by enhancing histone acetylation for gene expression by inhibiting HDAC; in addition, in a carnitine palmitoyl transferase 1A (CPT1A)-dependent manner, butyrate can also facilitate iTreg differentiation by increasing fatty acid oxidation (122). In a rheumatoid arthritis model, butyrate significantly increased systemic Treg cells and reduced Th17 cells by inhibiting the expression of proinflammatory cytokines, including IL-17A, IL-1β and IL-6, and promoting anti-inflammatory IL-10 expression (131), and it is suggested that butyrate may have the same effects in OA. Campbell et al. revealed that 3β-hydroxydeoxycholic acid (isoDCA), a kind of secondary BA, can enhance the induction of colonic Treg cells by acting on DCs in a conserved noncoding sequence 1 (CNS1)-dependent manner (120). Overall, more studies are needed to uncover the role of TFH cells, Treg cells and Th17 cells in the intestinal microbiome and OA, and SCFAs can modulate Treg cell levels and Th17 cell differentiation, which can be novel targets for future management of OA.

4.2 B cells in the gut microbiota and OA

B cells are known to generate immunoglobulins and to regulate immunity. As immunosuppressive cells, regulatory B cells (Bregs) can maintain immunological tolerance via IL-10, IL-35, and TGF-β1 (132). Bregs can inhibit the activation of Th1 cells, Th17 cells and CD8+ T cells; regulate the differentiation of macrophages and DCs; and facilitate the induction of Treg cells (133–135). Studies have reported that oligoclonal B cells infiltrate the joint synovium of people with OA, indicating that an antigen-presented immune response might contribute to the progression of OA (136). Sun et al. revealed that IL-10 could induce B cells, and B cells were detected in the synovial fluid in OA. Compared with rheumatoid arthritis patients, in the joint synovium of OA patients, the frequency of IL-10+ B cells was higher, while the total number of IL-10+ cells in synovial B cells was lower (137). Phenotypical analysis showed that the IL-10+ B cells were IgM+CD27+ and they expressed more IL-10 and inhibited IFN-γ expression and the proliferation of autologous T cells via IL-10. IgM+CD27+ B cells in the synovial fluid were negatively associated with the severity of OA (137). Rosser et al. reported that SCFAs were reduced in rheumatoid arthritis patients and arthritic mice in comparison with healthy people and in mice, and butyrate supplementation significantly alleviated the arthritis degree (138). Supplementation with butyrate amplified AhR activation in Bregs, which relieved arthritis (138). Similarly, Yao et al. also found that SCFAs, including valerate, butyrate, propionate and acetate, were reduced in rheumatoid arthritis patients, and the amounts of butyrate, propionate and acetate were positively correlated with the frequency of Bregs instead of Tregs in the peripheral blood (139). Treatment with acetate, propionate and butyrate mitigated symptoms of arthritis, increased the frequency of Bregs and reduced the frequency of transitional B and follicular B cells in collagen-induced mouse arthritis via the FFA2 receptor (139). Similarly, Zou et al. reported that treatment with SCFAs could upregulate Bregs and ameliorate clinical scores of arthritis in collagen-induced mouse arthritis in a manner dependent on HDAC inhibitory activity (123). Taken together, the immunosuppressive role of Bregs in rheumatoid arthritis and autoimmune arthritis suggest that the gut microbiota may regulate Bregs in OA in a similar way as described above. Bregs may be a novel therapeutic choice for OA, and more *in vivo* and *in vitro* studies are needed.

5 Gut microbiome modulation as a new choice for OA treatment

The main characteristics of OA are chronic and progressive joint cartilage destruction and osteophyte proliferation, which ultimately result in chronic disability and a crucial burden on health care systems globally. To date, the most recognized clinical therapy for OA is palliative treatment, which ameliorates the symptoms of OA (140). Presently, many factors have been reported to act in the initiation and pathogenesis of OA; however, the intestinal microbiome is considered to be a novel pathogenic factor for osteoarthritis, and related mechanisms have been elucidated (67). Furthermore, some studies have suggested that modulation of the gut microbiota can be a new treatment for OA. Thus far, interventions consisting of prebiotics, probiotics, diet, nutraceuticals, exercise and fecal microbiota transplantation (FMT) for the intestinal microbiome in OA have been proposed (Figure 4).

5.1 Prebiotics

The selective utilization of prebiotics by the host's gut microbiome is good for health, and the major components of prebiotics are part of carbohydrate groups, mostly oligosaccharides; however, prebiotics are not limited to carbohydrates only (141). According to the study by Schott et al., administration of oligofructose, a kind of prebiotic, restored the imbalance in the intestinal microflora in obese mice by increasing key commensal microflora, particularly beneficial Bifidobacteria, suppressed the downstream inflammatory responses in the local and systemic circulation and eventually alleviated OA-related joint cartilage injury (72). Oligofructose supplementation altered microbial populations in the gut of both lean and obese mice, probably due to the phylum Actinobacteria. The obese mice showed a reduced Bacteroidetes/Firmicutes ratio and a lack of Actinobacteria, while oligofructose could partially change the Bacteroidetes/Firmicutes ratio and increase the amount of Actinobacteria in obese mice, which is primarily associated with the abundance of Bifidobacteria. Oligofructose supplementation can induce Bifidobacteria to decrease gut permeability, increase the expression of tight junction proteins and inhibit inflammation. In addition, oligofructose reduced proinflammatory cytokines, such as MCP-1 and IL-12, and increased the anti-inflammatory cytokine IL-10 levels in obese mice. Additionally, oligofructose suppressed the migration of obesity-related macrophages to the joint synovium and reduced the proinflammatory cytokine MCP-1 levels in obese mouse joints; however, the number of tissue macrophages was not changed, indicating the role of oligofructose in the control of macrophage migration in the pathogenesis of OA. Similarly, Rios et al. observed that early intervention with oligofructose supplementation could reverse the harmful effects of a high-fat/high-sucrose (HFS) diet on joint damage in a rat OA model and could significantly ameliorate insulin resistance, restore gut microbiota dysbiosis and decrease endotoxemia (142). After oligofructose supplementation, Rios and colleagues observed an expansion of Bifidobacterium, Bacteroides/Prevotella and Roseburia and a decrease in the abundances of Akkermansia muciniphila, Methanobrevibacter, Faecalibacterium prausnitzii, Clostridium cluster I and Clostridium cluster IV. Bifidobacterium, Bacteroides/Prevotella and Roseburia were positively associated with cartilage protection, while Akkermansia muciniphila, Faecalibacterium prausnitzii, and Clostridium cluster IV were positively associated with cartilage damage. However, for rats with 12 weeks of exposure to a HFS diet, prebiotics plus exercise could not reverse the already established knee damage (143). Collectively, oligofructose can play a prophylactic role in OA development; however, oligofructose cannot repair OA-induced

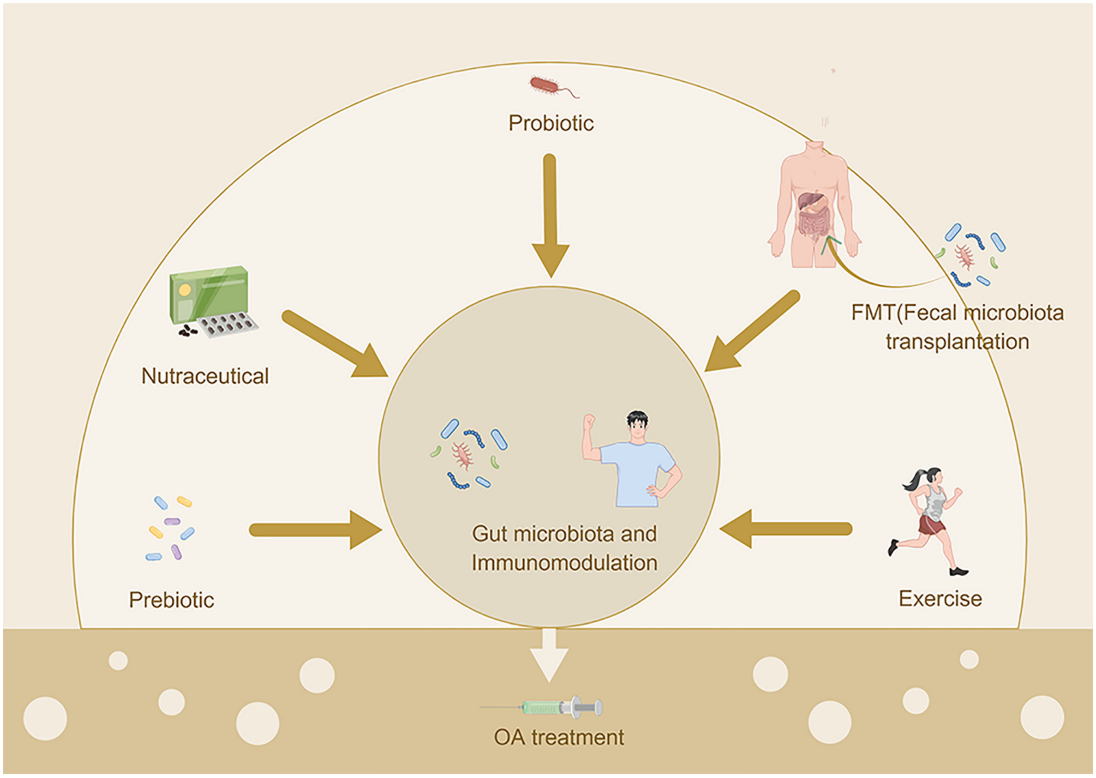

FIGURE 4
Therapeutic strategies for gut microbiota dysbiosis in OA patients.

cartilage damage. To date, no human studies have been performed, and future studies on prebiotics and OA in humans are needed to determine the appropriate time and dose of prebiotic supplementation in people with high risk factors for OA.

5.2 Probiotics

Probiotics, composed of live microorganisms, play a crucial role in the modulation of the intestinal microflora by enhancing the generation of antimicrobial compounds as well as immunoglobins and reducing the release of bacterial endotoxins (144, 145). Lactobacillus casei are the most studied probiotics. Lei and colleagues conducted a randomized double-blind, placebo-controlled clinical trial with a sample size of 537 individuals with OA (146), which is the most convincing evidence. The authors reported that after 6 months of Lactobacillus casei Shirota supplementation, both the VAS and WOMAC scores were significantly reduced compared with those in the placebo control group, accompanied by a significantly decreased serum level of high sensitivity C-reactive protein (hs-CRP), and serum hs-CRP levels were significantly associated with VAS and WOMAC scores, indicating that oral administration of Lactobacillus casei might be a new option for clinical therapy in knee osteoarthritis (146). Similarly, Sullivan et al. reported that oral Lactobacillus acidophilus consumption dramatically relieved joint pain, prevented cartilage degradation, expanded beneficial bacteria such as Akkermansia muciniphila and Lachnospiraceae, and lowered the alpha diversity in the gut compared to those in the vehicle group (147). In addition, Lactobacillus acidophilus supplementation suppressed proinflammatory cytokines, including NF-κB, TNF-α, and IL-1β, and increased anti-inflammatory IL-10, suggesting that Lactobacillus acidophilus may be a safe OA disease-modifying drug. In addition to lactic acid bacteria, Sim et al. revealed that tyndallized Clostridium butyricum could protect the knee synovium and joint cartilage, increase the weight-bearing distribution by ≥20%, and dramatically reduce the number of fibrous tissues by inhibiting inflammatory factors (IL-6, leukotriene B4, Cox 2) and MMP production and increasing anti-inflammatory cytokine INF-γ (148). Henrotin et al. reported that oral consumption of lyophilized inactivated culture from Bifidobacterium longum for 12 weeks reduced the structural damage of joint cartilage, synovial inflammation and type II collagen degradation in spontaneous OA pig model, indicating a potential preventive role of Bifidobacterium longum in the progression of OA (149). According to the research by Li et al., oral supplementation with Clostridium butyricum could relieve OA pain, ameliorate cartilage damage and inhibit synovial hyperplasia by downregulating TNF-α and IL-1β in OA cartilage and synovium of rat models (150). Lin and colleagues revealed that Lactobacillus plantarum improved joint mechanical load, alleviated cartilage damage, and reduced synovial inflammation by inhibiting inflammatory factors (TNF-α, IL-1β) in OA cartilage and synovium

of the anterior cruciate ligament transection-induced rat models (151). Streptococcus thermophilus (TCI633), a newly discovered bacterium in human milk, can produce hyaluronate in the gastrointestinal tract and widely reduce the inflammatory responses of synovial tissues and joint cartilage structural lesions in a dose-dependent manner in rat models. In addition, TCI633 can efficiently increase the production of type II collagen and decrease the apoptosis of chondrocytes in cartilage (152). Furthermore, Lyu et al. conducted a clinical RCT with 80 individuals for 12 weeks, and they observed no significant improvements in serum C-reactive protein and serum collagen type II C-telopeptide (sCTX-II) between the TCI633 group and the control group, which may be influenced by Kellgren/Lawrence grading, small sample size and a short observational time; however, the distinct WOMAC scores in the TCI633 group indicated that TCI633 might slow the pathological process of knee OA (153). In addition to cartilage protection, Taye et al. conducted an N-of-1 trial and showed that probiotic intervention was associated with lower pain scores, suggesting the role of probiotics in pain relief in OA patients (154).

So et al. revealed that coadministration of Lactobacillus casei and type II collagen/glucosamine could significantly relieve the pain of OA and reduce cartilage destruction in a rat OA model compared to type II collagen/glucosamine or Lactobacillus casei alone; furthermore, oral supplementation with Lactobacillus casei and type II collagen/glucosamine could significantly inhibit proinflammatory cytokines, such as TNF-α, IFN-γ, IL-17A, IL-12B, IL-12A, IL-6, IL-2, and IL-1β, and upregulate anti-inflammatory cytokines, including TGF-β, IL-10, and IL-4, in CD4+ T cells (128). In addition, administration of Lactobacillus casei and type II collagen/glucosamine significantly decreased inflammatory molecules, such as Cox-2, TNF-α, IL-6 and IL-1β, and MMPs, in synovial fibroblasts and chondrocytes in comparison with type II collagen/glucosamine or Lactobacillus casei alone. Korotkyi et al. performed an animal test to verify the effect of a multistrain probiotic with or without chondroitin sulfate addition on the expression of Col2a1, Tgfb1 and Ptgs2 in a monoiodoacetate-induced rat OA model (155). In a rat OA model, the expression of the proinflammatory cytokines Tgfb1 and Ptgs2 was upregulated, while the expression of the anti-inflammatory cytokine Col2a1 was downregulated. Separate supplementation with probiotics or chondroitin sulfate significantly suppressed the expression of Tgfb1 and Ptgs2 and increased Col2a1 expression. Compared with separate application, application of both showed significantly more alterations in the expression of Col2a1, Tgfb1 and Ptgs2 (155). In addition, Korotkyi et al. discovered that the separate application of probiotics and chondroitin sulfate slightly reduced OA scores, limited chondrocyte death and decreased subchondral bone resorption in rats; however, no significant decreases in the expression of TNF-α, NF-κB, TLR 2 and TLR 4 were detected (156). Combined treatment with probiotics and chondroitin sulfate significantly reduced OA scores and decreased the expression of TNF-α, NF-κB, TLR 2 and TLR 4 in synovial cells and chondrocytes (156). Korotkyi et al. conducted further investigation on the efficiency of probiotics and chondroitin sulfate separately or in combination in OA and observed that chondroitin sulfate and multistrain probiotics could regulate the NF-κB inflammatory signalling pathway mediated by TLR 2/4 and reduce the metabolism of cartilage, indicating that probiotics amplify the beneficial role of chondroitin sulfate in attenuating osteoarthritis (157). Wang et al. revealed that Akkermansia muciniphila, a gut commensal probiotic bacterial species, determined the role of chondroitin sulfate in OA (158). An optimum level of Akkermansia muciniphila can thicken the intestinal mucosa and activate mucosal immunity to prevent pathogen infiltration, while an overabundance of Akkermansia muciniphila can cause mucin degradation, damage to the colonic mucosa and severe leakage of endotoxin. Akkermansia muciniphila can compete with sulfatase-secreting bacteria and sulfate-reducing bacteria, which are expanded by oral chondroitin sulfate in the distal gut. In the presence of optimum levels of Akkermansia muciniphila, chondroitin sulfate can ameliorate OA; otherwise, chondroitin sulfate can aggravate OA (158). Therefore, the role of chondroitin sulfate in OA is affected by the gut microbiome, indicating that elderly patients or immunosuppressed patients may not benefit from oral chondroitin sulfate, and the combination of chondroitin sulfate with probiotics may show better improvements in OA patients. Jhun and colleagues found that the combination of L. acidophilus, vitamin B, and curcumin suppressed Th17 cell differentiation and maintained the Treg cell population by phosphorylating STAT3; at the same time, the combination suppressed the proinflammatory Th17-related cytokine IL-17 level and increased the anti-inflammatory Treg-related cytokine IL-10 level in human peripheral blood mononuclear cells (117). Moreover, the combination of L. acidophilus, vitamin B, and curcumin alleviated pain, preserved joint cartilage, increased the anabolic enzyme TIMP13, decreased the catabolic enzyme MMP13 and reduced the levels of proinflammatory cytokines, such as MCP-1, TNF-α, IL-17 and IL-1β, in the monosodium iodoacetate-induced rat OA model. In addition, the combination upregulated TIMP13 and downregulated MMP13 in human chondrocytes. The combination of probiotic complex (multiple Lactobacillus, Bifdobacterium, Streptococcus species), rosavin plus zinc exhibited anti-inflammatory and antioxidant properties, relieved pain, and improved cartilage destruction by inhibiting the production of catabolic factors (MMP3, TIMP3) as well as proinflammatory cytokines (TNF-α, IL-6) and increasing the anti-inflammatory cytokine IL-10 in rat models (159).

In sum, probiotics can mitigate the development of OA by relieving pain, inhibiting inflammatory responses and restoring the imbalance in the intestinal microbiome. In addition, the synergistic effect of probiotics and other pharmaceuticals on the gut microbiota and OA is better than their separate application. To better treat OA, large sample size clinical studies of probiotics and OA are necessary.

5.3 Nutraceuticals

Glucosamine sulfate and chondroitin sulfate are common medicines used to relieve the symptoms of OA in clinical therapy and are recommended by the European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases; in

contrast, chondroitin sulfate and glucosamine sulfate are not recommended for OA by the Osteoarthritis Research Society International (160). The intestinal microbiome can affect the metabolism of glucosamine sulfate and chondroitin sulfate in the gastrointestinal tract, so oral administration of glucosamine sulfate and chondroitin sulfate is limited. By analyzing the degradation products of CSA (the main component of chondroitin sulfate pharmakon) of the intestinal microbiome from six healthy humans, Shang et al. observed that each subject's microflora exhibited different degrading activities, but in all cases, the end products contained ΔUA-GalNAc4S, suggesting that chondroitin sulfate could be easily degraded to various degrees by many microbial communities, which may contribute to the poor bioavailability and different effects of chondroitin sulfate during the management of OA patients (161). In addition, Liu et al. investigated the impact of chondroitin sulfate disaccharides on the content and function of the intestinal microflora in mice under stressed conditions and in healthy mice. They found that chondroitin sulfate disaccharides can reduce blood LPS levels and increase the levels of fecal total SCFAs, especially butyrate. Additionally, chondroitin sulfate supplementation reduced the Proteobacteria abundance and increased that of Bacteroidetes in the gut, suggesting that chondroitin sulfate is a bioactive nutraceutical that inhibits inflammation and protects the gut (162). As we mentioned above, chondroitin sulfate can ameliorate OA with the assistance of Akkermansia muciniphila (158). In combination with beneficial gut microbiota, chondroitin sulfate can significantly decrease the expression of proinflammatory cytokines, such as Ptgs2 and Tgfb1, and increase proinflammatory Col2a1 expression in rat OA models (155). Recently, Zhang et al. investigated the effect of type II collagen peptides, cartilage powder from chicken and chondroitin sulfate in rat OA models and showed that chondroitin sulfate exhibited the best therapeutic effect for OA, as it dramatically improved joint cartilage morphology and decreased OA scores by reducing inflammatory cytokine levels in the serum or synovial fluid, such as prostaglandin E2, TNF-α, IL-1β, IL-6, and IL-17, and increasing the abundance of Bacteroidetes (163). For glucosamine, Sicard et al. reported that the mucin sugar N-acetyl-glucosamine could decrease biofilm formation of Escherichia coli by influencing the virulence properties of pathogenic E. coli, which help maintain the gut barrier (164). According to the study by Coulson et al., oral administration of glucosamine sulfate or green lipped mussel extract can improve symptoms of OA via the regulation of the components, metabolism and immunity of the intestinal microbiome; notably, the decrease in Clostridia was associated with improvements in the symptoms of OA, and Clostridia can induce the generation of Th17 cells to inhibit inflammation (165). Shmagel et al. performed a systematic review on the effects of glucosamine sulfate or chondroitin sulfate on intestinal microbial composition in humans and animals and reported that chondroitin sulfate could increase the amount of the genus Bacteroides in human and mouse intestines, whereas evidence of glucosamine sulfate on the gut microbiome was limited (166). Pycnogenol, a proprietary extract from pine bark, can be metabolized by the intestinal microbiome and alleviate the symptoms of OA via anti-inflammatory, antioxidative and chondroprotective effects. In addition, oral administration of pycnogenol could reduce gastrointestinal complications and hospitalization of people with OA by sparing the use of nonsteroidal anti-inflammatory drugs (167). In summary, nutraceuticals can modulate the gut microbiota and mitigate the symptoms of OA, whereas nutraceuticals can be affected in the gut by the intestinal microbiome. More studies on nutraceuticals are warranted for them to serve as a novel option for OA treatment.

5.4 Exercise

Exercise has been proven to alleviate pain and ameliorate joint function in OA patients (168). In addition to the reduction in mechanical load on subchondral bone and cartilage, exercises can increase the amount of beneficial microbial species, enrich the diversity of the microflora and improve the development of symbiotic bacteria, therefore providing benefits to the host (169). Some studies have reported that physical exercise impacts the content of the intestinal microbiome, such as increasing the ratio of Bacteroidetes-Firmicutes, enhancing the immune function of the intestinal mucosa, improving the profile of BAs and increasing the generation of SCFAs, such as butyrate, acetate and propionate (170), which are beneficial for mitigating symptoms in OA patients. As mentioned above, interventions with aerobic exercise and prebiotic fiber separately or in combination significantly protected knee joints from damage in rat OA models, suggesting that exercise may influence the progression of OA in coordination with the gut microbiota (142). To further interpret the significant synergistic effect of nutraceuticals and exercise in dealing with osteoarthritis, de Sire et al. summarized that synergistic physiogenomic and nutrigenomic treatment could reduce and slow down the complicated pathological characteristics of OA through apoptotic, proinflammatory and anti-inflammatory signals (171). Recently, Li et al. reported that wheel-running exercise enriched the diversity of the intestinal microbiome, modified the intestinal microbiome, reduced the amount of LPS in synovial fluids and blood, reduced the expression of MMP-13 and TLR4 and ameliorated cartilage damage in rat OA models, suggesting that moderate exercise is a novel therapeutic option for preventing and treating obesity-related OA (172). Whole body vibration (WBV), a novel kind of neuromuscular technique, uses the vibration generated by a vibration platform to improve the bioactivity of muscle groups. According to the research by Yu et al., M1 macrophages were polarized to M2 macrophages through the induction of WBV, and WBV could alter the fecal microbiome in diabetic mice (173). In addition, Song et al. reported novel impacts of WBV on the gut microbiome and immunity (174). They observed significantly increased levels of CD4 and CD25 positive lymphocytes and enhanced differentiation of Treg cells in the WBV group. Further microbiome analysis revealed that the amount of Lactobacillus animalis was dramatically elevated as a result of vibration application in mice, while Lactobacillus sanfranciscensis and Lactobacillus paraplantarum were increased in humans. Moreover, Lactobacillus spp. was associated with Treg cell differentiation in humans and mice (174). In addition, studies on the application of WBV in knee OA have been summarized. Furthermore, Wang et al. summarized possible mechanisms of WBV in knee OA, such as bone microstructure improvement, joint cartilage degeneration delay and the modulation of

inflammatory responses, which provide ideas for the future application of WBV in the management of knee OA (174). To date, studies regarding exercise, the gut microbiota and OA are scarce, and more relevant studies are warranted to interpret the precise mechanisms and guide the application of exercise in OA treatment.

5.5 Fecal microbiota transplantation

To address diseases correlated with the intestinal microbiome, fecal microbiota transplantation (FMT) has been introduced, where the nature of FMT is to transfer a healthy subject's feces into another's distal gastrointestinal tract (175). Notably, during the treatment for recurrent Clostridium difficile infection, FMT has been proven to be a dramatic success, and a relevant standard of fecal preparation for FMT was reported (176). Given its excellent manifestation in eradicating Clostridium difficile infection, researchers have explored the application of FMT in some diseases, including allergic diseases, autoimmune diseases, neurodevelopmental disorders, metabolic syndrome, irritable bowel syndrome and inflammatory disease (177), and after a trial in inflammatory bowel disease, FMT was considered to be a possible therapeutic option (178). As mentioned above, the gut microbiota is considered a virtual organ with 4 broad functions, namely, playing roles in nutrition and metabolism, epithelial cell differentiation and proliferation, immunomodulation and pathogenic resistance and clearance (12). Several studies have revealed that enteric dysbacteriosis was linked to the onset and development of OA; moreover, FMT was considered to be an important method in the manipulation of gut dysbiosis (179); hence, the hypothesis of FMT application in OA has been proposed. Huang et al. conducted a clinical trial to transfer human fecal samples from a group with knee osteoarthritis with metabolic syndrome, a group with knee osteoarthritis without metabolic syndrome and a healthy control group into germ-free mice 2 weeks prior to surgically induced OA by meniscal/ligamentous injury (20). In germ-free mice without transplantsation, minimal signs of OA and synovitis were observed. In mice transplanted with fecal samples from subjects with knee osteoarthritis with metabolic syndrome, Huang et al. found higher cartilage damage scores, higher levels of serum inflammatory factors (IL-1β, IL-6 and macrophage inflammatory protein-1α), higher serum LPS levels, higher intestinal permeability and a lower α diversity of the gut microbiome. By analyzing the correlation between the gut bacterial genera, cartilage histology scores and inflammatory factors, the enrichment of Fusobacterium and Faecalibacterium and the reduced abundance of Ruminococcaceae were significantly associated with both higher cartilage histology scores and greater inflammatory factor levels, indicating the direct gut microbiome-knee osteoarthritis connection and the possibility of gut microbiome targeted intervention. Given the modification of FMT in the intestinal microbiome, the application of FMT for OA treatment is possible. However, relevant studies are rare, and problems including donor selection and filtration, standards of fecal preparation, indications and contraindications and recipient monitoring are still need to be taken into consideration.

6 Conclusions and perspectives

Currently, the incidence rate and disability rate of OA are very high, which makes OA a crucial public health problem globally. The pathogenesis of OA needs to be fully elucidated to develop effective means for preventing and treating OA. The gut microbiome is one of the risk factors correlated with OA; however, the causal effect of the gut microbiome and OA is still controversial. As mentioned above, the gut microbiome interacts with risk factors for OA, such as obesity, estrogen, age, sex, diet, metabolic syndrome, genetics, inflammation, mechanical loading and exercise. In this manuscript, we have summarized the potential immune mechanisms of the intestinal microbiome during the onset and progression of knee osteoarthritis and relevant studies on the immunomodulation of the gut microbiome in the management of OA. To investigate the pathogenesis and uncover the immune mechanisms between OA and the gut microbiota, more research on the distribution and composition of the intestinal microbiome and immune cells in synovial fluids and synovial tissues is warranted. Moreover, the alterations in intestinal microflora in individuals with OA need further investigation to identify the specific pathogens involved in the immune responses. In addition, more research is required to identify the potential common pathways and the synergistic effect between prebiotics, probiotics, nutraceuticals and exercise in the immune modulation of the intestinal microflora. Due to the enormous development of metabolomics, transcriptomics and next-generation sequencing, future studies should include more novel interventions on immune cell modifications and gene regulation on specific gut microbiota related to OA, and it is likely that more studies will uncover the relationship between distinct cell subgroups and the gut microbiota in OA. Additionally, signals associated with enteric dysbacteriosis and immune modulation in OA patients will be identified, and novel therapies targeting the gut microbiota and immunomodulation will be proposed to prevent the progression of OA.

Author contributions

CS and JM conceived the study. CS, XZ, and TG screened the literatures and wrote the review. JM reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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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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TLR7/TLR8 activation and susceptibility genes synergize to breach gut barrier in a mouse model of lupus

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Background: Mounting evidence suggests that increased gut permeability, or leaky gut, and the resulting translocation of pathobionts or their metabolites contributes to the pathogenesis of Systemic Lupus Erythematosus. However, the mechanisms underlying the induction of gut leakage remain unclear. In this study, we examined the effect of a treatment with a TLR7/8 agonist in the B6.*Sle1.Sle2.Sle3* triple congenic (TC) mouse, a spontaneous mouse model of lupus without gut leakage.

Materials and methods: Lupus-prone mice (TC), TC.*Rag1*$^{-/-}$ mice that lack B and T cells, and congenic B6 healthy controls were treated with R848. Gut barrier integrity was assessed by measuring FITC-dextran in the serum following oral gavage. Claudin-1 and PECAM1 expression as well as the extent of CD45^{+} immune cells, B220^{+} B cells, CD3^{+} T cells and CD11b^{+} myeloid cells were measured in the ileum by immunofluorescence. NKp46^{+} cells were measured in the ileum and colon by immunofluorescence. Immune cells in the ileum were also analyzed by flow cytometry.

Results: R848 decreased gut barrier integrity in TC but not in congenic control B6 mice. Immunofluorescence staining of the ileum showed a reduced expression of the tight junction protein Claudin-1, endothelial cell tight junction PECAM1, as well as an increased infiltration of immune cells, including B cells and CD11b^{+} cells, in R848-treated TC as compared to untreated control mice. However, NKp46^{+} cells which play critical role in maintaining gut barrier integrity, had a lower frequency in treated TC mice. Flow cytometry showed an increased frequency of plasma cells, dendritic cells and macrophages along with a decreased frequency of NK cells in R848 treated TC mice lamina propria. In addition, we showed that the R848 treatment did not induce gut leakage in TC.*Rag1*$^{-/-}$ mice that lack mature T and B cells.

Conclusions: These results demonstrate that TLR7/8 activation induces a leaky gut in lupus-prone mice, which is mediated by adaptive immune responses. TLR7/8 activation is however not sufficient to breach gut barrier integrity in non-autoimmune mice.

KEYWORDS

lupus, TLR7/8 activation, gut barrier, lymphocytes, NKp46^{+} cells

1 Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the production of autoantibodies and tissue damage induced by immune complex deposition (1). Genetic as well as environmental factors contribute to the initiation and progression of disease. Loss of gut barrier integrity has been reported in SLE patients as well as in several mouse models of the disease, and it has been proposed that microbial translocation out of the gut contributes to lupus pathogenesis (2). Bacterial components have been detected in the blood of lupus patients (3, 4). In addition, *Enterococcus gallinarium and Lactobacillus reuteri* are pathobionts that have been detected in the internal organs of two mouse models lupus (5, 6). *E. gallinarum* was also detected in the liver of patients with autoimmune hepatitis, and human hepatocytes cultured with *E. gallinarum* produced multiple autoimmune-promoting factors, including type I IFN (5). Since whole bacteria or their components are strong proinflammatory mediators, their presence in internal organs and the circulation may trigger inflammation, resulting in the loss of host immune tolerance or the amplification of an existing autoimmune activation. The mechanisms responsible for gut leakage in SLE are not well-understood, although it is recognized that the balance of anti- and pro-inflammatory factors is critical to maintain gut function and integrity (7). It has been proposed that specific bacteria enriched in the gut of SLE patients may directly contribute to the loss of barrier integrity. In support of this hypothesis, gut leakage was induced in gnotobiotic non-autoimmune mice by the colonization with a strain of *Ruminococcus gnavus* that blooms in patients with lupus nephritis (8). The inflammatory milieu that develops in the gut of SLE patients may also contribute to the loss of barrier integrity.

Toll like receptors (TLRs) play a crucial role in the recognition of pathogen-associated molecular patterns (PAMPs) and the activation of the immune response. It is well known that interactions between TLRs and gut microbiota help to maintain gut homeostasis. Overstimulation of TLRs may disturb a balanced composition of the gut microbiome (9), which may in turn promote gut permeability. TLR activation has also been implicated both in the loss and the maintenance of the gut integrity in mice. TLR4 activation by LPS increased the colonic paracellular permeability while activating TLR2 alleviated gut leakage (10). In addition, intracolonic activation of TLR7 increased gut permeability without altering the expression of tight junction proteins, suggesting that other mechanisms were involved (11).

TLR7 overactivation is tightly linked to lupus pathogenesis, with a gain of function mutation resulting in a monogenic pediatric SLE (12) and *Tlr7* duplication in the *Yaa* locus or transgenic expression resulting in lupus phenotypes in mice (13). Moreover, a topical treatment with Resiquimod (R848), a TLR7/8 agonist, induces lupus-like manifestations in non-autoimmune mice (14). The two lupus-prone mouse strains in which the spontaneous translocation of pathobionts has been documented either carry the *Yaa* locus (5) or a *Tlr7* transgene (6). However, whether TLR7 activation played a role in this pathogenic trigger was not specifically addressed. We have shown that the B6.*Sle1.Sle2.Sle3* triple congenic (TC) mouse model of lupus presents a gut bacterial dysbiosis that induces autoimmune activation upon transfer into non-autoimmune congenic control C57BL/6 (B6) mice (15). Gut permeability is not impaired in this model, suggesting that other mechanisms, including an altered microbial tryptophan metabolism, are responsible for the dysbiotic autoimmune activation (15, 16). We have shown that topical R848 greatly accelerated disease in TC mice, including the development of cardiovascular pathology that did not develop in R848-treated B6 mice (17). This indicated that a lupus-prone genetic background potentiates the inflammatory consequences of the activation of the TLR7/8 pathway. Using this model, we found in this study that activation of the TLR7/8 pathway impaired gut permeability, but only in the presence of a lupus-prone genetic background. We also showed that this TLR7/8-induced inflammation of the gut did not occur in Rag1-deficient TC mice. TLR7/8 activation remodeled the distribution of immune cells in the gut epithelium with an increase of dendritic cells (DCs), macrophages and plasma cells, and a loss of natural killer (NK) cells. These results indicate that the activation of the TLR7/8 pathway that frequently occurs in lupus pathogenesis is likely to contribute to the loss of gut barrier integrity, and that it depends on the presence of lymphocytes and the expression of lupus-susceptibility genes.

2 Materials and methods

2.1 Mice and treatment

C57Bl/6J (B6) were purchased from The Jackson Laboratories. B6.*Sle1.Sle2.Sle3* (TC) and TC.*Rag1*$^{-/-}$ mice have been described previously (18, 19). All mice were bred and maintained at the University of Florida in specific pathogen-free conditions. Both males and females were used with gender and age-matched controls for each experiment. Mice from each strain used in this study were strictly maintained with littermates or mice from the same strain within the same group (i.e. less than 4 weeks apart). This housing policy was to avoid transfer of autoimmune activation or attenuation that occurs between B6 and TC mice that share their microbiome (15). rTC mice between 6 and 12 weeks of age, before they produce anti-dsDNA IgG (referred to as "pre-autoimmune"), along with B6 mice were treated with 100 µg resiquimod (R848; Tocris) in 100 µl acetone (Thermo Fisher Scientific) by topical application to the right ear three times a week for 2 weeks. Control mice were left untreated. The tissues were harvested 2 days after the last treatment. Before euthanasia, the gut permeability was evaluated by gavaging mice fasted for 4 h with 5 mg FITC-dextran 4000 (Sigma-Aldrich) in 200 µl 1x PBS, which was quantified in the serum 2 h later by flow cytometry. This study was carried out in accordance with the guidelines from the Guide for the Care and Use of Laboratory Animals of the Animal Welfare Act and the National Institutes of Health. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Florida.

2.2 Intestinal tissue harvest and immunostaining

Ileum and colon were prepared as "Swiss roll" before being embed in OCT medium (Fisher Scientific) and snap-frozen at – 80 °C. 7 μm thick sections mounted on histology slides were put at room temperature for 20 min before being placed into PBS to dissolve the OCT. The sections were fixed with cold acetone for 10 min., washed 3 times wash, and blocked with 10% normal rat serum (Equitech-Bio) in PBS for 30 min. The sections were then stained with antibodies to CD45 (1:25 dilution; Biolegend 30-F11), B220 (1:50; Southern Biotech RA3-6B2), CD3 (1:50; eBioscience 145-2C11), Claudin-1 (1:100 dilution; Invitrogen MH25), NKp46 (1:100 dilution; R&D Systems MAB22252), and PECAM1 (1:50 dilution; BD Biosciences MEC13.3). Fluorescence intensity was analyzed using ImageJ.

2.3 Flow cytometry

Single-cell suspensions were isolated from the gut by using the lamina propria dissociation kit with gentleMACS tissue dissociator (Miltenyi Biotech). Cells were stained in 2.5% FBS and 0.05% sodium azide in PBS. Fluorochrome-conjugated antibodies were as follows: B220 (RA3-6B2), CD11b (M1/70), CD11c (HL3), CD3e (145-2C11), CD95 (Jo2), CD19 (eBio1D3), CD8a (53-6.7), Ly6G (1A8) and Siglec-F (E50-2440) were purchased from BD Biosciences. CD4 (RM4-5), CD138 (281–2), MHCII (M5/114.15.2), NK.1.1 (PK 136), CD49b (HMa2), IL-10 (JES5-16E3), IL-17 (TC11-18h10.1), F4/80 (BM8) and IFN-γ (XMG1.2) were purchased from BioLegend. Foxp3 (FJK-16S), GL-7 (GL-7), CD45 (104) and PDCA-1 (eBio927) were purchased from eBioscience. Dead cells were excluded with fixable viability dye (eFluor780; Thermo Fisher Scientific). Intracellular staining was performed with a fixation/permeabilization kit (eBioscience). For cytokine detection, splenocytes were stimulated with the Leukocyte Activation Cocktail (BD Biosciences) at 37 ^{0}C for 4 h. All samples were acquired on an LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star). Gating strategies are shown in Figures S1, S2. Cell counts for each cell population were calculated based on the splenocyte cell counts measured with a Cellometer Auto 2000 (Nexcelom) and the frequencies of these populations based on the gates shown in Figures S1, S2.

2.4 Detection of IL-6 by ELISA

Serum IL-6 was quantitated with the IL-6 ELISA kit (BD Biosciences) according to manufacturer's instructions. Serum samples were diluted 1:50 and assayed in duplicate. The absorbance was detected with the Promega GloMax® Explorer microplate reader at 450 nm.

2.5 Statistics

Statistical analyses were performed with the Graphpad Prism 9.0 software. Differences between groups were evaluated by one-way ANOVA with correction for multiple tests, or unpaired t tests, as indicated in the text. Unless specified, all tests are two-tailed. Results were expressed as means ± standard deviation. The levels of statistical significance were set at *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ and ****: $P < 0.0001$.

3 Results

3.1 Topical TLR7/8 activation enhanced gut permeability in lupus-prone TC mice

To assess the effect of TLR7/8 activation on gut integrity, we treated pre-autoimmune TC and age-matched control B6 mice with R848 applicated to the ear for 2 weeks as previously described (17). Untreated mice were used as controls. At the end of the treatment, the amount of FITC-dextran in the serum following oral gavage was measured to assess gut permeability. R848 increased the level of FITC-dextran in the serum of TC mice but not in B6 mice (Figure 1A). Claudin-1, one of the tight junction proteins that maintains the intestinal barrier, was quantified in the ileum by immunofluorescence. Claudin-1 levels were lower in untreated TC than untreated B6 mice, and they were further reduced by the R848 treatment (Figures 1B, C). Similarly, the expression of PECAM1, a tight junction protein between endothelial cells, was also decreased by R848 treatment in the blood and/or lymphatic vessels in the intestinal lamina propria (Figures 1D, E). The treatment induced a heavy CD45$^+$ immune cell infiltration in the gut, albeit without statistical difference between the strains. Taken together, the combination of TLR7/TLR8 activation and lupus susceptibility genes expressed in TC mice functionally breached the gut barrier and decreased the expression of tight junction proteins.

3.2 Both innate and adaptive immune cells were recruited by TLR7/8 activation to the ileum of TC mice

We used immunofluorescence to characterize the massive immune cell infiltration induced by the R848 treatment in the ileum of TC mice (Figure 2A). A significantly higher number of B220$^+$ B cells was detected in the lamina propria of treated mice, with notably most of them located towards the bottom of the villi (Figure 2B). CD3$^+$ T cells were distributed evenly across the lamina propria and the R848 treatment did not change their number (Figure 2C). The number of CD11b$^+$ cells aggregated in the lamina propria was higher in R848-treated than control TC mice (Figure 2D). We also investigated the NKp46$^+$ cell population, which includes a subset of NK cells and innate lymphoid cells (ILC), and plays a critical role in the maintenance of gut barrier integrity (20, 21). A high number of NKp46$^+$ cells were found in the colon of TC mice that was decreased by the R848 treatment (Figure 2E). NKp46$^+$ cells were not detected by histology in the ileum of TC mice (data not shown). Overall, TLR7/8 activation mediated the infiltration of B cells and innate immune cells in the gut of TC mice but decreased NKp46$^+$ cells.

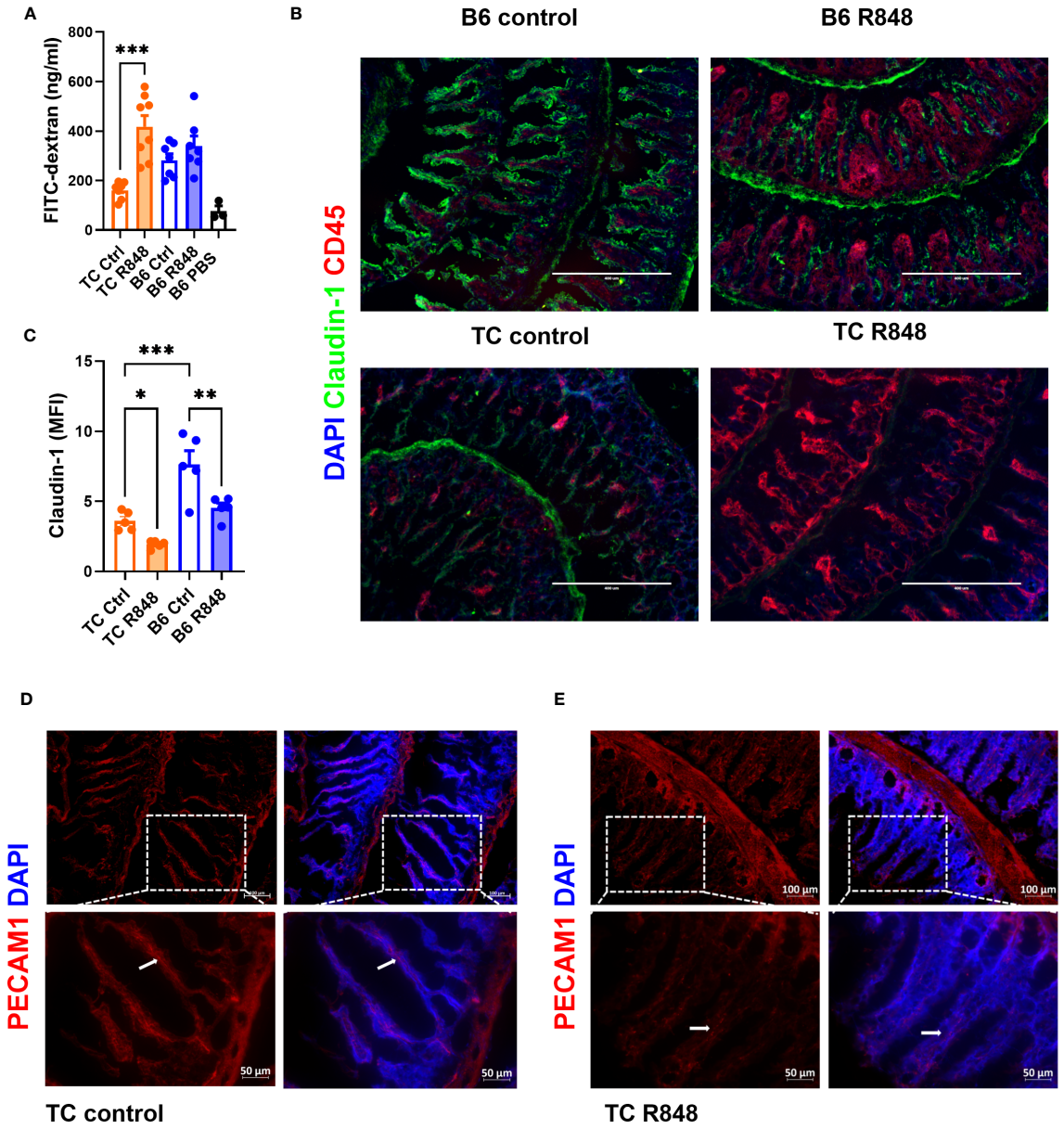

FIGURE 1
TLR7/8 activation enhanced gut permeability in lupus-prone TC mice. **(A)** FITC-dextran concentration in the serum of TC or B6 mice with or without R848 treatment. The results were from two cohorts of mice. **(B)** Representative immunostainings of tight junction protein Claudin-1 and immune cell marker CD45 in the ileum of TC and B6 mice with or without R848 treatment. **(C)** Quantification of Claudin-1 in the ileum of the TC or B6 mice with or without R848 treatment. **(D, E)** Representative immunostainings of endothelial tight junction protein PECAM1 (red) in the ileum of control **(D)** and treated **(E)** TC mice. The dashed boxes indicate the location of the higher magnification images shown below. Arrows point to PECAM1 labeling. N = 3 - 5. Mean + SEM compared with 1-way ANOVA with multiple-comparison tests. *: P < 0.05; **: P < 0.01; ***: P < 0.001.

The cells in the lamina propria mice were further compared between treated and control TC mice by flow cytometry. A higher frequency of macrophages and conventional dendritic cells (DCs) was found in treated TC mice (Figures 3A, B, Figure S3A, B). In comparison, the treatment reduced the frequency of both plasmacytoid dendritic cells (pDCs) and inflammatory pDCs (ipDCs) gated as defined in (22, 23) and shown in Figure S2 (Figures 3C, D, Figures S3C, D). Since pDCs are the main producers of type I IFN in response to TLR7 activation (24), it is possible that R848 induced their recruitment and migration outside the gut. Similarly, the frequency of total $CD4^+$ T cells and regulatory T (Treg) cells among $CD45^+$ cells was reduced by the R848 treatment (Figures 3E, F, Figures S3D, E). The R848 treatment did not affect however the frequency of Treg cells among $CD4^+$ T cells in TC mice (Figure S4A). In addition, the frequency of $CD8^+$ T cells followed the same trend as total $CD4^+$ T cells (Figure S4B). There was a trend of reduced numbers of $CD4^+$ and $CD8^+$ T cells in the lamina propria, but the differences were not significant (Table 1). The R848 treatment also decreased the frequency of B cells while significantly increasing the frequency of plasma cells (Figures 3G, H, Figures S3F, G), consistent with TLR7/8 activation. Finally, the R848 treatment virtually eliminated NK cells from the

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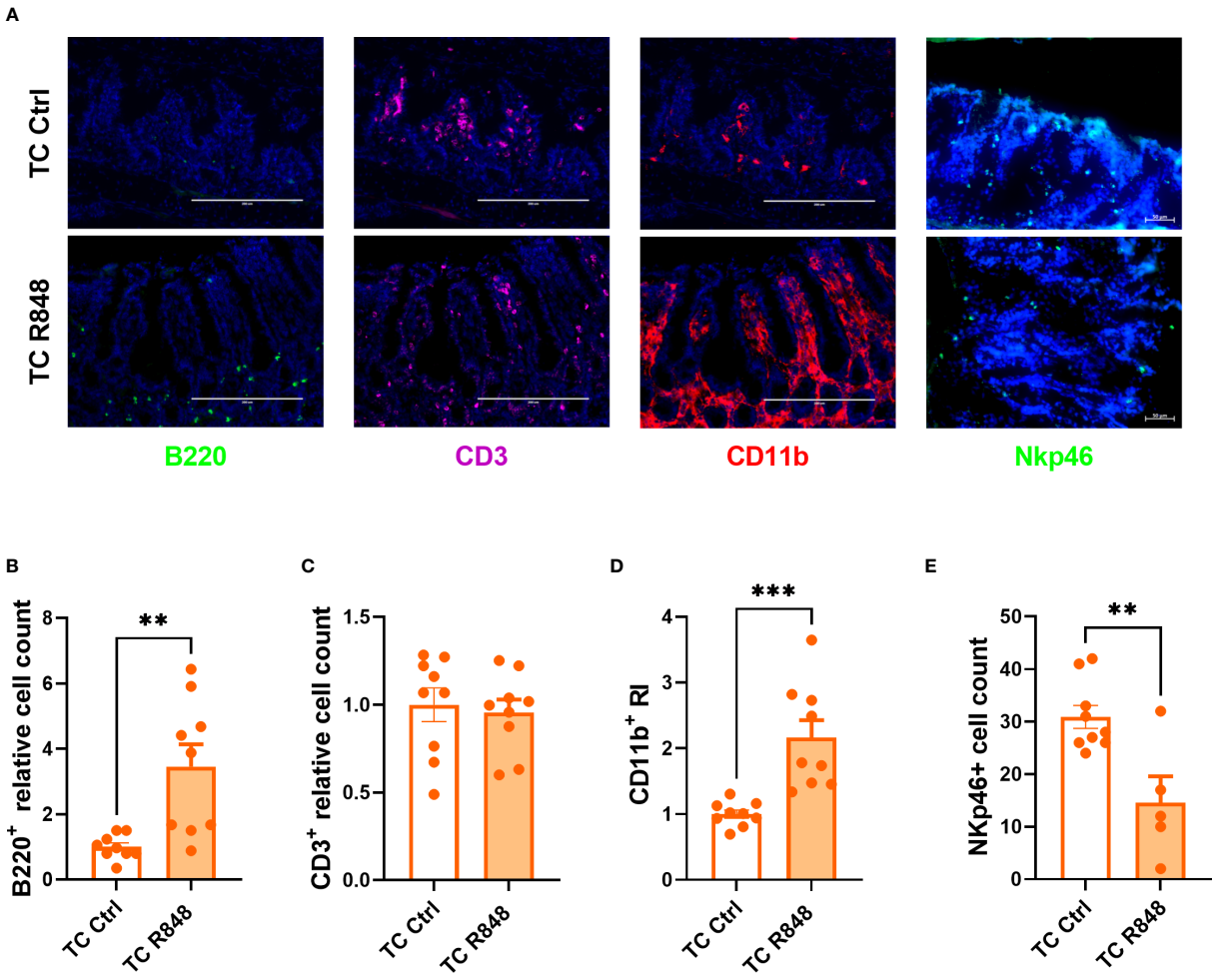

FIGURE 2
Immune cell recruitment by TLR7/8 activation to the gut of TC mice. Representative images **(A)** and quantitation **(B–E)** of immunofluorescence staining of B220$^+$, CD3$^+$, CD11b$^+$ **(B–D)** and NKp46$^+$ cells in the ileum and colon **(E)** of TC mice with or without R848 treatment. The relative cell counts in panel **(B, C)** were calculated relative to the mean of control groups. N = 3. Mean + SEM compared with t tests. **: P < 0.01; ***: P < 0.001.

ileum of TC mice (Figure 3I, Figure S3H). The frequency of neutrophils and eosinophils in the ileum of TC mice were not affected by the R848 treatment (Figures S4C-F). Similar results were obtained with cell numbers (Table 1). These observations show a complex pattern of modifications of immune cells in the gut of TC mice by TLR7/8 activation.

3.3 Lymphocytes are required for R848-mediated gut permeability in TC mice

The inflammatory cascade induced by either immune complexes or the activation of innate cells causes tissue injury that can decrease gut integrity (25). To dissect the relative roles of innate and adaptive immunity in R848-mediated gut leakage in TC mice, we evaluated B and T-cell deficient TC.*Rag1*$^{-/-}$ mice. The R848 treatment did not increase the amount of FITC-dextran leaked in the serum of TC.*Rag1*$^{-/-}$ mice as it did for TC mice (Figure 4A), suggesting that B cells, T cells, or both were involved in the TLR7/8-mediated gut leakage in TC mice. We also evaluated the level of serum IL-6, which can be produced by B and T cells, and is elevated in lupus (26). High levels of IL-6 have been associated with decreased gut barrier integrity, including in a model of lupus (27, 28). Il-6 levels were similar between groups (Figure S5), excluding a casual role of IL-6 on gut leakage in this model. Immunofluorescence staining showed that the R848 treatment failed to recruit CD45$^+$ immune cells to the gut of TC. Rag1$^{-/-}$ mice as it did in TC mice. Moreover, the expression of Claudin-1 was significantly higher in TC. *Rag1*$^{-/-}$ than TC ileums, although it was decreased by the R848 treatment in both strains (Figures 4B, C). The results suggest that adaptive immunity is required in TLR7/8 signaling pathway activation mediated gut leakage in TC mice.

4 Discussion

TLR7/8 activation impaired gut integrity in lupus-prone TC mice but not in congenic controls based on the FITC-dextran assay, and it lowered the expression of Claudin-1 between gut epithelial

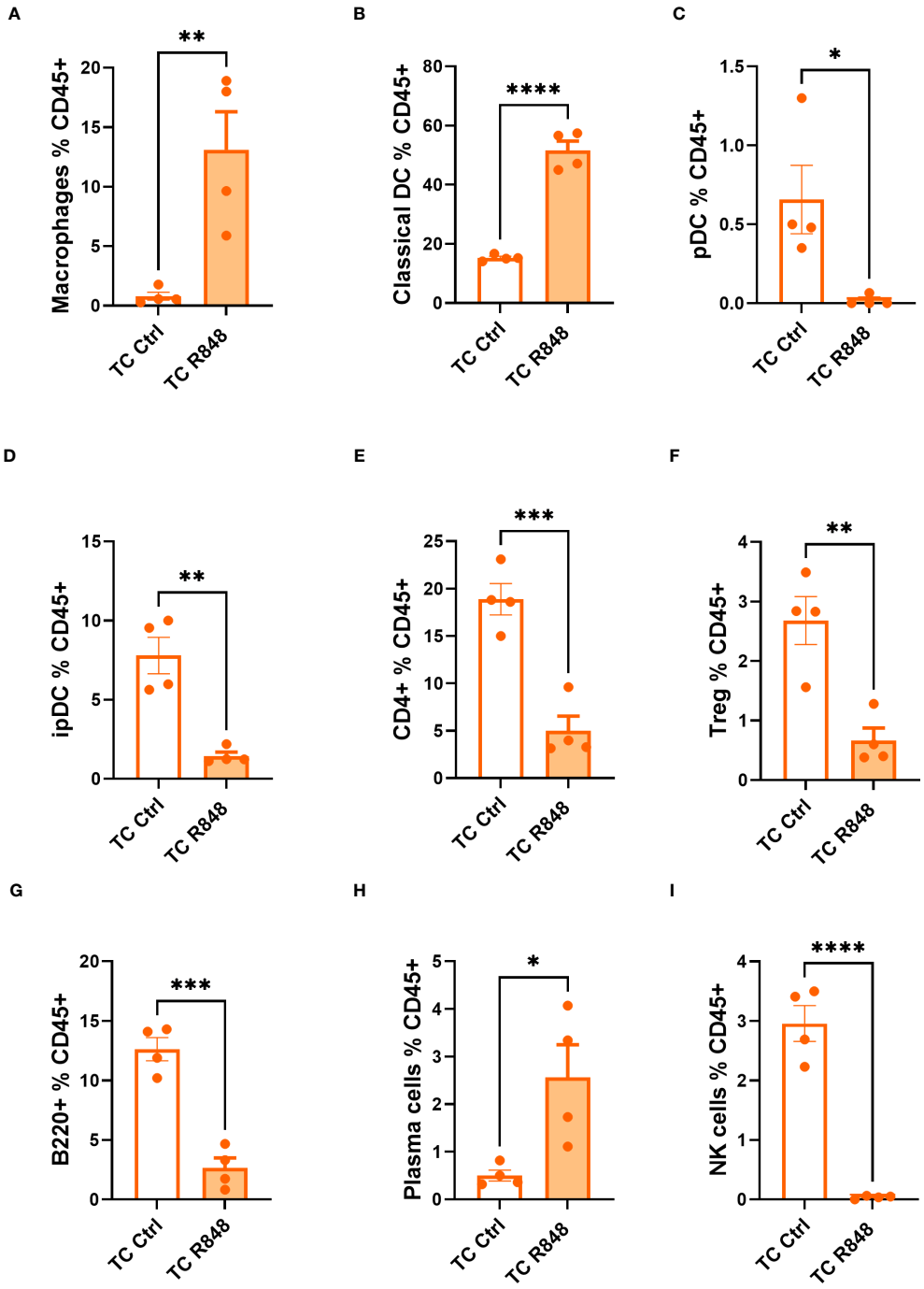

FIGURE 3
Immunophenotyping of the ileum lamina propria comparing control (Ctrl) and treated (R848) TC mice. Frequency of macrophages **(A)**, cDCs **(B)**, pDCs **(C)**, ipDCs **(D)**, $CD4^+$ T cells **(E)** and Treg cells **(F)** out of $CD45^+$ cells. The frequency of B cells **(G)**, plasma cells **(H)**, and NK cells out of $CD45^+$ cells **(I)**. N = 4. Mean + SEM compared with t tests. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.

cells and PECAM1 between blood/lymphatic endothelial cells. These results demonstrated that TLR7/8 activation compromises gut barrier integrity in mice that express lupus susceptibility genes, but it is not sufficient on a non-autoimmune genetic background. Since a control group of mice treated with acetone alone was not included in the study, these results should however be interpreted with caution. Mechanistically, the lupus genetic background could license the breach of gut barrier integrity in response to TLR7/8 activation through the gut dysbiosis that is present in TC mice, and that can transfer autoimmune activation in non-autoimmune mice (15). It is conceivable that the TC gut microbiota was further modified by TLR7/8 activation to expand bacteria that breach the epithelial barrier. $NKp46^+$ cells, which are critical in maintaining barrier integrity depend on the microbiota to differentiate (29). A TRL7/8 induced alteration of the TC microbiota could result in the observed decreased $NKp46^+$ cell frequency, which in turn, may fail

TABLE 1 Immune cells count in the lamina propria of treated and control TC mice.

	TC Ctrl	TC R848
cDCs	324.00 ± 56.65	999.30 ± 262.8*
pDCs	13.75 ± 4.48	0.25 ± 0.25*
ipDCs	180.80 ± 54.87	30.50 ± 12.55*
$CD4^+$ T cells	301.00 ± 63.02	111.00 ± 68.38
$CD8^+$ T cells	110.80 ± 28.46	52.25 ± 37.18
Treg cells	43.75 ± 10.72	14.75 ± 9.11
$B220^+$ cells	287.50 ± 68.54	82.50 ± 31.17*
Plasma cells	11.50 ± 4.63	72.25 ± 16.54*
NK cells	69.00 ± 18.61	1.25 ± 0.63*
Macrophages	18.25 ± 9.707	331.00 ± 129.80
Neutrophils	9.75 ± 1.44	5.75 ± 1.80
Eosinophils	3.50 ± 1.32	18.00 ± 6.65

N = 4. Mean + SEM compared with t tests. *: P < 0.05.

to maintain barrier integrity. Testing these hypotheses will require at minimum to assess gut permeability in control mice after fecal microbiome transfers from R848-treated TC mice.

The lupus genetic background could also license the breach of gut barrier integrity in response to TLR7/8 activation through the immune system. TC mice present a heightened immune activation at baseline (18) and in response to TLR7/8 activation (17). Our group has reported an enhanced expression of type 1 interferon stimulated genes in the heart of R848-treated TC mice (17). Considering the dual roles of type 1 interferon on gut inflammation and injury (30), the activation of this pathway in TC mice may partially explain the increased gut leakage in this model. TLR7 and TLR8 are widely expressed in gut myeloid cells, lymphocytes, and dendritic cells, as well as intestinal epithelial cells (IEC), with the highest expression levels in the latter two populations (31). The finding that the R848 treatment does not induce gut leakage in TC.*Rag1*$^{-/-}$ mice strongly suggests that the activation of B cells and/or T cells by TLR7/8 pathway is directly or indirectly required for the induction of gut leakage. By histology, we found that the R848 treatment increased the numbers of B cells and $CD11b^+$ myeloid cells, but not $CD3^+$ T cells in the ileum of TC mice. Flow cytometry showed an increased frequency of plasma cells but a decreased frequency of B cells. Previous studies have demonstrated that R848 stimulation could initiate B cell proliferation, upregulation of costimulatory molecule expression and antibody production (32). Our observations suggest that the R848-treatment triggers a robust differentiation into plasma cells in the gut of TC mice. Antibodies or autoantibodies may regulate gut homeostasis through their interactions with bacteria (33). Secretory Immunoglobulin A (SIgA) derived from intestinal plasma cells binds to pathogenic microbes, preventing their expansion and keeping the gut homeostasis (34). However, antibodies produced by plasma cells in the lamina propria may form immune complexes that may induce tissue damage and increase gut permeability. In support of this hypothesis, the number of IgA-producing plasma cells was positively correlated with the gut permeability in human duodenal biopsies (35).

Flow cytometry also showed a global decrease in T cell frequency in the ileum of R848-treated TC mice, including in Treg cell frequency relative to the total immune infiltrate. The discrepancy showing a similar number of T cells by immunofluorescence and a decreased by flow cytometry deserves to be examined further in futures studies. Flow cytometry is likely to be more reliable as more quantitative. Treg cells have been proposed to be central to the interplay between the host and microbial milieu. These cells are involved in promoting gut barrier integrity and a balanced interaction with gut microbiota–derived short-chain fatty acids (SCFAs) (36). The decreased number of Treg cells in R848-treated TC mice may contribute to an enhanced gut inflammation, leading to deleterious gut integrity, although their frequency relative to effector T cells was not changed. However, Treg cells in the gut are heterogenous in their origin, function and interactions with the microbiome (37). A more detailed analysis will be therefore necessary to assess whether a loss of Treg cells in R848-treated TC mice plays a role in their gut leakage. Several T cell-derived cytokines contribute to the maintenance of gut integrity, including IL-10, IL-17 and IL-22 (38–40). The decreased frequency of TC $CD4^+$ T cells in the gut by TLR7/8 activation may skew the cytokine environment. Ultimately, this altered cytokine milieu may affect gut integrity directly or modify other cell populations through downstream signaling pathway and eventually make an impact on gut barrier function.

The gut myeloid populations of TC mice showed a heterogeneous response to R848 treatment, which included an increased frequency of macrophages and cDCs. R848 activates the NF-κB pathway (41) as well as the production of IL-1β and IL-18 (42), and promotes an M1 phenotype (43), all of which are likely to amplify inflammation or cause tissue damage (44). cDCs work as professional antigen-presenting cells for T cell priming. Interactions of resting immature DCs with TLR ligands leads to a cascade of pro-inflammatory cytokines and skewing of T cell responses. Interactions between cDCs and bacteria may alter cDC activation and indirectly shape $CD4^+$ T cell differentiation and effector functions. Specific components of the gut microbiota isolated from patients with celiac disease altered DC maturation and their interactions with epithelial cells, leading to gut permeability (45). In a model of gut inflammation, DC activation and aberrant distribution induced by acute antigen uptake has been correlated to an enhanced gut permeability (46). In the small intestine, lamina propria dendritic cells (LPDCs) induce Treg cell differentiation. Over-reactivated LPDCs may lose their tolerogenic function, leading to a decrease of Treg cell number (47). In the present study, an enhanced DC activity may explain the reduced number of Treg cells, both of which may contribute to an increased inflammatory state. At same time, a decreased pDC frequency was observed in the gut of R848-treated TC mice. We have shown that R848 expanded the pDC population in the spleen of TC mice to a greater extent than in B6 spleens (17). TLR7 activation suppressed the expression of CCR9, the gut homing receptor, on pDCs (48). This observation may at least partially explain the decreased frequency of pDCs in the gut of R848-treated TC mice.

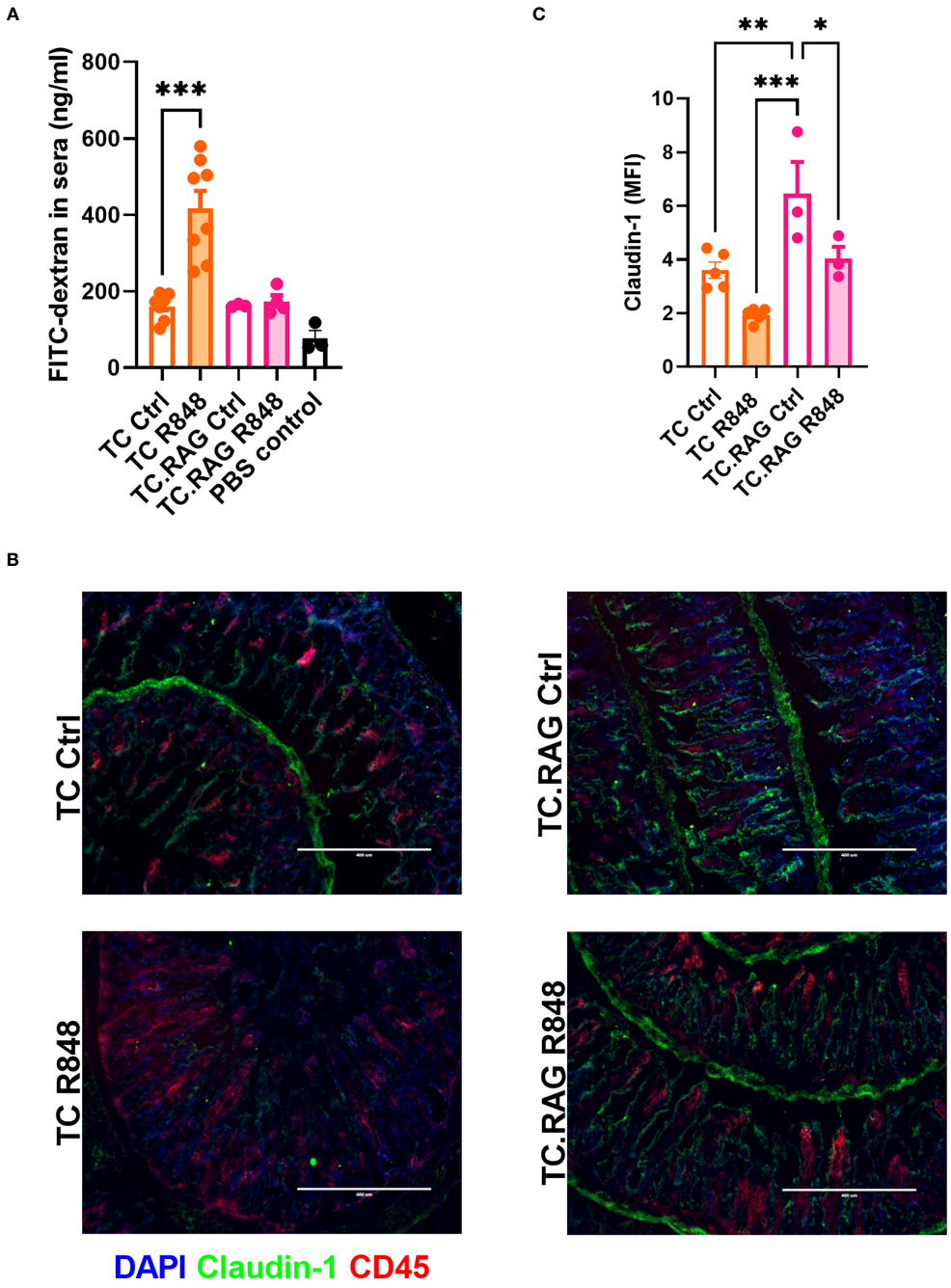

FIGURE 4
Adaptive immunity is required in R848-mediated gut permeability in TC mice. **(A)** FITC-dextran concentration in the serum of TC or TC. Rag1$^{-/-}$ mice with or without R848 treatment. **(B)** Representative immunostainings of tight junction protein Claudin-1 and immune cell marker CD45 in the ileum of TC and TC. Rag1$^{-/-}$ mice with or without R848 treatment. **(C)** Quantification of Claudin-1 protein. N = 3 - 5. Mean + SEM compared with t tests. *: P < 0.05; **: P < 0.01; ***: P < 0.001.

Finally, we observed that the R848 treatment greatly reduced the NKp46^{+} cell population in the colon and the NK cells in the ileum of TC mice. Cytokines, including IL-22, that are produced by NK cells and ILCs are involved in the regulation of various immune cells maintaining the integrity of the gut mucosa (49). ILC3 cells have been related to the gut integrity maintenance (50). Altered gut permeability was associated to a reduced NK cell number in schizophrenia patients (51). Therefore, the decreased number of NKp46^{+} cells in the gut of R848-treated TC mice may partially explain the increased gut permeability. It should be noted that although the results obtained with TC.*Rag1*$^{-/-}$ mice indicate that B and/or T cells play a major role in breaching the gut barrier in response to R848, cDCs, macrophages, and NKp46^{+} cells may play an indirect role by altering the lymphocyte populations. For instance, cytokines produced by T and B cells may modify activation of DCs and macrophages. Which immune cell population individually or collectively contribute to gut permeability needs to be addressed with selective elimination with lineage specific antibodies.

In summary, we showed that the activation of the TLR7/8 pathway, a common feature of lupus pathogenesis, may contribute to impaired gut barrier integrity but only in the presence of lupus genetic susceptibility. It would be of great interest to evaluate whether SLE patients with an activated TLR7/8 pathway, most likely measured as its downstream type I IFN activity, present with an increased incidence of leaky gut. The interpretation of such a study may however be difficult because such patients tend to also present with a higher disease activity and inflammation, which may be a contributing factor to leaky gut independently from TLR7/8.

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 reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida (IACUC 202009466).

Author contributions

LMa, MT, JB, AE, and LMo designed the experiments and analyzed results. LMa, MT, JB, and AE conducted experiments. LMa and LMo wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

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SUPPLEMENTARY FIGURE 1
Gating strategies for lamina propria immunophenotyping.

SUPPLEMENTARY FIGURE 2
Gating strategy for dendritic cell populations.

SUPPLEMENTARY FIGURE 3
Representative FACS plots for macrophage **(A)**, cDCs**(B)**, pDCs & ipDCs **(C)**, CD4+ & CD8+ **(D)**, Treg **(E)**, B cells **(F)**, plasma cells & plasma blast cells **(G)** and NK cells **(H)**.

SUPPLEMENTARY FIGURE 4
Frequency of Treg **(A)**, $CD8^+$ T cells **(B)**, neutrophils **(C)** and eosinophils **(D)** in the ileum of TC mice with or without R848 treatment and representative FACS plots for neutrophils **(E)** and eosinophils **(F)**. N = 4. Mean + SEM.

SUPPLEMENTARY FIGURE 5
Serum IL-6 levels in B6, TC or Tc. $Rag^{-/-}$ mice with or without R848 treatment. N = 3 - 4. Mean + SEM.

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Arecoline aggravates acute ulcerative colitis in mice by affecting intestinal microbiota and serum metabolites

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Arecoline is an alkaloid extracted from betel nut, which has various pharmacological effects. In the present study, we showed that arecoline aggravated experimental acute ulcerative colitis (UC) induced by dextran sodium sulfate (DSS) in mice. We measured body weight and colon length, evaluated disease activity index, colon pathology sections, and levels of colonic inflammatory factors. Arecoline exacerbated the clinical signs of UC and the colonic inflammatory response in mice. The results of 16S rRNA sequencing of fecal samples showed a significant decrease in the percentage of probiotic bacteria *Ligilactobacillus*, *Limosilactobacillus* and *Lactobacillus* and a significant increase in the percentage of conditionally pathogenic bacteria *Odoribacter* and *Bacteroides* after arecoline treatment. Serum untargeted metabolomics showed that arecoline intervention reduced the levels of ergothioneine, pentostatin, diadenosine tetraphosphate and other metabolites and modulated nicotinate and nicotinamide metabolism, metabolic pathways, glyoxylate and dicarboxylate metabolism, and other metabolic pathways of intestinal microorganisms. According to the combined microbial and metabolite analysis, arecoline influences metabolite levels by modulating the intestinal microbiota. In summary, it was found that arecoline treatment exacerbated colonic injury and intestinal inflammatory responses in UC mice, disrupted the host's intestinal flora, and affected changes in flora metabolites, thereby exacerbating the development of colonic inflammation. Therefore, the consumption of betel nut can be associated with the risk of aggravating UC.

KEYWORDS

arecoline, ulcerative colitis, gut microbiota, metabolomics, inflammatory responses

Introduction

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD) that has become a globally widespread and disease that poses a serious health risk given its increasing incidence (1). UC is a chronic idiopathic inflammatory disease with common symptoms including blood in the stool and diarrhea and mainly involves the colon and rectum. Its

pathogenesis includes environmental factors, an unhealthy lifestyle, immune imbalance and dysbiosis of the gut microbiota (2, 3).Meanwhile, UC is also closely related to colorectal cancer, and studies have shown that long-term UC increases the risk of developing colorectal cancer (4).

Gut microbiota are closely related to the development of UC, and dysbiosis of the gut flora can increase the severity of UC (5). Studies have shown that a significant increase in the abundance of *Bacteroidota* and a relative decrease in the abundance of *Firmicutes* in the intestines of UC patients and reduced ratio of *Firmicutes* to *Bacteroides* (F/B) contribute to the development of UC (6).5-aminosalicylate sodium (5-ASA), cyclosporine, and glucocorticoids are the traditional drugs used to treat UC. However, these drugs are associated with serious side effects. Probiotics have fewer side effects than traditional drugs and can treat and alleviate the occurrence of UC (7). The probiotic *Lactobacillus plantarum* HNU082 (Lp082) was found to protect the mucosal barrier of the intestine, modulate the gut microbiota and reduce the symptoms of intestinal inflammation for the purpose of treating UC (8). Furthermore, live and pasteurized *Akkermansia muciniphila* effectively alleviated the symptoms of colitis in mice by promoting the proliferation of beneficial intestinal bacteria, increasing the production of short-chain fatty acids and suppressing intestinal inflammation (9).

Herbal extracts have also been extensively studied for their ability to inhibit the development of UC in mice. For example, the polyphenol extract of *Thymus vulgaris L.* could protect the intestinal epithelial barrier, modulate the intestinal microbiota, and inhibit the TLR4/NF-κB-NLRP3 inflammatory vesicle pathway to improve UC in mice (10). Intestinal flora metabolites are closely related to the maintenance of the intestinal barrier and the balance of the intestinal immune microenvironment. Short-chain fatty acids (SCFAs) have been shown to have immunomodulatory effects by reducing the production of pro-inflammatory factors to reduce the inflammatory response and mitigate the development of UC (11, 12).

Arecoline is the active ingredient of betel nut, and its pharmacological action has important effects on the nervous, cardiovascular, endocrine, and digestive systems (13–15). Some studies have shown that arecoline can increase inflammatory cell infiltration in the oral epithelium and affect the synthesis of various inflammatory mediators, thus leading to the development of oral epithelial fibrosis (16, 17). More importantly, long-term stimulation by arecoline may lead to further development of oral squamous cell carcinoma (18, 19). One study found that consumption of betel nut induces the peripheral blood mononuclear cells (PBMC) to secrete inflammatory cytokines such as IL-1β, IL-8, TNF-α, and IL-6, leading to their increased circulation (20). In long-term studies, arecoline has great research significance for its role in the body (21). However, it remains unexplored whether the development of UC is affected by arecoline.

In this study, the effect of arecoline on dextran sodium sulfate (DSS)-induced UC was investigated. We assessed the clinical signs by observing the colonic length, weight change, and disease activity index of mice, and the inflammatory response based on their colonic pathological changes and the concentrations of colonic tissue inflammatory factors. We also performed 16S rRNA sequencing on fecal samples and untargeted metabolomics analysis on serum samples to explore the specific mechanism of arecoline action on UC. The results suggest that arecoline may exacerbate DSS-induced UC by affecting the intestinal microbiota and its metabolites.

Materials and methods

Animal experimental design

We purchased 18 C57BL/6 male mice (age: 6–8 weeks) from Jiangsu Jicuiyaokang Biotechnology Co (Nanjing, China). The mice were housed at the Jiangxi Academy of Sciences, Royo Biotech Co., Ltd. (Nanchang, China). They were housed at a temperature of 25°C with a 24-h light-dark cycle. Mice had *ad libitum* access to food and water. After 7 days of acclimation, the mice were divided into three groups (n=6 per group): (1) Control group that was allowed *ad libitum* access to food and water; (2) DSS group: *ad libitum* diet was allowed for 1–7 days and 3% DSS (Meilunbio, Dalian) was added to the drinking water for 8–14 days; (3) Arec group: ad libitum diet was allowed for 1–7 days, 3% DSS was added to the drinking water for 8–14 days, and 5 mg/kg arecoline (Yuanye, Shanghai) was administered daily by tube feeding.

Colonic length and histological analysis

Mice were killed after anesthesia, and their colon was dissected out and its length measured. Colon samples were preserved and fixed in 4% paraformaldehyde solution and then embedded in paraffin. The colon tissue was cut into 4-μm–thick sections, and then the colon sections were stained using hematoxylin and eosin (H&E). The stained slides were examined under an inverted microscope and photographed.

Assessment of disease activity index

Throughout the experiment, the mice were weighed daily and fecal conditions were recorded. The stool properties and body weight were scored together, and the scores were summed and recorded as the DAI (22). Assessment was performed according to the following scoring criteria:

weight change: 0, no weight loss; 1, weight loss of 1–5%; 2, weight loss of 5–10%; 3, weight loss of 10–15%; 4: weight loss of >15%.

stool consistency: 0, normal; 2, dilute stool; 4, diarrhea.

blood in the stool: 0, no blood; 2, visual pellet bleeding; 4, severe bloody stool and blood around the anus.

Enzyme-linked immunosorbent assay

Colon tissue stored at -80°C was thawed and mixed with phosphate-buffered saline (PBS) at a volume of 1:9 by weight and placed in a tissue grinder for homogenization, followed by centrifugation at 3000×g for 4 min at 5°C. We quantified the supernatant after centrifugation for quantitative ELISA (Shenkebio, Wuhan) of TNF-α, IL-1β, and IL-6 according to the manufacturer's instructions.

16S rRNA sequencing

Mice fecal samples were collected after excretion and stored immediately in liquid nitrogen. We extracted genomic DNA by the Cetyltrimethylammonium Bromide (CTAB) method. The purity and concentration of DNA was assessed using 2% agarose gel electrophoresis; the DNA samples were diluted in sterile water to 1 ng/μl. After electrophoretic detection, the DNA was purified using magnetic beads and quantified by enzyme marker. Electrophoretic detection was again performed using 2% agarose gels, followed by recovery of target bands using Qiagen's gel recovery kit (Qiagen, Germany). We used TruSeq® DNA PCR-Free sample preparation kit to construct libraries. Libraries were constructed and quantified by Qubit and Q-PCR, and the qualified libraries were sequenced using NovaSeq6000. 16S rRNA sequencing was provided by Metware Biotechnology Co., Ltd. (Wuhan, China).

The data was efficiently processed by FLASH (v1.2.11) and Qiime (v1.9.1). The valid data were clustered using the Uparse algorithm (USEARCH v7) to assign sequences with a similarity ≥97% to the same operational taxonomic units (OTUs). Taxonomic information was annotated using the Mothur algorithm of the SILVA v138.1 (http://www.arb-silva.de/) database. The Shannon index, Simpson index, ACE index, and Chao1 index were calculated using R software (v4.1.2) and QIIME (v1.9.1) to assess alpha diversity. We also performed principal co-ordinate analysis (PCoA) and analysis of similarities (ANOSIM) to assess beta diversity using QIIME and R software. Last, LEfSe analysis using LEfSe software was used to screen for LDA score ≥4.

Untargeted metabolomics

After thawing and vortexing the samples, 50 μL sample and 300 μL extract containing the internal standard (acetonitrile: methanol=1:4, V/V) were added to the centrifuge tube. The sample was again vortexed for 3 min and centrifuged at 12,000 rpm for 10 min at 4°C. Then, the 200 μL supernatant was collected and placed at -20°C for 30 min, followed by re-centrifuging at 12,000 rpm for 3 min at 4°C. This supernatant was collected for LC-MS analysis (Metware Biotechnology Co., Ltd., Wuhan, China). Samples were collected and analyzed according to the LC-MS system machine instructions. Meanwhile, we annotated the identified metabolites using the Kyoto Encyclopedia of Genes and Genomes (KEGG) compound database (http://www.kegg.jp/kegg/compound/) and then mapped the annotated metabolites to the KEGG pathway database (http://www.kegg.jp/kegg/pathway.html). Orthogonal partial least squares discriminant analysis of the three groups of differential serum metabolites was performed by R software. VIP (VIP>1) and P-value (P<0.05, Student's *t*-test) were used for differential metabolite screening in both groups. For multiple group analysis, VIP (VIP>1) and P-value (P-value<0.05, ANOVA) were used for differential metabolite screening.

Statistical analysis

Data are expressed as arithmetic mean ± standard error of the mean (SEM). Data were analyzed and counted using GraphPad Prism 8.0. Statistically significant differences between the groups were assessed by one-way analysis of variance (ANOVA). Bivariate correlations were calculated using Pearson's r coefficient. Heat maps were constructed using R software (v4.1.2). We investigated the correlation between gut microbiota and serum metabolites using Spearman's correlation analysis. P<0.05 was considered to indicate statistically significant differences.

Results

Effect of arecoline on clinical symptoms of colitis in mice

We used 3% DSS solution to induce colitis in mice to establish the UC model, which was used to study the effect of arecoline on colitis. Details of the study groups are presented in Figure 1A. By measuring the colon length of the three groups of mice, we found that mice in the DSS group had a shorter colon length than those in the control group (P<0.01); however, the shortening of the colon was more severe in the Arec group (P<0.001) (Figures 1B, C). With respect to weight loss, there was a significant difference in weight between the arecoline and control groups at day 14 (P<0.01); furthermore, arecoline exacerbated the weight loss in the DSS group mice (Figure 1D). We used the DAI scores to analyze body weight change and fecal properties (blood in stool and stool consistency) in all mice, and arecoline aggravated the clinical signs of DSS-induced acute ulcerative colitis in mice (Figure 1E).

Effect of arecoline on the inflammatory response in mice colon

We observed by HE staining of colonic sections that the colonic structure was intact in the control group and severely disrupted in the DSS group, with destruction of the crypt and disappearance of glands, more severe disruption of the intestinal structure, and intestinal inflammation. The Arec group showed more aggregation of neutrophils than the other two groups (Figures 2A). The results of ELISA showed that the levels of inflammatory factors IL-6 (P<0.001), TNF-α (P<0.001), and IL-1β (P<0.001) were significantly higher in the colonic tissue of the Arec group than the DSS group (Figures 2B–D).

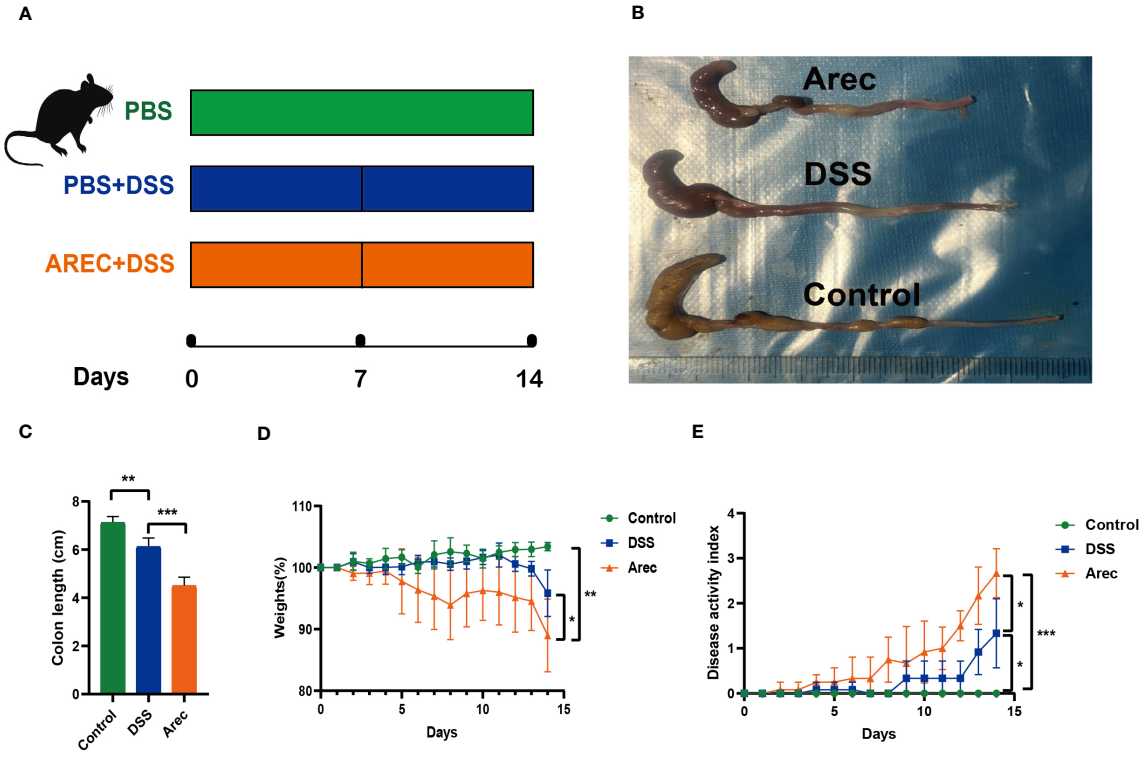

FIGURE 1
The effect of arecoline on clinical symptoms in mice with DSS-induced UC. **(A)** Establishment of a mouse UC model and treatment of arecoline. **(B)** Representative images of the colon in the Arec, DSS, and control groups of mice. **(C)** Colonic length in mice. **(D)** Body weight changes of the three groups of mice during the experiment. **(E)** Disease activity index of the three groups of mice. P<0.05 was statistically significant. *P<0.05, **P<0.01, and ***P<0.001.

Effect of arecoline on intestinal microbiota

It has been shown that the intestinal microbiota is related to digestion, absorption, metabolism, immunity, and other functions, and that dysbiosis of the intestinal flora is closely related to the occurrence of many diseases such as tumors, diarrhea, obesity, and cardiovascular and cerebrovascular diseases (23–25). Disruptions in the balance of the gut microbiota have also been reported to have many links to the development of UC (26, 27). Therefore, to investigate the effects of arecoline and DSS on intestinal flora, we sequenced 16S rRNA in fecal samples from all three groups and analyzed the alpha and beta diversity indices of the mouse intestinal microbiota. The Arec group had increased gut microbiota diversity as compared to the DSS and control groups according to the Shannon, Simpson, and ACE indices. According to the Chao1 index, the DSS group showed increased diversity of gut microbiota and the Arec group showed decreased diversity of gut microbiota compared to the control group (Table 1). OTU-based weighted UniFrac distance PCoA analysis showed significant differences in the gut microbiota composition of the three groups of mice (P<0.001) (Figure 3A). ANOSIM showed the same results, with significant differences between the control, DSS, and Arec groups (Figures 3B–D). At the phylum level, the Arec group showed an increase in the relative abundance of Bacteroidota and Firmicutes and a decrease in the relative abundance of Proteobacteria and Actinobacteria compared to the DSS group. The relative abundance of Proteobacteria and Bacteroidota increased in the DSS group compared to the control group, while the relative abundance of Firmicutes decreased significantly (Figure 3E). At the genus level, the relative abundance of *Bacteroides* and *Odoribacter* increased in the Arec group compared to the DSS group, while the abundance of *Parasutterella*, *Allobaculum*, *Ligilactobacillus*, *Lactobacillus*, and *Limosilactobacillus* decreased significantly (Figure 3F).

In the analysis of LEfSe results, the taxonomic phylogram showed that the main composition of the intestinal microbiota in the three groups of mice was Bacteroidota, Firmicutes, and Proteobacteria (Figure 4A). The LDA scores for the abundance of taxonomic units indicated that *Bacteroides* in the Arec group had higher LDA scores than in the DSS and control groups. By contrast, the control group was dominated by *Lactobacillus* (Figure 4B). As shown in Figures 4C–F, the population of *Bacteroides* and *Bacteroides acidifaciens* was significantly increased in the Arec group compared to the other two groups, while the population of *Ligilactobacillus* and *Lactobacillus* almost disappeared. Therefore, elevated abundance of *Bacteroides* and reduced abundance of *Lactobacillus* may be the reason for arecoline treatment leading to more severe colonic inflammation.

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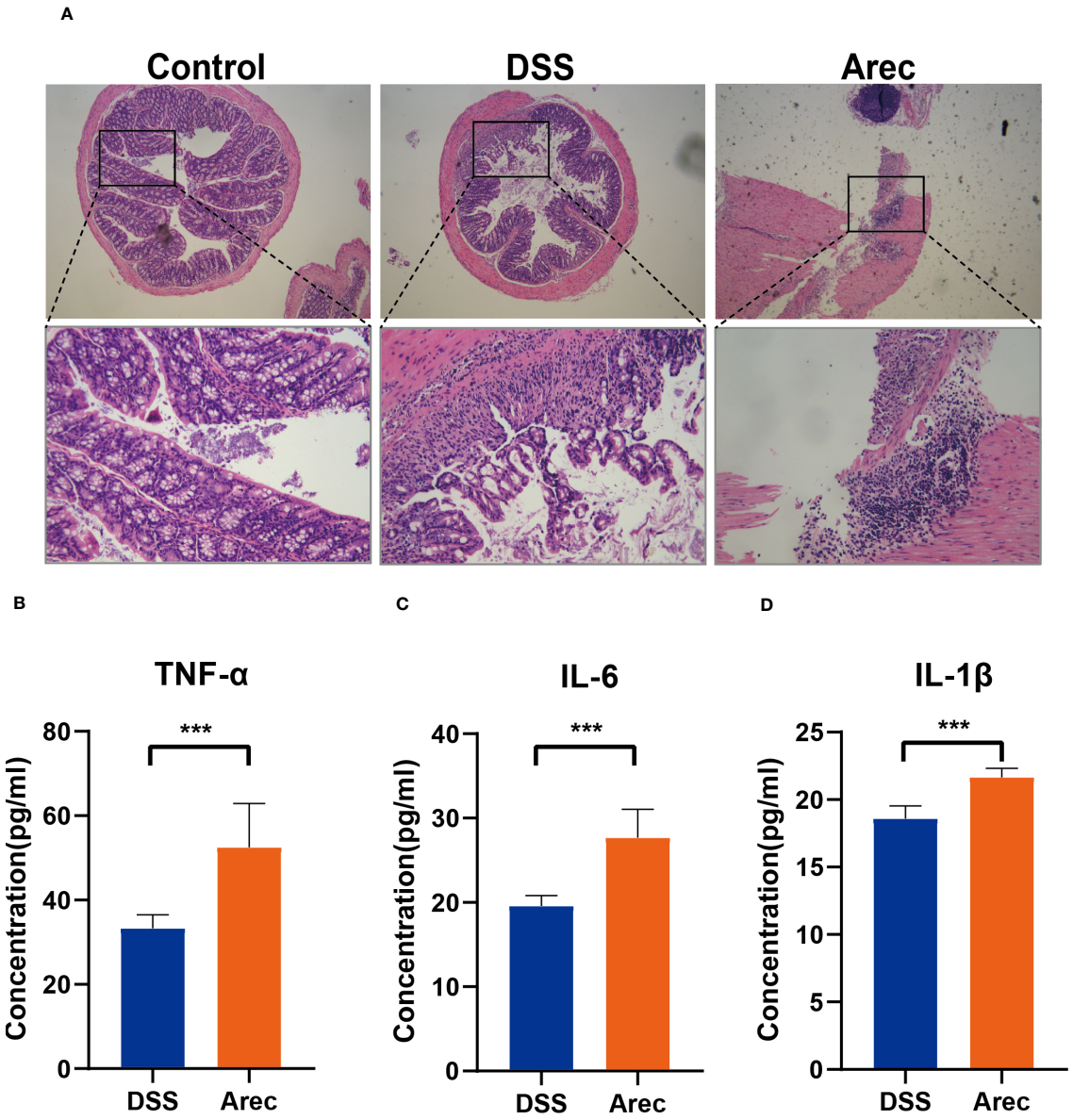

FIGURE 2
Histopathological sections of mouse colon with changes in inflammatory factors. **(A)** H&E stained images of mouse colon tissue (magnification 40×, 200×). **(B–D)** The concentration of inflammatory factors including TNF-α, IL-6, and IL-1β in the colonic tissue of mice in the DSS and Arec groups. P<0.05 was statistically significant. ***P<0.001.

Effect of arecoline on the metabolism of intestinal flora

The metabolites of the intestinal microbiota regulate all bodily functions. We analyzed the differential metabolites of the three groups of mice sera by performing untargeted metabolomics assays on their sera. In the positive ion mode, OPLS-DA showed a significant separation of metabolites in the three groups (Figure 5A). In addition, the metabolites were significantly separated between the DSS and control groups (Figure 5B) and between the Arec and DSS groups (Figure 5C). As shown in Figure 5D, the clustering analysis of different metabolites between

TABLE 1 Alpha diversity parameters assessed by the Shannon, Simpson, Chao1, and ACE indices.

Group	Shannon	Simpson	Chao1	ACE
Arec	6.275	0.945	833.626	834.783
DSS	5.839	0.962	783.965	793.998
Control	5.562	0.947	765.866	782.761

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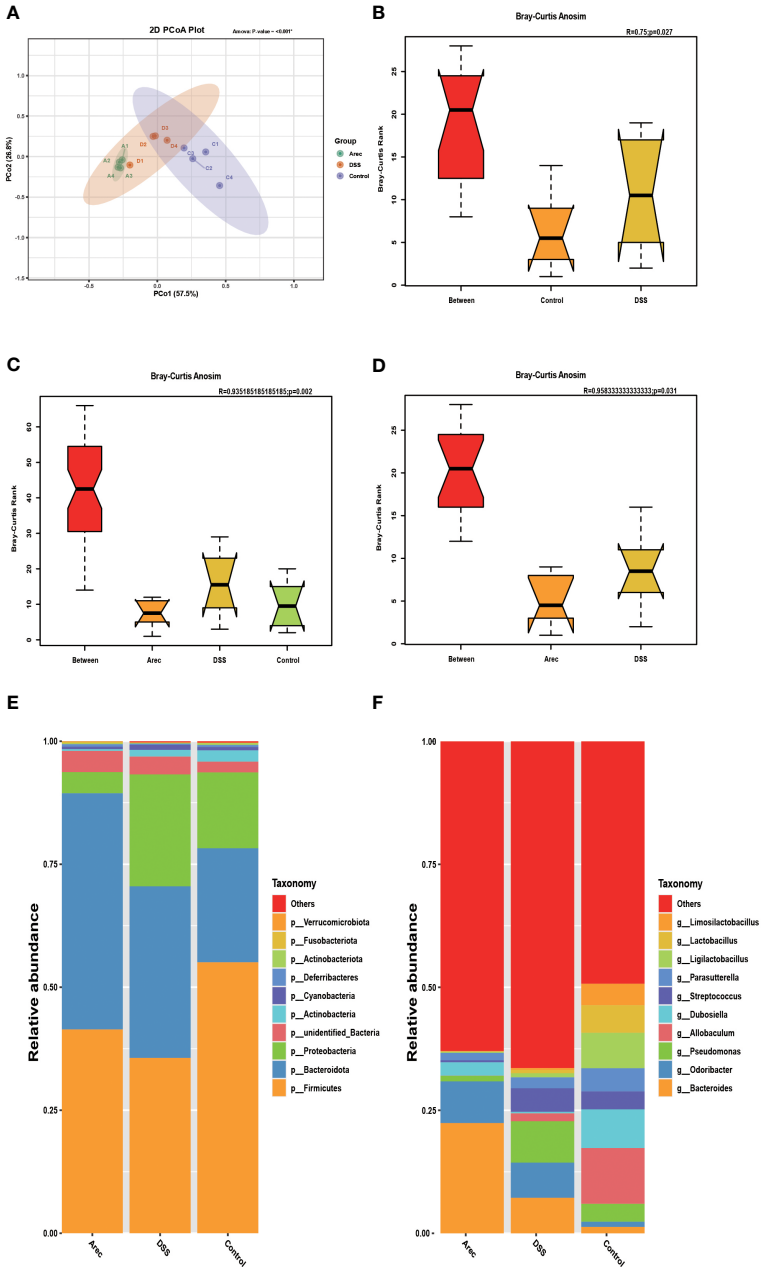

FIGURE 3
Arecoline and DSS interventions affect changes in intestinal flora. **(A)** PCoA analysis of OTU-based weighted UniFrac distances showed microbial differences between the groups. **(B–D)** OTU-based ANOSIM inter-group variation analysis. **(E)** Gut microbial composition of the three groups of mice at the portal level. **(F)** Intestinal microbial composition of the three groups of mice at the genus level. P<0.05 was statistically significant.

the Arec group and DSS group in positive-ion mode showed that there were 114 differential metabolites between the two groups, with 25 up-regulated and 89 down-regulated ones. As shown in Figure 5E, the top 20 metabolic pathways including Nicotinate and nicotinamide metabolism, Metabolic pathways, and Glyoxylate and dicarboxylate metabolism were obtained using KEGG pathway enrichment analysis based on the different metabolite results between the Arec and DSS groups.

We performed a joint analysis for intestinal flora and differential metabolites. Pentostatin, nifedipine, LPC (20:1/0:0), ergothioneine, and other metabolites increased with increasing abundance of Proteobacteria and Firmicutes and decreased with increasing abundance of Bacteroidota and Actinobacteria (Figure 6).

Discussion

Ulcerative colitis is a chronic idiopathic inflammatory disease caused by an imbalance of immune mechanisms and environmental factors, which often manifests clinically as diarrhea, bleeding, and malabsorption, and is a significant threat to people's physical and mental health (2, 3). The clinical signs and pathological changes in

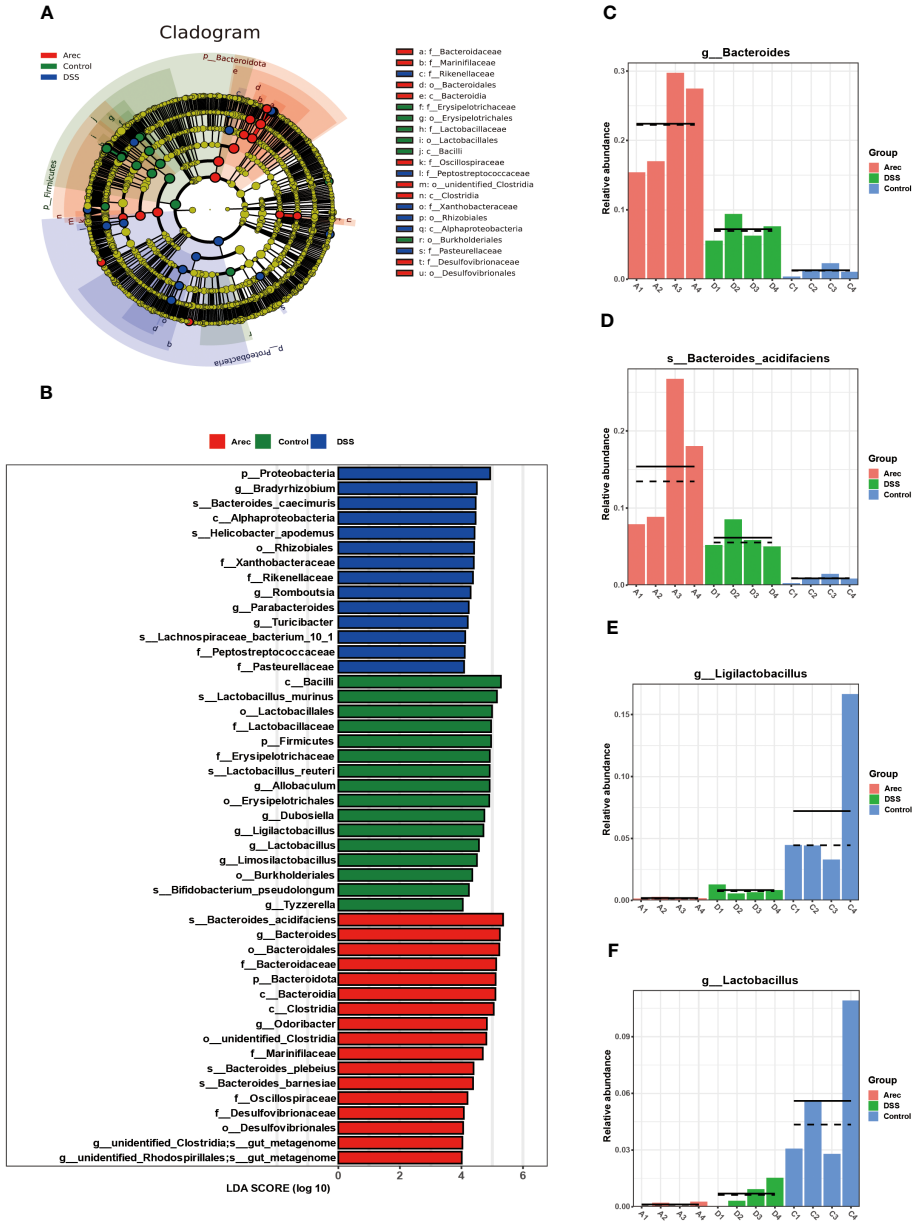

FIGURE 4
Results of LEfSe analysis of gut microbiota in the Arec, DSS, and control groups. **(A)** Taxonomic cladogram showing the main constituent taxa of the three groups of mouse intestinal microorganisms. **(B)** The distribution of LDA values in the histogram shows the species with LDA score greater than 4. **(C–F)** Difference maps of representative intestinal microorganisms three groups of mice.

mice in the DSS group after 7 days of induction with 3% DSS indicated the success of our UC model. Our experiments revealed that 5 mg/kg arecoline aggravated the symptoms of colitis induced by DSS, and the Arec group showed higher DAI scores and showed more severe weight loss as well as shortening of the colon than the DSS and control groups. Pathological examination of the intestinal tract of mice showed that the DSS group had reduced crypts, mucosal breakdown, and inflammatory cell infiltration; moreover, the breakdown of the intestinal barrier and inflammatory cell infiltration were more severe with the addition of arecoline.

We also detected a significant increase in pro-inflammatory factors (IL-6, IL-1β, and TNF-α) in the Arec group by ELISA. Among them, the pro-inflammatory effect of IL-6 stems from its ability to prevent T-cell apoptosis, but it also affects the pro-ablative and repair functions of the epithelial barrier (28). TNF-α promotes T-cell proliferation and differentiation and aggravates intestinal inflammation (29). Studies have shown that elevated IL-1β correlates with the severity of acute inflammation and that inhibition of IL-1β expression alleviates the symptoms of colitis (30). These experimental results suggest that arecoline promotes the expression of inflammatory factors and aggravates UC; therefore, we investigated its mechanism of action in more depth.

As the study of UC has become more advanced, a large number of studies have shown that intestinal microorganisms play a central

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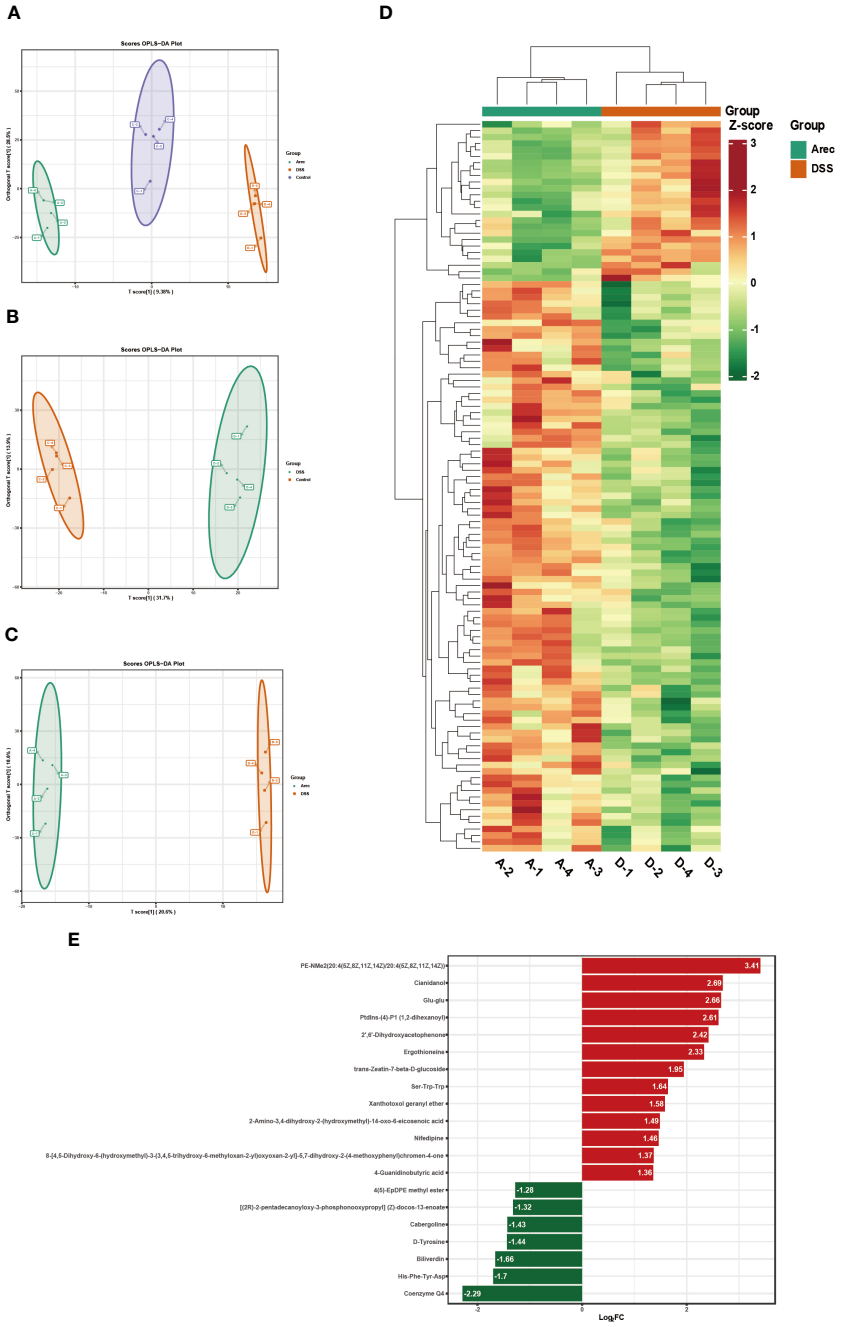

FIGURE 5
Arecoline regulates serum metabolism in positive-ion mode. **(A–C)** OPLS-DA based analysis showing differences among the Arec, DSS, and control groups; differences between the DSS and control groups, and differences between the Arec and DSS groups. **(D)** Heat map reflects the differential metabolites between the Arec and DSS groups (high levels in red, low levels in green). **(E)** KEGG enrichment maps of differential metabolites in the Arec and DSS groups.

role in the development of UC (31, 32). Hence, we collected fecal samples from mice for 16S rRNA sequencing to examine their intestinal flora composition. The sequencing results showed significant differences in the gut microbial composition of the three groups. At the phylum level, the relative abundance of Proteobacteria was significantly increased after DSS treatment compared to the control group, consistent with previous studies. The study showed that the ratio of Firmicutes to Bacteroides (F/B) was reduced in the gut of UC patients (33), consistent with our experimental results, and the reduction of F/B was more significant with the addition of arecoline, which aggravated the dysbiosis of the intestinal flora caused by DSS.

At the genus level, arecoline exacerbated the effect of DSS on beneficial bacterial genera in the intestinal tract. *Lactobacillus* is a well-known beneficial genus in the intestine and has a protective effect on the gut in colitis (34, 35). The abundance of *Lactobacillus* was significantly reduced in the DSS group relative to the control group, and almost disappeared after the use of arecoline.

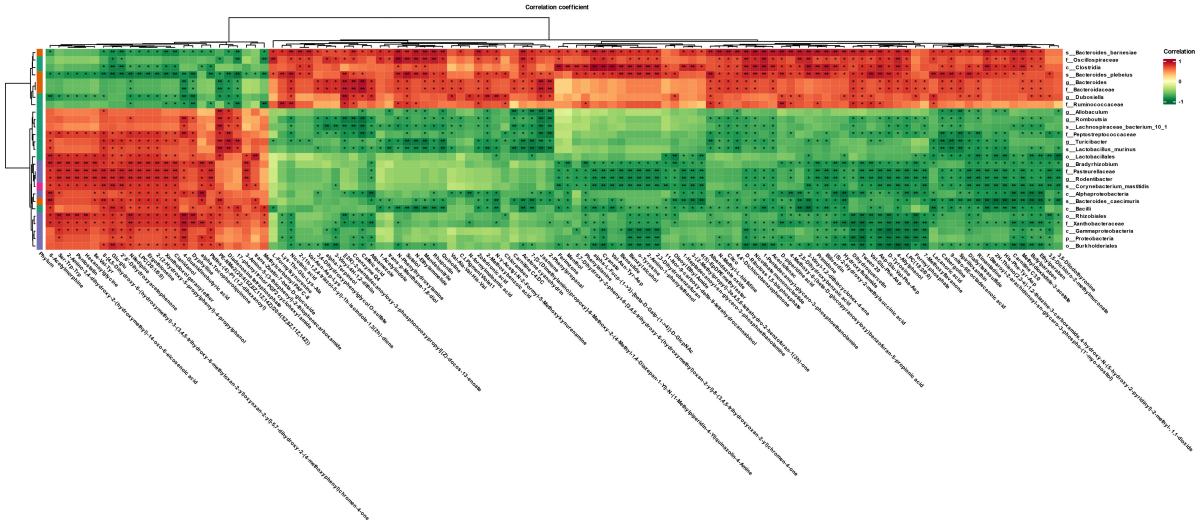

FIGURE 6
Heat map showing gut microbes associated with differential metabolites in the Arec and DSS groups (red indicates high levels, green indicates low levels). *P<0.05 and **P<0.01.

Furthermore, the abundance of *Limosilactobacillus* (36, 37) and *Ligilactobacillus* (38, 39), which have antimicrobial and immune as well as intestinal barrier-enhancing abilities, were inhibited by DSS. *Limosilactobacillus* has been shown to influence the NF-κB signaling pathway to inhibit inflammation and prevent the development and progression of colitis (40). In addition, *Limosilactobacillus* also increases the induction and production of PD-1$^+$ T follicular helper cell-dependent IgA, which alters the gut microbiome and prevents DSS-induced colitis and intestinal ecological dysbiosis (41). These results suggest that arecoline further exacerbates DSS-induced colitis by decreasing the abundance of beneficial bacterial genera in the mouse intestine, thereby exacerbating the development of colitis.

In addition, the relative abundance of *Bacteroides* in the intestine of DSS-treated mice was significantly increased in our assay, which is consistent with previous results of studies regarding the potential role of symbiotic *Bacteroides* in the induction of colonic inflammation in mice (42). It has also been reported that enterotoxigenic *Bacteroides fragilis* (ETBF) in *Bacteroides* increases the permeability of intestinal epithelial cells and disrupts epithelial barrier function by secreting enterotoxins, while ETBF colonization leads to acute or chronic inflammation of the intestine (43, 44). Therefore, based on our experimental results, it can be inferred that an increase in the relative abundance of *Bacteroides* may lead to the development of UC. The abundance of *Odoribacter* significantly increased with the use of DSS in our study, which was contrary to previous studies which reported that *Odoribacter* was more abundant in healthy individuals than in those with colitis (45). However, there are also many studies suggesting that *Odoribacter* may act as a conditional pathogenic agent and that its increased relative abundance may be positively associated with the occurrence of UC (46, 47). These results suggest that arecoline increases the relative abundance of conditionally pathogenic bacteria, thereby exacerbating DSS-induced colitis.

We analyzed the main components of intestinal microbiota of the three groups of mice by LEfSe analysis, in which the probiotic *Lactobacillus* obtained a higher score in the control group and the conditional pathogenic bacteria *Bacteroides* was higher in the Arec group. These results further corroborate our inference.

Intestinal microbiota can regulate immunity, maintain the intestinal environment, and provide energy to the body through its metabolites (48). For example, tryptophan and its metabolites can alleviate intestinal inflammation by reducing pro-inflammatory factors such as IL-22, and IL-17 (49). The production of SCFAs is essential for intestinal integrity, as they regulate intestinal pH and influence intestinal mucus production, thereby increasing mucosal immune function (50). In our study, metabolites such as ergothioneine, pentostatin, LPC (20:1/0:0), Glu-glu, and diadenosine tetraphosphate were reduced after arecoline treatment when compared with the DSS group. Ergothioneine is a common oxidant with strong antioxidant activity. It has been shown that oral administration of ergothioneine can increase the level of anti-inflammatory factors in the body and inhibit the TLR4/MyD88/NF-κB signaling pathway, thus protecting from colonic shortening and intestinal pathological damage in UC (51). Ergothioneine is thought to be a metabolite of the probiotic bacterium *Limosilactobacillus*, and the elevation of ergothioneine was positively correlated with *Limosilactobacillus* abundance (52). This is also consistent with the results of our combined gut microbial and metabolite analysis, wherein the relative population of *Limosilactobacillus* in the Arec group nearly disappeared, and arecoline may have reduced the amount of ergothioneine in mice by reducing the abundance of *Limosilactobacillus*. Pentostatin is a purine antimetabolite commonly used in the treatment of malignancies, which inhibits adenosine deaminase. Studies have shown that pentostatin can affect the release of pro-inflammatory factors and attenuate the effects of IL-10-/colitis (53). Our combined microbial and metabolite report showed that

pentostatin levels increased with Proteobacteria and Firmicutes and decreased with Bacteroidota. Therefore, the decrease in pentostatin likely indicates that arecoline treatment increased the abundance of Bacteroidota and decreased the abundance of Proteobacteria. Diadenosine tetraphosphate is a metabolite produced under stressful conditions such as hypoxia and injury, and is widely found in prokaryotic and eukaryotic organisms for its role in regulating immune responses, gene expression repair, and DNA replication and synthesis (54). When DSS induced stress in mice, diadenosine tetraphosphate was secreted. However, the use of arecoline reduced the serum levels of diadenosine tetraphosphate, and according to our combined microbial and metabolite analysis report, it is possible that arecoline reduced the abundance of *Allobaculum* and *Lactobacillus* and increased the abundance of *Bacteroides*, resulting in a decrease of diadenosine tetraphosphate in mice. According to the metabolic pathway enrichment analysis based on the differential metabolite results, arecoline may aggravate UC in mice by modulating gut microbiota to regulate nicotinate and nicotinamide metabolism, metabolic pathways, and glyoxylate and dicarboxylate metabolism, which are important metabolic pathways. In summary, we suggest that arecoline regulates serum metabolite production by directly affecting the abundance of intestinal microbiota.

Conclusion

Our study shows that arecoline can aggravate the colonic damage of DSS-induced UC and increase the release of inflammatory factors. Based on our gut microbial sequencing results, we can conclude that UC in arecoline-treated aggravated mice may be mediated by modulation of the gut microbiota resulting in a decrease in the abundance of beneficial intestinal genera and an increase in the abundance of conditionally pathogenic bacteria. Moreover, the combined microbial and metabolite analysis showed that gut microbes were significantly associated with differential serum metabolites, and arecoline exacerbated UC in mice by affecting the abundance of intestinal flora that regulated serum metabolite concentrations. However, the exact mechanism of arecoline action needs to be further confirmed by targeted metabolomics. In addition, further studies are needed to investigate the effects of arecoline on patients with inflammatory bowel disease.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA977598.

Ethics statement

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee of Nanchang Royo Biotech Co., Ltd.

Author contributions

DL and BY supervised the project and designed this study. HZ and TD performed the experiments and organized the manuscript. YC and WY conducted the data analysis. JR revised the manuscript. All authors contributed to the article and approved the submitted version.

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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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Causality between inflammatory bowel disease and the cerebral cortex: insights from Mendelian randomization and integrated bioinformatics analysis

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Background: Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), is a chronic, progressive, and recurrent intestinal condition that poses a significant global health burden. The high prevalence of neuropsychiatric comorbidities in IBD necessitates the development of targeted management strategies.

Methods: Leveraging genetic data from genome-wide association studies and Immunochip genotype analyses of nearly 150,000 individuals, we conducted a two-sample Mendelian randomization study to elucidate the driving force of IBD, UC, and CD on cortical reshaping. Genetic variants mediating the causality were collected to disclose the biological pathways linking intestinal inflammation to brain dysfunction.

Results: Here, 115, 69, and 98 instrumental variables genetically predicted IBD, UC, and CD. We found that CD significantly decreased the surface area of the temporal pole gyrus ($\beta = -0.946$ mm^2, $P = 0.005$, false discovery rate-$P = 0.085$). Additionally, we identified suggestive variations in cortical surface area and thickness induced by exposure across eight functional gyri. The top 10 variant-matched genes were *STAT3*, *FOS*, *NFKB1*, *JAK2*, *STAT4*, *TYK2*, *SMAD3*, *IL12B*, *MYC*, *and CCL2*, which are interconnected in the interaction network and play a role in inflammatory and immune processes.

Conclusion: We explore the causality between intestinal inflammation and altered cortical morphology. It is likely that neuroinflammation-induced damage, impaired neurological function, and persistent nociceptive input lead to morphological changes in the cerebral cortex, which may trigger neuropsychiatric disorders.

KEYWORDS

inflammatory bowel disease, cerebral cortex, causality, Mendelian randomization, integrated bioinformatics

1 Introduction

Inflammatory bowel disease (IBD) encompasses a range of chronic nonspecific inflammatory disorders affecting the intestine and extraintestinal organs (1). The two primary phenotypes of IBD are ulcerative colitis (UC) and Crohn's disease (CD), which are differentiated based on clinical characteristics. Historically, IBD has been predominantly observed in individuals of European descent. However, there has been a significant increase in the incidence of IBD among Americans and non-white populations, leading to a global health burden (2).

More than 20% of IBD patients suffer from mental disorders (3), while the prevalence of combined neurodegenerative diseases is at least 1.14-fold higher than that in healthy populations or patients with other long-standing illnesses (4). The correlation between chronic gut inflammation and neuropsychologic abnormalities has been proactively initiated these years, spawning the concept of the gut–brain axis—a bidirectional communication network between the intestine and the brain (5, 6). Animal experiments have demonstrated reduced neurogenesis and inhibited neuronal progenitor proliferation in mice with induced colitis (7, 8). Similarly, clinical trials targeting IBD populations with neuropsychiatric symptoms revealed regionally morphologic changes in the gray matter, making them promising neuroimaging biomarkers (9, 10). Taken together, these findings suggest that IBD can trigger structural variations in the brain, particularly in the cerebral cortex, which is the foundation for advanced neural activity. However, there are still gaps in our understanding of the causal relationship between IBD and the cerebral cortex due to incomplete translation of animal models to humans. Moreover, limited sample sizes and confounding factors in clinical cohorts present challenges when drawing definitive conclusions.

As an epidemiologic approach, Mendelian randomization (MR) allows for assessing causal relationships between specific exposure and outcome traits by randomly allocating genetic variants as instrumental variables (11, 12), which is applicable in etiological studies. Recently, MR has been employed to identify genetic overlap between intestinal inflammation and brain function (13). The application of MR in the field of the gut–brain axis has yielded valuable insights into potential therapeutic targets for related diseases and has sparked further investigations. Compared with traditional observations and randomized controlled trials, MR eliminates the drawbacks of potential confounding effects, inverse causality, and high execution difficulty.

Using publicly available genome-wide association studies (GWASs), we conducted a two-sample MR analysis to investigate the effect of IBD, UC, and CD on the cortical structure. This study aims to identify underlying factors that contribute to neuropsychiatric symptoms in patients with IBD and facilitate the discovery of the gut–brain axis. By exploring the mechanisms underlying cortical structural modifications, we hope to gain new insights that can inform the development of pharmaceutical therapies.

2 Methods

2.1 Data sources

2.1.1 Exposure: inflammatory bowel disease

The GWAS summary statistics for IBD, UC, and CD were obtained from a trans-ethnic association study conducted by Liu et al. (14), who aggregated GWASs and Immunochip genotype data from a total of 86,640 European participants (38,155 cases; 48,485 controls) as well as 9,846 non-European ancestry participants (Supplementary Table S1). We downloaded the compiled information from the NHGRI-EBI GWAS catalog (15), which serves as a comprehensive repository of freely accessible bioinformatics resources.

2.1.2 Outcome: cerebral cortex structure

The GWAS summary statistics of the cerebral cortex were derived from a structural magnetic resonance imaging (MRI)-based study that included 51,665 European individuals (Supplementary Table S2). Grasby et al. (16) comprehensively analyzed the entire cortex. They

Abbreviations: IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; MR, Mendelian randomization; GWAS, genome-wide association study; MRI, magnetic resonance imaging; SA, surface area; TH, thickness; SNP, single nucleotide polymorphism; IVW, inverse-variance weighted; V2G, variant-to-gene; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate; JAK-STAT, Janus kinase-signal transducer and activator of transcription.

developed 138 distinctive phenotypes [total surface area (SA) and average thickness (TH), 34 globally controlled cortical SA and TH, 34 non-globally controlled cortical SA and TH]. We employed the GWAS data for these 138 cortical traits to explore whether IBD, UC, and CD causally induce cortical structural changes.

Our study only extracted GWAS summary statistics from published studies, which were also publicly accessible. No extra ethical approval or informed consent was required.

2.2 Selection of genetic instruments

In essence, MR involves using a genome-wide single nucleotide polymorphism (SNP) as an instrumental variable to validate the exposure–outcome association. The beta values and standard errors of SNPs for each trait are selected for MR analysis. A qualified instrumental variant must adhere to the following principles (11): 1) it should be strongly correlated with the exposure data at a test threshold of 5×10^{-8}; 2) it should not be directly associated with the outcome trait, indicating that any observed causality is solely driven by the exposure; and 3) it should independently establish exposure–outcome causality, excluding the influence of confounding factors (Figure 1A).

For our study, we applied three main hypotheses to select genetic variants (Figure 1B). Firstly, we excluded SNPs that did not reach genome-wide significance at a threshold of 5×10^{-8} in the respective summarized dataset for the exposure phenotypes. We also removed SNPs in high linkage disequilibrium with an r^2 value of 0.001 and a physical distance of 10,000 kb from the index variant. Additionally, we calculated F-statistics for candidate SNPs and retained those with an F-statistic exceeding 10 (17, 18). After incorporating the outcome data, instruments significantly associated with the cerebral cortex at a significance level of 5×10^{-8} were excluded.

To establish a direct causal IBD–brain relationship, we accounted for potential confounding factors such as obesity, body mass index, type 1 and type 2 diabetes mellitus, hypertension, smoking, drinking, and neuropsychiatric diseases. We employed PhenoScanner, an online database of human genotype–phenotype associations (www.phenoscanner.medschl.cam.ac.uk), to identify and exclude confounder SNPs from the genetic variants (*P*-value: 1×10^{-5}, r^2: 0.8, European, reference: GRCh37).

2.3 Analyses of causal effect

Outlier SNPs should be identified and removed using the MR-PRESSO method prior to conducting the MR analysis. In our study, a two-sample MR analysis was employed to extrapolate the extent and direction of the effect of IBD on brain structure. Five strategies were utilized: MR-Egger, random-effect inverse-variance weighted (IVW), weighted median, simple mode, and weighted mode. The MR-Egger method accommodates the presence of horizontal pleiotropy from genetic variants, provided that it is uncorrelated with the instrument–exposure correlation (19). The weighted median-based MR approach remains unbiased as long as at least half of the instrumental variables are non-pleiotropic (20). The mode-based approaches cluster similar variants and derive estimates based on the specific aggregation with the highest number of SNPs (21). We have opted to endorse the IVW method among these approaches, as it conducts a meta-analysis of the Wald ratio across all instrumental SNPs (22). Meanwhile, if the results from the remaining four methods are consistent with those generated by IVW, they can be employed to supplement IVW estimates.

Once causality is established, heterogeneity and pleiotropy tests are required to confirm the findings' reliability. The estimate is non-heterogenetic if the *P*-value from Cochran's Q test exceeds 0.05. MR-Egger intercept test (*P*-value threshold of 0.05) and leave-one-out plot are utilized to verify the presence of horizontal pleiotropy. Additionally, a funnel plot evaluates if directional pleiotropy is presented. To reinforce the credibility of our results, we employed an online calculator to assess the power of MR analyses (https://sb452.shinyapps.io/power/).

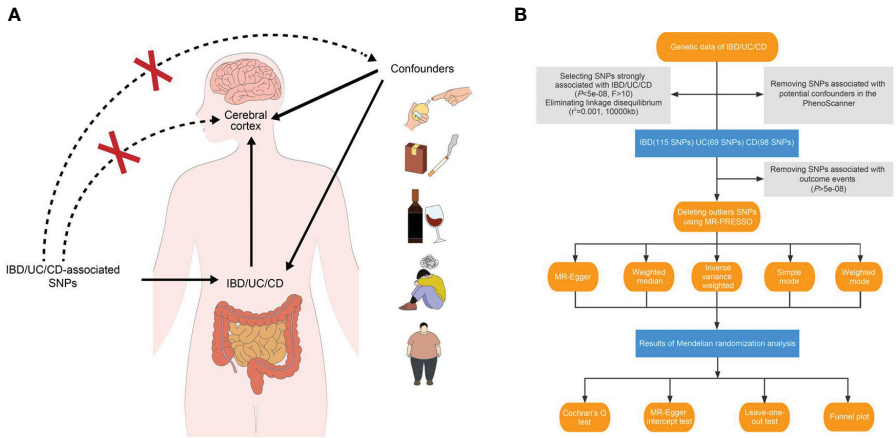

FIGURE 1
Study design overview. **(A)** Three essential hypotheses of Mendelian randomization in our study. **(B)** Flowchart of Mendelian randomization analysis disclosing the underlying role of IBD, UC, and CD in the altered brain structure. IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; SNP, single nucleotide polymorphism.

2.4 Function exploration of mediator genes

We curated and consolidated mediator instruments that establish a connection between IBD and the cerebral cortex. Open Targets Genetics (https://genetics.opentargets.org/) is a variant-centric tool that integrates functional genomics data and quantitative trait loci from multiple heterogeneous sources to generate overall variant-to-gene (V2G) scores, which enables us to prioritize candidate genes. Genes with the highest overall V2G scores were selected and annotated using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. Those pathways with less than five genes and more than 500 genes should be removed before the enrichment analysis.

Using bioinformatics approaches, we further established interaction networks and tracked hub nodes that regulate other genes in various physiological processes. These efforts were valuable in detecting shared loci with the cerebral cortex in several loci confirmed to be associated with IBD, thereby providing fresh insights into targeted therapies.

2.5 Statistical analysis

R studio (version 4.1.2) was utilized for conducting MR analyses and function annotation. The TwoSampleMR package (version 0.5.6) was employed for the MR analysis, while org.Hs.eg.db (version 3.16.0) and clusterProfiler (version 4.2.2) packages were used for species annotation and subsequent pathway enrichment.

To address the issue of repeated calculations within the same datasets, we implemented the Benjamini–Hochberg procedure to control the false discovery rate (FDR). Significant estimates were those with a P-value < 0.05 and an FDR-adjusted P-value < 0.1. We considered results with a P-value < 0.05 but an FDR-adjusted P-value > 0.1 to be suggestive in nature.

Protein–protein interactions were investigated using STRING (Search Tool for the Retrieval of Interacting Genes), a database of known and predicted interactions between proteins. The complex networks were visualized and integrated with Cytoscape (version 3.9.1), while candidate genes were ranked based on their degree centrality values calculated by the cytoHubba plugin. Hub genes were defined as those with the top 10 highest degree values.

3 Results

3.1 IBD causally affects brain cortical structure

We obtained robust instruments with average F-values surpassing 300 as genetic substitutes for IBD, UC, and CD. We then utilized PhenoScanner to detect and eliminate genetic variants shared with confounders. Ultimately, we employed 115, 69, and 98 SNPs to genetically predict IBD, UC, and CD (Supplementary Tables S3-S5). An outlier SNP, rs72924296, was excluded before estimating the causality between IBD and the globally adjusted TH of the postcentral gyrus. No outliers were detected in other phenotypic pairs. Details of SNPs used in every MR analysis are presented in Supplementary Table S6.

Figure 2 shows 12 significant and suggestive IVW-derived estimates. Following FDR correction, we realized that CD significantly decreased the globally adjusted SA of the temporal pole gyrus ($\beta = -0.946$ mm^2, $P = 0.005$, FDR-$P = 0.085$). We have also identified 11 suggestive findings indicating regionally cortical variations induced by chronic colitis with IVW-derived P-values < 0.05. In terms of the affected areas, we observed that the SA was reduced in three functional gyri (inferior parietal, inferior temporal, and temporal pole) as well as a variably changed TH in the frontal pole, postcentral, caudal middle frontal, lateral orbitofrontal, and superior frontal (Figure 3). Our research findings showed that IBD and CD tended to promote changes in the SA and TH across distinct brain regions, whereas UC primarily affected the inferior parietal lobe by diminishing its SA. Supplementary Figure S1 presents scatter plots illustrating the causal relationships.

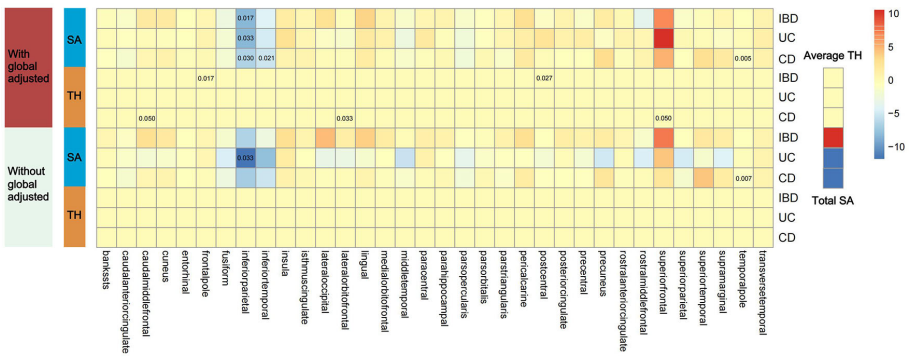

FIGURE 2
Presentation of IVW estimates derived from the IBD–cortex relationship. The color of each block represents the IVW-produced beta values, and the 12 numbers printed on the blocks are IVW-generated P-values. IVW, inverse-variance weighted; IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; SA, surface area; TH, thickness.

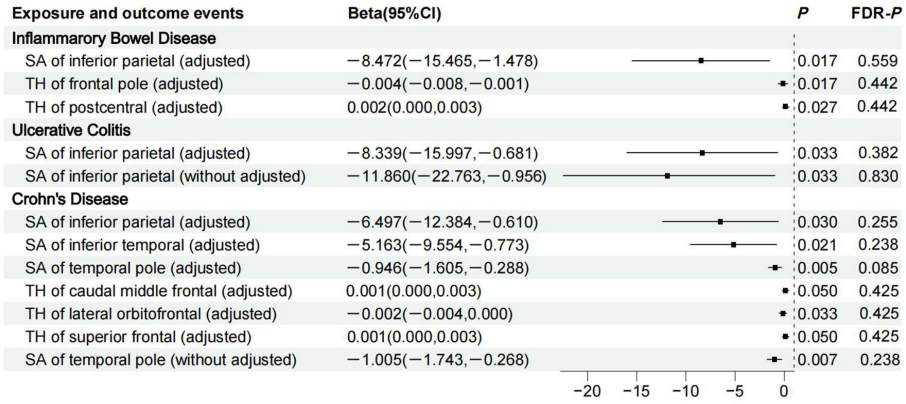

FIGURE 3
Details of causality between 12 phenotype pairs. IVW estimates from significant and suggestive causality between IBD, UC, and CD and regionally cortical SA and TH. IVW, inverse-variance weighted; IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; SA, surface area; TH, thickness; CI, confidence interval; FDR, false discovery rate.

Due to regional interactions, the structural variations of the entire cortex triggered by IBD, UC, and CD were found to be offset. No genetic evidence supported a causal relationship between IBD and the total SA or the average TH (β_{SA} = 27.885 mm^2, P_{SA} = 0.810; β_{TH} = 1.793 × 10^{-4} mm, P_{TH} = 0.846). Genetic analyses suggested that neither UC nor CD had significant effects on the global SA and TH measurements (β_{SA} = −140.892 mm^2, P_{SA} = 0.387 for UC; β_{TH} = 5.812 × 10^{-5} mm, P_{TH} = 0.953 for UC; β_{SA} = −40.268 mm^2, P_{SA} = 0.715 for CD; β_{TH} = −0.001 mm, P_{TH} = 0.428 for CD).

All MR-Egger intercept *P*-values exceeding 0.05 and nearly deviation-free leave-one-out plots indicated that horizontal pleiotropy was effectively controlled, providing evidence for a direct causal relationship between the gut and the cerebral cortex (Supplementary Figure S2). We observed heterogeneity in the causal effects of IBD and CD on the postcentral and superior frontal gyri (Supplementary Table S7), which was deemed acceptable given the use of a random-effects model. Visually symmetric funnel plots provided evidence against directional pleiotropy (Supplementary Figure S3) (23). The power of MR analyses in different pairs was 100% at an alpha rate of 5%.

3.2 Genetic architecture mediating the causal effects of IBD

We identified dominant instrumental loci in IBD–cortex causality and mapped them to the gene database. The distribution of 1,126 instruments is shown in detail in Supplementary Figure S4. After removing duplicates, we finally matched 195 independent SNPs to 140 independent genes with the highest V2G scores (Supplementary Table S8).

GO biological process analysis indicated that these mediators were mainly concentrated in "mononuclear cell differentiation," "regulation of T cell activation," "lymphocyte differentiation," "regulation of leukocyte cell-cell adhesion," and "leukocyte cell-cell adhesion" (Figures 4A, B). In GO cellular component analysis, causal genes were particularly enriched in "RNA polymerase II transcription regulator complex," "interleukin-23 receptor complex," "heteromeric SMAD protein complex," "SMAD protein complex," and "presynaptic cytosol." Regarding GO molecular function analysis, the top 5 significantly enriched terms were "cytokine receptor binding," "DNA-binding transcription factor binding," "RNA polymerase II-specific DNA-binding transcription factor binding," "DNA-binding transcription repressor activity," and "cytokine receptor activity." The critical KEGG-enriched pathways were "Th17 cell differentiation," "Th1 and Th2 cell differentiation," "Cytokine–cytokine receptor interaction," "Inflammatory bowel disease," and "Hepatitis B" (Figures 4C, D).

The interaction networks demonstrated interconnections among the variant-associated genes (Figure 5A). Degree centrality values were calculated (Figure 5B), and the hub genes identified were *STAT3*, *FOS*, *NFKB1*, *JAK2*, *STAT4*, *TYK2*, *SMAD3*, *IL12B*, *MYC*, and *CCL2* (Figure 5C). The shared genetic architecture between IBD and the cerebral cortex was implicated in cytokine regulation and interaction, immune cell differentiation and activation, and immune response modulation.

4 Discussion

Despite the high stability and heritability of structural parameters during cortical development, multiple genetic and environmental factors likely influence the postnatal remodeling of the cerebral cortex. In our study, we utilized genetic variants obtained from large-scale GWAS datasets to disclose the promoting impact of IBD, UC, and CD on the altered cortical SA and TH. This ongoing project elucidates the regionally specific morphological changes of the human brain induced by IBD, UC, and CD and their underlying mechanisms using MR and

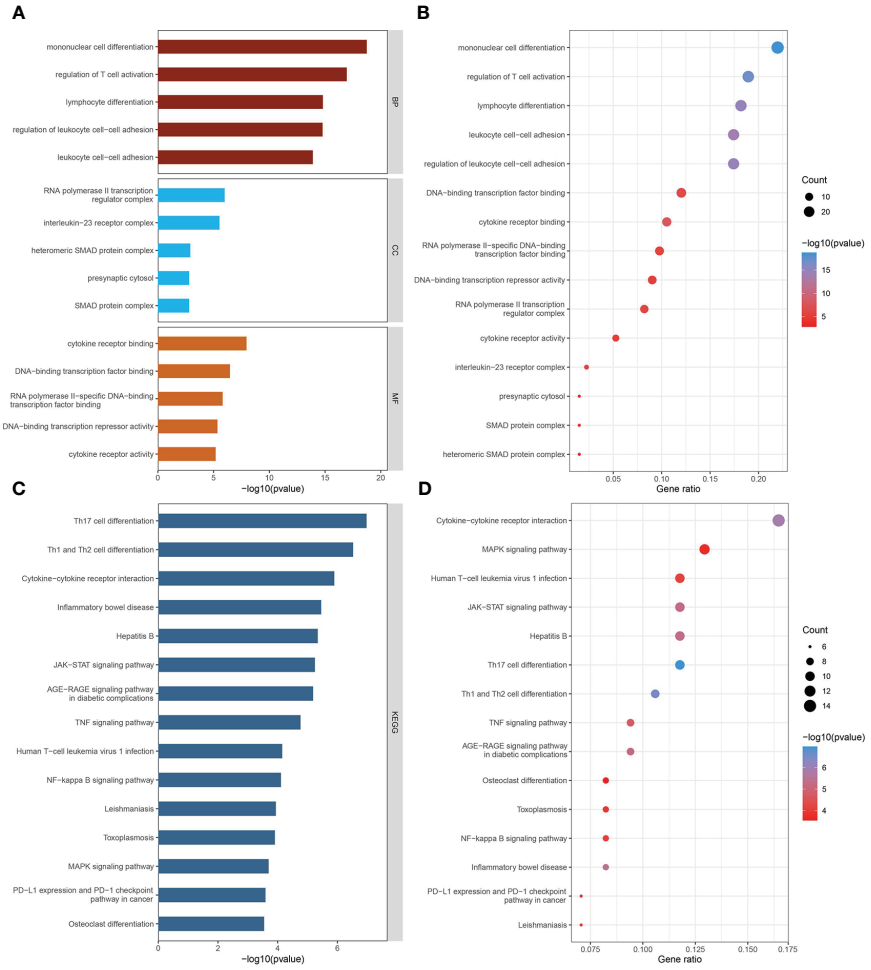

FIGURE 4
The biological functions of mediator genes. **(A, B)** The top 15 GO terms are presented in three categories: BP, CC, and MF. **(C, D)** The top 15 KEGG-enriched pathways are demonstrated. GO-term and KEGG-pathway enrichment analysis was conducted based on overrepresentation analysis, while the background gene list was the entire human genome. GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.

bioinformatics approaches. This research provides a theoretical foundation for comprehending the higher prevalence of neuropsychiatric disorders and enriches our knowledge of the gut–brain axis.

MR estimates suggest that CD significantly reduces the SA of the temporal pole, a region long associated with socioemotional function. We have also identified a potentially decreased SA of the inferior parietal lobe in individuals with IBD, UC, and CD. It is reported that anomalies in the anatomical structure of the temporal pole can lead to unstable mood states (24). The inferior parietal lobe is known to play a crucial role in attention, language, and social functioning, serving as a central neural substrate for various cognitive operations (25). General cognitive ability is positively associated with an expanded SA of the evolutionary inferior parietal region (26), while a decreased cortical SA indicates a higher susceptibility to neuropsychiatric conditions (27–29). Our findings have shed light on the role of cortical reshaping in neuropsychiatric disorders induced by intestinal inflammation, encouraging the clinical utility of brain MRI. However, a previous observation has shown that a thinner TH of the inferior parietal cortex was observed in populations at greater risk of psychosis rather than a diminished SA (30). This phenomenon may be attributed to a heterogeneous population without definitive psychiatric diagnoses.

The theory of radial units posits that increased cell number resulting from neurogenic divisions is responsible for cortical TH growth (31). We found that IBD thickened the postcentral cortex, which seems complicated to understand, since the region is traditionally known as a crucial somatosensory cortex perceiving general bodily rather than visceral sensations (32). The postcentral cortex is believed to contain a visceral information region in rats (33). Furthermore, visceral perception has been confirmed to be directly relevant to regional activity and information flow around the postcentral gyrus (34). Therefore, visceral perception seems structurally or functionally relevant to the postcentral gyrus. We then hypothesize that prolonged mucosal inflammation in patients

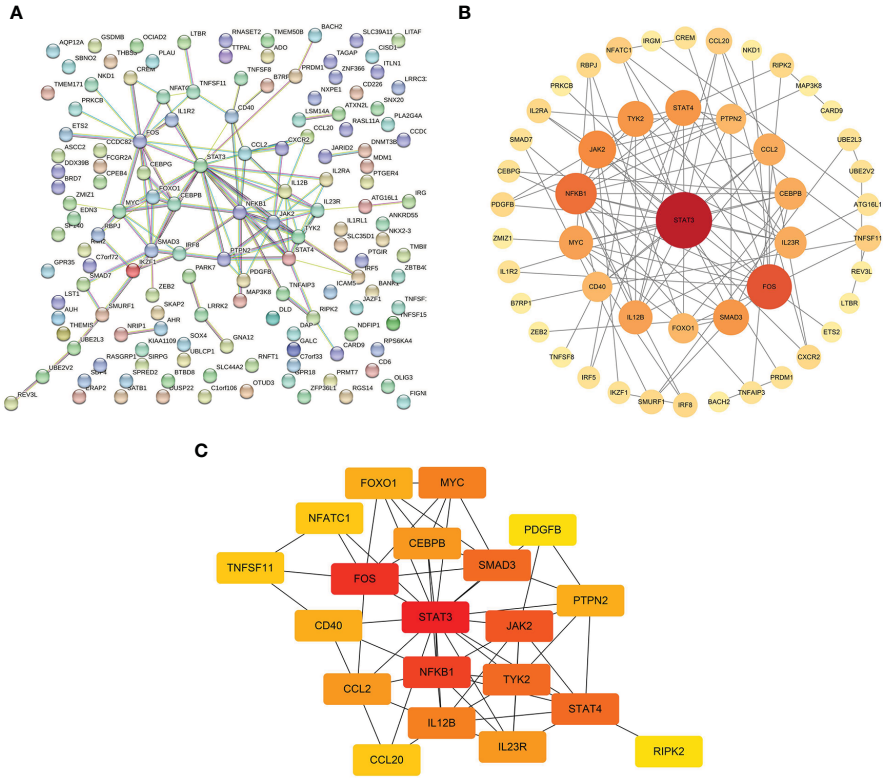

FIGURE 5
Interactions of mediator genes and hub gene identification. **(A)** Interaction network of target genes was constructed using the STRING database. **(B)** After excluding the isolated genes, other genes are ranked based on degree values and listed in descending order of importance, reflected in circle size and color shade, from red to orange to yellow. **(C)** Genes with the top 20 highest degree values are listed, of which hub genes are *STAT3*, *FOS*, *NFKB1*, *JAK2*, *STAT4*, *TYK2*, *SMAD3*, *IL12B*, *MYC*, and *CCL2*. STRING, Search Tool for the Retrieval of Interacting Genes.

with IBD may lead to enhanced sensory input from the viscera, further activating neurogenic divisions and thickening the postcentral gyrus. Compared with patients with irritable bowel syndrome and healthy controls, the TH of the postcentral was considerably increased and positively associated with symptom duration in patients with colitis, thus supporting our proposed hypotheses (35). Meanwhile, we assume that a thickened postcentral may not be the primary cause of neuropsychiatric disorders in IBD patients as what constitutes the underlying pathophysiological mechanisms of cognitive impairment is its thinning structure (36). Further investigations are required to determine whether changes in specific cortices, such as the inferior parietal and postcentral regions, are implicated in psychosis triggered by chronic colitis.

Genetic overlap between IBD and the cerebral cortex accounts for alterations in cortical structure. A significant portion (40%) of identified hub genes were enriched in the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway, which is known to disrupt intestinal homeostasis and contribute to the development of IBD by generating multiple cytokines (37). In the brain, the aberrantly activated JAK-STAT pathway regulates pro-inflammatory gene transcription and elicits neuroinflammation (38). We propose that neuroinflammation, triggered by the JAK-STAT pathway, is a crucial agent responsible for the observed causality. Chronic gut inflammation resulting from mucosal immunity promotes the translocation of inflammatory mediators from peripheral tissues to the central nervous system, where neuroglial cells are recruited and induce neuroinflammation (39, 40). Neuroinflammation, in turn, significantly downregulates the expression of brain-derived neurotrophic factors, leading to neuronal degeneration, apoptosis, and dystrophy, ultimately resulting in altered cortical structure (41). The identification of *FOS* and *NFKB1* in the interaction network supports our assumptions. As an immediate early gene, the expression of *FOS* indicates neuronal activation to multiple stimuli (42), which has also been confirmed as a master regulator of autoimmunity and inflammation in the central nervous system (43). Given that *NFKB1* fuels neuroinflammation (44), its presence emphasizes the role of neuroinflammation in cortical morphology changes.

Other genetic loci in the gene network offer novel insights into mechanisms of cortical alterations. Genes such as *CEBPB*, *PTPN2*, and *NFATC1* exert neuroprotective effects by alleviating inflammatory responses and facilitating neurological function repair (45–47). *PDGFB* is a protein-encoding gene responsible for maintaining the integrity of the blood–brain barrier by activating pericytes, and its mutation facilitates the neuroinflammatory

cascade in the brain (48). Additionally, mutant *PDGFB* has been associated with a neurodegenerative condition characterized by extensive calcification of brain areas, including the cerebral cortex (49).

This study innovatively adopted a combination of MR analysis, functional enrichment, and protein–protein interaction network to investigate the correlation between IBD and the human cortex, offering novel insights into IBD-induced brain dysfunction. Due to the differential formation pattern of UC and CD, we have implemented genetic instruments that independently predicted IBD, UC, and CD, yielding more precise estimates. The most effective genetic instruments correlated with exposure events with F-statistics over 10 were selected, and we avoided disruptions from possible confounders in every causal analysis. These considerations ensure the robustness and reliability of the MR estimates presented in the study.

However, several limitations should be acknowledged. Firstly, due to the utilization of summary statistics, stratified discussions based on age, sex, disease activity, and subjective perception score were not feasible. Secondly, target genes can only be qualitatively analyzed due to the lack of expression data, thereby impeding measuring their upregulation and downregulation within the pathways. Finally, this study focused on individuals of European heritage; therefore, our observations could be more conclusive if supplemented with large-scale studies on individuals from diverse ethnic backgrounds.

5 Conclusion

By utilizing GWAS summary statistics, we have shed light on the causality between intestinal inflammation and altered cortical morphology, thereby facilitating the application of brain MRI in patients with IBD. Bioinformatics analyses showed that neuroinflammation-induced neuronal damage and impaired neurological repair likely trigger cortical atrophy, whereas continuous nociceptive input increases cortical TH. Further studies on the causality between IBD and cortex as well as disease-associated mechanisms are necessary for future advancement.

Data availability statement

All data are publicly available. The summarized inflammatory bowel disease data adopted in this paper are available from the GWAS Catalog (https://www.ebi.ac.uk/gwas/summary-statistics). The meta-analytic brain structure data presented in this paper are available from the ENIGMA consortium website (http://enigma.ini.usc.edu/research/download-enigma-gwas-results). Operation codes of two-sample MR could be obtained from the corresponding author upon a reasonable request.

Ethics statement

Our study only extracted GWAS summary statistics from published studies, which were also publicly accessible. No extra ethical approval or informed consent was required.

Author contributions

Research conception and design: YO, SH, and YP. Data collection and analysis: SH and YP. Figure and table production: SH, YP, and XC. Data verification: YO and XC. First drafting of article: SH and YP. All authors have revised the article and approved the submission

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1175873/full#supplementary-material

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