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Oral nanomedicine for modulating immunity, intestinal barrier functions, and gut microbiome

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ABSTRACT

The gastrointestinal tract (GIT) affects not only local diseases in the GIT but also various systemic diseases. Factors that can affect the health and disease of both GIT and the human body include 1) the mucosal immune system composed of the gut-associated lymphoid tissues and the lamina propria, 2) the intestinal barrier composed of mucus and intestinal epithelium, and 3) the gut microbiota. Selective delivery of drugs, including antigens, immune-modulators, intestinal barrier enhancers, and gut-microbiome manipulators, has shown promising results for oral vaccines, immune tolerance, treatment of inflammatory bowel diseases, and other systemic diseases, including cancer. However, physicochemical and biological barriers of the GIT present significant challenges for successful translation. With the advances of novel nanomaterials, oral nanomedicine has emerged as an attractive option to not only overcome these barriers but also to selectively deliver drugs to the target sites in GIT. In this review, we discuss the GIT factors and physicochemical and biological barriers in the GIT. Furthermore, we present the recent progress of oral nanomedicine for oral vaccines, immune tolerance, and anti-inflammation therapies. We also discuss recent advances in oral nanomedicine designed to fortify the intestinal barrier functions and modulate the gut microbiota and microbial metabolites. Finally, we opine about the future directions of oral nano-immunotherapy.

Contents

Abbreviations: APCs, Antigen-presenting cells; ARF, ADP ribosylation factor 6; C1orf106, Chromosome 1 open reading frame 106; CRC, Colorectal cancer; CTB, Cholera toxin B subunit; CX3CR1, CX3C chemokine receptor 1; CyD1, Cyclin D1; DCs, Dendritic cells; DSS, Dextran sodium sulfate; EAE, Experimental autoimmune encephalomyelitis; F.VIII, Coagulation factor VIII; GAA, Acid alpha-glucosidase; GALT, the gut-associated lymphoid tissues; GIT, Gastrointestinal tract; HABN, Hyaluronic acid-bilirubin nanoparticles; HCC, Hepatocellular carcinoma; IBD, Inflammatory bowel diseases; ICAM-1, Intercellular adhesion molecule 1; Ig, Immunoglobulin; ILC, Innate lymphoid cells; LNs, Lymph nodes; M−cells, Microfold cells; mLN, Mesentric lymph node; MS, Multiple sclerosis; NK cells, Natural killer cells; NPs, Nanoparticles; PEG, Polyethylene glycol; PRRs, Pattern recognition receptors; TfR, Transferrin receptor; TGF-β, Transforming growth factor-beta; TLRs, Toll like receptors; TNF-α, Tumor necrosis factor-alpha; Tregs, Regulatory T-cells.

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1. Introduction

The gastrointestinal tract (GIT) is the largest interface in the body that is in direct contact with the external environment [1,2]. GIT affects not only local diseases in GIT (e.g., inflammatory bowel disease, IBD) but also various systemic diseases (e.g., diabetes and cancer) [3–8]. Thus, therapeutic approaches targeted to GIT have broad applications. When developing therapeutics for GIT, the following factors should be taken into account. 1) The mucosal immune system in GIT is composed of the GALT (the gut-associated lymphoid tissues including Peyer's patches and isolated lymphoid follicles), lamina propria, and intestinal epithelium [9–11]. 2) The intestinal barrier is composed of mucus and intestinal epithelium [2,8], and 3) there is a large number ($>10^{14}$) and variety (~10,000 species) of gut commensal microbes colonized in GIT [12–13]. GALT composed of Peyer's patches and lymphoid follicles is a major site for inducing immune responses against antigens trafficked from the gut lumen. After adaptive immune system is activated via the inductive processes in GALT, activated B-cells and T-cells reach the gut lamina propria and other systemic mucosal regions to establish protective immunity [14–16]. On the other hand, when an antigen is processed in the lamina propria, the general immunological consequence is an immune tolerance to that specific antigen [11,17]. Under the status of certain diseases (e.g., IBD, diabetes, and colorectal cancer), intestinal barrier functions are disrupted, resulting in over-reactive and dysregulated mucosal immune responses against gut-associated antigens [8,18,19]. Notably, the gut microbiota closely interacts with the intestinal barrier and mucosal immune system and provides microbial antigens and metabolites [4,6,8,19,20]. In addition, the composition and dysbiosis of gut microbiota, along with dysfunction of the intestinal barrier and the immune system, have great influences on the development, severity, and treatment of various GIT and systemic diseases [4,6,8,19,20]. Therefore, there is strong rationale for developing GIT-targeted therapeutics that can modulate the GIT factors.

Compared with other routes of administration, oral immunotherapy has clear advantages for modulating the GIT factors, including the ease of targeting GIT, limited systemic drug exposure, simple self-administration, and patient compliance [10]. The major goals and challenges of oral immunotherapies include the following (Fig. 1). 1) Antigens employed in oral vaccine applications should be targeted to microfold (M) cells, which are epithelial cells specialized in transporting antigens from the lumen to lymphoid tissues, such as Peyer's patches [10]. Immune cells in Peyer's patches would then elicit antigen-specific immune responses and establish protective immunity in the GI mucosal surfaces as well as in the systemic compartments. 2) On the other hand, antigens taken up in the lamina propria are generally known to induce systemic and mucosal immune tolerance, and this forms the basis of experimental oral immunotherapies against autoimmune diseases and allergies [21]. 3) Furthermore, dysregulated lamina propria is associated with IBD and other GIT disorders; thus, the lamina propria as a potential target for oral immunotherapies [22]. 4) Moreover, disrupted intestinal barrier functions result in numerous pathologies, including IBD. Therefore, the recovery and reinforcement of the intestinal barrier functions are viable therapeutic approaches against GIT disorders [8]. 5) Lastly, emerging evidence indicates that dysbiosis of gut microbiota is linked with numerous diseases, including colitis, diabetes, and colorectal cancer [20,23–25]. Thus, strategies that can restore the healthy gut microbiome have gained much attention as a new form of oral immunotherapy.

To achieve these goals, various therapeutics are under development. In the case of small molecule drugs, only a fraction of orally administered drugs typically reach the target sites in the GIT, while a large portion of the dose is excreted or systemically absorbed, often resulting in toxic systemic exposure and serious side effects [26,27]. For instance, methylprednisolone can cause thymic involution, insomnia, depression, bone density loss, and moon face, while the side effects of mesalamine include itching, muscle or join pain, swelling of any part of the body, chest pain, heartburn, and dizziness [28,29]. In contrast, macromolecules administered orally have limited systemic absorption due to their large sizes [30]. However, they are prone to structural changes, degradation, and loss of bioactivity by the harsh conditions of GIT, including the pH variation and proteolytic enzymes [31,32]. Therefore, there is a great need for new oral drug delivery systems that can effectively protect their cargo from the harsh conditions of the GIT and selectively deliver drugs to desired target sites.

Nanomedicine has attracted much attention due to the abilities of nanoparticles (NPs) to protect cargo molecules from external stresses, deliver drugs to target tissues, and sustain drug release [33]. To achieve these goals, oral nanomedicine should overcome multiple physicochemical and biological barriers in the GIT, including the highly acidic environment in the stomach, pH variation and proteolytic enzymes along the GIT [31,32]. In the case of NPs with weak acid or base groups, it should be taken into consideration that the pH variation will affect the ionizable groups and morphologies of NPs along the GIT [10]. For NPs targeted to Peyer's patches or lamina propria, the intestinal epithelium and its mucus-secreting layers are critical barriers to overcome [34,35]. Thus, it may be suitable to design NPs that adhere to the mucus layer and penetrate the intestinal epithelium. On the other hand, for NPs targeted to the small intestines where the majority of absorption processes occur, the short residence time (3–4 h) in the small intestine present additional challenges for the design and development of small intestine-targeting NPs [36]. Lastly, the design criteria for oral nanomedicine should take into account the properties of target tissues in healthy versus pathological states. For example, since the key features of IBD are leaky inflamed epithelium and loss of mucus layers [8,19,22], oral nanomedicine for treating IBD may passively reach the disrupted intestinal barriers and the lamina propria. In contrast, oral

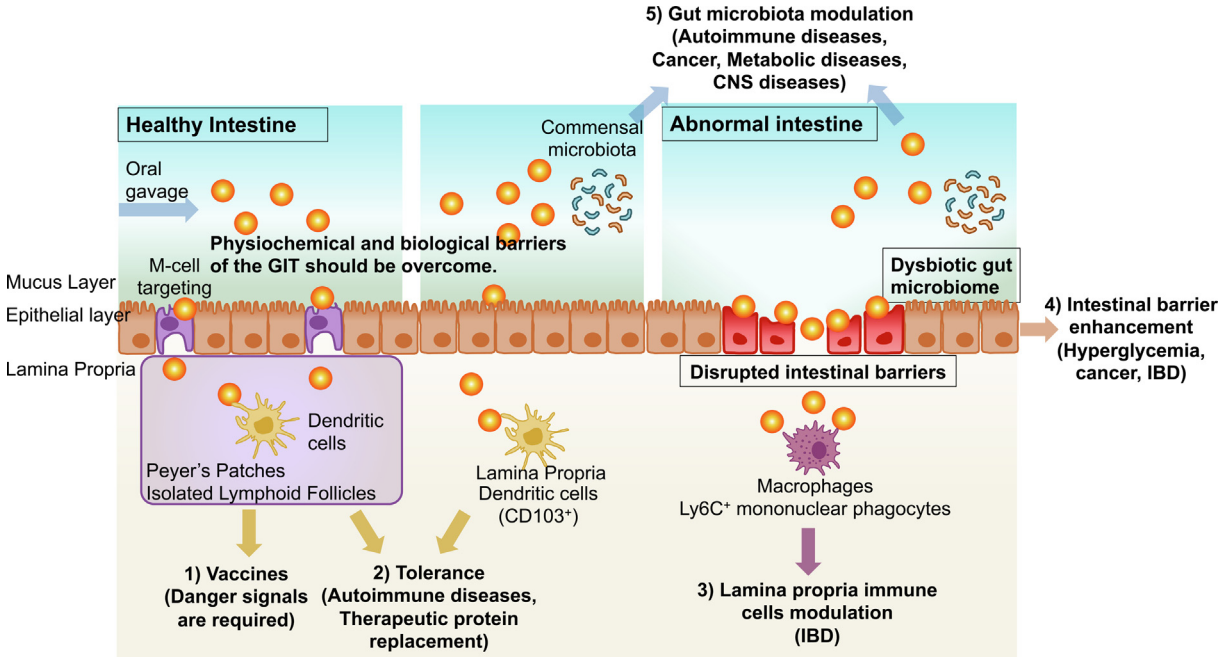

Fig. 1. Biomedical applications of oral nano-immunotherapy. Oral nano-immunotherapy serves as the therapeutic platform for **1**) oral vaccines, 2) immune tolerance, 3) treatment of inflammatory bowel diseases, 4) enhancing the intestinal barrier, and 5) modulating the gut microbiota.

nanomedicine intended to modulate the gut microbiome should target the mucus layer since most commensal microbes reside in the mucosal layer in the gut lumen.

In this review paper, we first present the key GIT factors, including the immune system, intestinal barrier, and gut microbiota, and we review physicochemical and biological barriers of the GIT that oral nanomedicine needs to overcome. We also discuss recent progress in oral nanomedicine designed for oral vaccines, oral tolerance, and anti-inflammation applications. Lastly, we highlight the recent developments in oral nanomedicine for modulating the intestinal barrier functions and gut microbiota as a new therapeutic approach against various diseases.

2. Interactions between the immune system, intestinal barrier, and gut microbiome.

2.1. The GIT immune system

It is estimated that GIT harbors up to 70% of lymphocytes in the body, and thus, GIT is the largest immunological organ [1,2]. The intestinal immune system can be divided into inductive and effector sites. Inductive sites include GALT (e.g., Peyer's patches and isolated lymphoid follicles) and gut-draining mesenteric lymph nodes (mLNs). The main effector sites are epithelium and lamina propria that contains large populations of activated T-cells and antibody-secreting plasma cells as well as innate immune cells, such as macrophages and dendritic cells (DCs) [9–11]. The gastrointestinal immune system is constantly challenged with antigens from the lumen and therefore must be able to distinguish which antigens should be tolerated (e.g., self-antigens, food, symbiotic microbes) or not (e.g., pathogens, toxins) [11]. Typically, the intestinal immune environment (intestinal epithelium and lamina propria) is immunosuppressive due to the high levels of anti-inflammatory factors, such as IL-10, transforming growth factor (TGF)- β, and retinoic acid [11,37].

In the intestinal innate immune system, the outermost sentinel is the intestinal epithelium that lines the intestine and serves as a physical barrier between the luminal contents and the host immune system [38]. The intestinal epithelium actively contributes to the innate immune responses and senses microbial organisms via pattern recognition receptors (PRRs), such as Toll like receptors (TLRs) [38]. Furthermore, the epithelium expresses the major histocompatibility complexes and serves as antigen-presenting cells [38]. Macrophages also has important housekeeping roles, including clearance of apoptotic or senescent cells, tissue remodeling, and maintenance of the immunoregulatory gut environment [39]. Under steady state conditions, Ly6C^{high} monocytes) constitutively enter the intestinal mucosa (especially in the lamina propria) and differentiate locally into anti-inflammatory mature CX3C chemokine receptor 1(CX3CR1)^{high} F4/80^{+} macrophages that express scavenger receptors and major histocompatibility complex-II. They are hyporesponsive to pro-inflammatory stimuli but highly phagocytic against invading commensals or pathogens [39,40]. Furthermore, the resident macrophages help CD103^{+} DCs to induce oral tolerance by directly sampling the luminal contents via dendrites extended between the cells of the intestinal epithelial barrier [41]. CX3CR1^{high} macrophages also produce a large amounts of IL-10 which enhances the secondary expansion of regulatory T cells (Tregs) in the mucosa and may also condition newly arrived monocytes [40]. In turn, TGF-β generated by Tregs may condition newly extravasated monocytes. Resident macrophages can also help maintain the epithelial integrity by secreting prostaglandin E2, which contributes to physiological tissue remodeling [42].

The gut homeostasis can be perturbed by inflammation or infection. The epithelium plays an important role in the initial colonization and sensing of an infection by PRRs such that it transduces the signal to other innate cells that, in turn, amplify the response [38]. When homeostasis is perturbed by inflammation or infection, the normal pattern of monocyte differentiation is disrupted, leading to the accumulation of potent pro-inflammatory effector Ly6C^{high} monocytes and CX3CR1^{int} macrophages [40]. Macrophages sense the perturbation through TLRs and activate intracellular pro-inflammatory signals. Resident monocytes contribute to the recruitment of neutrophils through production of macrophage-

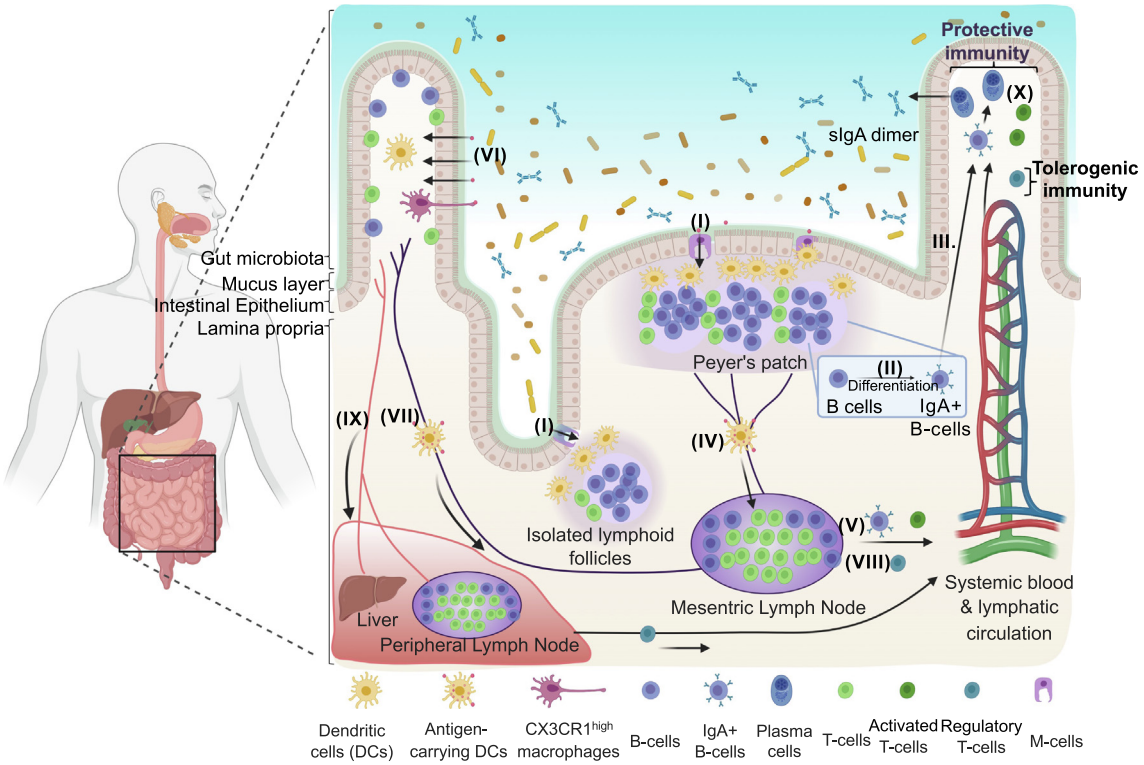

Fig. 2. Immune system in the gastrointestinal tract. (I) Specialized M cells in the epithelium overlying GALT mediates transcellular transport of antigens to DCs. (II) B-cells are activated to immunoglobulin-secreting cells that (III) migrate to the lamina propria and systemic mucosal effector sites and differentiate into IgA-secreting plasma cells. (IV) Antigen-carrying DCs migrate to mLNs to activate B-cells and T-cells, which (V) migrate to distant effector sites through the lymphatic system. (VI) Antigens can be recognized by DCs through diffusion through epithelial tight junctions, transfer across epithelial cells by transcellular routes, exosome-mediated delivery, or capturing from CX3CR1 high macrophages. (VII) Oral tolerance is initiated by antigen-carrying $CD103^+$ DCs that induce Tregs, leading to (VIII) their dissemination to distant effector sites. (IX) Antigens also reach to the liver and peripheral lymph nodes. (X) Oral immunotherapy could trigger protective immunity or tolerogenic immunity, depending on the target cells and local signals. Created by BioRender.com.

derived chemokines [43]. Neutrophils in the blood circulation can sense the chemoattractant gradient and extravasate the vascular endothelium to reach the intestinal lamina propria [43]. Neutrophils are equipped with elegant defense mechanisms, such as nicotinamide adenine dinucleotide phosphate, oxidase-mediated reactive oxygen species burst, antimicrobial peptides, myeloperoxidases, and neutrophil extracellular traps [43]. Neutrophils also contribute to the recruitment of other immune cells and facilitate mucosal healing by releasing mediators necessary for the resolution of inflammation [44]. Importantly, during inflammation, the resident $CX3CR1^{high}$ macrophages retain their anti-inflammatory characteristics, e.g. IL-10 production [39–40]. Classical monocytes and resident $CX3CR1^{high}$ macrophages play regulatory roles by controlling and removing the activated harmful neutrophils [39,40,43,44].

Amplification of the innate response can have adverse effects on the host. Although the above responses are clearly beneficial, excessive recruitment and accumulation of pro-inflammatory monocytes, activated neutrophils, and other innate immune cells in the intestine under pathological conditions is associated with mucosal injury and debilitating disease symptoms [39,43,45,46]. Furthermore, the production of tumor necrosis factor (TNF)-α from activated innate immune cells has an important role in protecting the host, but when produced in high quantities, or over extended periods of time, it contributes to tissue damage, including DNA damage [47]. As most cells expresses receptors for TNF-α, those can lead to an enhanced accumulation of reactive oxygen species and increased levels of chemokines and other pro-inflammatory responses. TNF-α has promiscuous effects, rendering it an effective therapeutic target in IBD patients [48].

As the primary inductive sites of adaptive immune responses, GALT and mLNs harbor memory B- and T-cells that subsequently migrate to the mucosal effector sites (both gut and systemic mucosal effector sites) via the lymphatic system and exert mucosal immune responses [14,15] (Fig. 2). GALT contains follicular B cell zones, inter-follicular T cell zones, and antigen-presenting cells (APCs), such as DCs and macrophages [49]. In organized tissues of GALT, such as Peyer's patches and isolated lymphoid follicles, specialized M cells in the epithelium overlying Peyer's patches and the lymphoid follicles play a pivotal role in transcellular transport of antigens [11,50]. M cells pass antigens to DCs that lie either below the epithelium or in a "pocket" created at the basolateral surface of M cells [11,50]. Antigens can also be directly taken up by DCs in the GALT from the lumen by their extended dendrites [51]. Afterwards, DCs process antigens and present antigenic fragments on their surfaces to activate naïve $CD4^+$ T-cells. Subsequently, $CD4^+$ helper T-cells interact with antigen-specific B-cells, leading to class switching of B-cells, which become immunoglobulin-secreting cells [10,52]. Activated B-cells leave Peyer's patches and the lymphoid follicles and reach distant effector sites (i.e., systemic mucosal and gut effector sites), leading to their differentiation and maturation into plasma cells [10,52]. Alternatively, antigen-carrying DCs themselves could also directly migrate to LNs, interact with germinal centers, and activate humoral and cellular responses, resulting in the migration of activated immune cells (B-cells and T-cells) to distant effector sites [10,52]. Notably, it remains unclear whether M cell-mediated antigen uptake into GALT has an important role in the induction of oral tolerance against soluble antigens [11,21,53–55]. Also, while it seems that antigen uptake by Peyer's patches and isolated

lymphoid follicles play a minor role in oral tolerance [11,21,53–55], they may be more important in modulation of immune responses to the gut microbiome [11]. In certain circumstances, low doses of antigens with multiple administrations or high doses of antigens without pro-inflammatory danger signals may cause oral tolerance in the GALT [21]. This should be considered in the development of oral vaccines and immunotherapies for oral tolerance.

The lamina propria (the gut effector site) harbors antigen-specific mucosal effector cells, such as immunoglobulin (Ig) A-producing plasma cells and memory B- and T-cells, and performs mucosal protective activities for maintaining homeostasis [14,16]. For example, IgA from plasma cells is transported across the epithelium by polymeric Ig receptors [56]. Secretory IgA antibodies prevent the attachment and colonization of pathogens at mucosal surfaces [56]. Other important effector mechanisms that contribute to the host defense against pathogens at lamina propria include locally produced IgM and IgG and mucosal cytotoxic T lymphocytes [57]. Under inflammation as in IBD, the lamina propria is altered, as characterized by increased frequency of activated immune cells [4,6,20,58], leakage of the intestinal barrier [8,19], penetration of pathogens or gut microbiota [4,6,20], and dysregulated immune responses [4,6,20,58]. This renders the lamina propria to be an inflamed environment prone for tissue damage, as observed in IBD [37,58].

On the other hand, the lamina propria could also serve as an inductive site for oral tolerance. Antigen uptake by $CD103^+$ DCs in the lamina propria induces oral tolerance to soluble antigens [11,17]. Antigens can be recognized by DCs [11] through 1) diffusion through the epithelial tight junctions [59], 2) transfer across epithelial cells by transcellular routes [59], 3) exosome-mediated delivery [60], and 4) capturing from CX3CR1 high macrophages [41]. Oral tolerance is initiated by antigen-carrying $CD103^+$ DCs migrating from lamina propria into mLNs [17,61]. In mLNs, retinoic acid (RA) produced from vitamin A by retinal dehydrogenase of DCs and local stromal cells induces the expression of gut-homing receptors $\alpha4\beta7$ integrin and C-C motif chemokine receptor 9 on antigen-specific T-cells, and TGF-β dependent differentiation of $Foxp3^+$ Tregs [62,63]. Tregs re-enter the intestinal lamina propria and undergo secondary expansion under the influence of IL-10 produced by $CX3CR1^{high}$ macrophages [64,65]. Subsequently, Tregs enter other immunologic regions via the lymphatic circulation and/or systemic blood circulation, establishing systemic oral tolerance [11,66]. Antigens taken up into Peyer's patches or the lamina propria may also reach the liver where sinusoidal endothelial cells, tolerogenic conventional DCs, or plasmacytoid DCs induce systemic tolerance [67–69]. When antigens reach peripheral lymph nodes and are presented by resident DCs in the absence of co-stimulation, systemic tolerance may also occur [11].

2.2. The intestinal barrier

The intestinal barrier is composed of a single layer of intestinal epithelial cells that participate in the induction and maintenance of innate immunity [2,8]. Additional intestinal cells include goblet cells, Paneth cells, M cells, intestinal epithelial stem cells, and enteroendocrine cells [34,70]. Among these, enterocytes, goblet cells, and M cells play crucial roles in gut protection, transport, and immunity [10,34,70,71]. The mucus layer on the GIT epithelium protects the body from the invasion of pathogenic threats [34,35] (Fig. 3). Mucus, primarily composed of mucins secreted by goblet cells, is essentially a hydrogel (N95% water) consisting of a mixture of proteins, carbohydrates, lipids, salts, and antibodies [35,72,73]. The shielding and lubricating functions of mucus have a crucial role in maintaining an intestinal homeostasis [74]. The mucus layer directly interfaces with the gut microbiota [75,76] and provides attachment sites to glycan-binding components of microorganisms, thus affecting the colonization of microorganisms [77,78]. Mucin glycans also serves as nutrition for mucus-associated bacteria, so-called 'mucolytic bacteria' [79], favoring their replication [80,81]. These leads to the selection of various commensal microbes that make up our gut microbiota [75,82]. Moreover, some commensal microbial species, such as *Lactobacillus spp* [83,84], *Bifidobacterium longum* [85], *Lactobacillus reuteri* [86], and *Akkermansia muciniphila* [87–92], promote the mucin production and increase the mucus layer thickness. Commensal bacteria provide protection against pathogenic microbes by increasing the mucus production and offering an additional intestinal barrier that prevents the adhesion of pathogenic microbes [93–94] (Fig. 3). Viscosity of the mucus layer also limits the motility of microbes [93], thus protecting the underlying epithelium against microbes. Collectively, the integrity of the mucus barrier is crucial for the protective functions of the GIT [75,80,82,93,94].

The intestinal barrier function is crucial to maintaining tissue homeostasis [8]. The connection between individual epithelial cells is held by a series of intercellular tight junctions, composed of junctional adhesion molecules, occludin, claudin, and tricelluin [95,96]. Tight junction is the apical junction along the lateral surface and is directly responsible for intestinal barrier functions [95,96]. When the integrity of the cell–cell junctions is disrupted, unrestricted passage of pathogens and molecules across the epithelial layers could occur [8]. Furthermore, the mucus layers and gut microbiota are also important for the intestinal barrier functions [75]. The intestinal epithelial barrier is constantly being challenged by the gut microbiota, food, and food-associated microbes, and dysregulated intestinal barrier functions, epithelial integrity, and cell–cell junctions are associated with diverse pathological states, IBD [7,97–99], autoimmune diseases [100], and systemic infection [101] (Fig. 3). Inactivation of the IBD susceptibility gene, C1orf106 (chromosome 1 open reading frame 106), decreased the intestinal barrier function, thereby triggering intestinal inflammation and IBD [99,102,103]. C1orf106 protein has an important role in maintaining an appropriate level of cytohesin 1 protein in mature epithelia. Cytohesins are activators of the Ras guanosine triphosphatase ARF (ADP ribosylation factor 6), which directs endocytic internalization of cadherins. Downregulation of the ARF6 activity is important in maintaining the stability of tight junctions. Depletion of C1orf106 led to an abnormally high amount of cytohesin and excessive ARF6 activation. This in turn increases cadherin endocytosis and tight junction permeability. The passage of bacterial components, debris, and other antigenic molecules through the leaky tight junction structures cause immune response, inflammation, and tissue damage. Also, a pathogenic bacterium, *Enterococcus gallinarum*, can induce intestinal barrier defects and translocate to LNs and liver, triggering autoimmune diseases, such as systemic lupus erythematosus [100]. In addition, hyperglycemia, associated with diabetes and other metabolic syndromes, can disrupt the intestinal barrier, leading to intestinal inflammation and infection [101]. Chronic hyperglycemia affects the barrier functions through metabolic and transcriptional reprogramming of the glucose transporter GLUT2 in intestinal epithelial cells, leading to the dissemination of bacterial byproducts and systemic inflammation [101]. Overall, these studies have shown the crucial roles of intestinal barrier functions in protection against IBD and other systemic diseases. However, the mechanism by which the intestinal barrier becomes leaky is unclear and requires further investigation.

2.3. The gut microbiota

The human body is inhabited by trillions of microorganisms comprised of bacteria, fungi, and virus [12]. It is estimated that

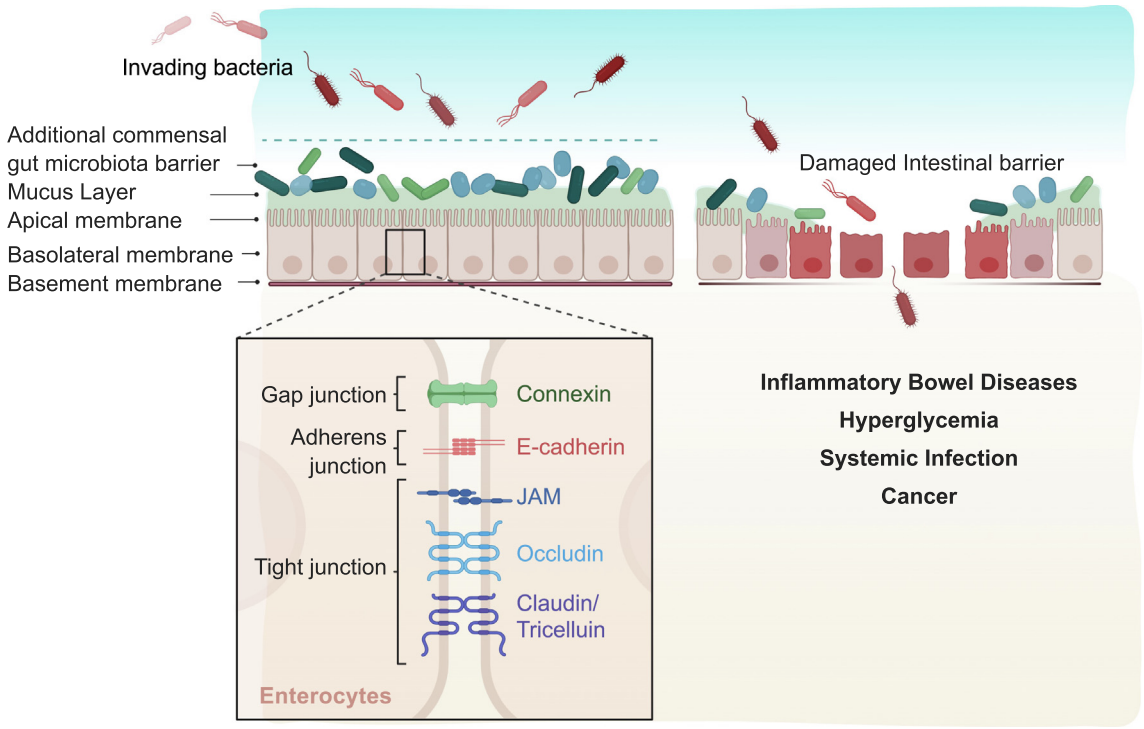

Fig. 3. Maintenance of the intestinal barrier functions. The intestinal barrier is composed of intestinal epithelium and mucosal layer. Epithelial cells are connected by a series of intercellular tight junctions, which are responsible for the intestinal barrier functions. The mucosal and gut microbiota layers protect the body from pathogens. Disrupted of intestinal barrier is associated with various diseases, including IBD, hyperglycemia, infection, and cancer. Created by BioRender.com.

more than 10^{14} microorganisms of ~ 10,000 bacterial species colonize the GIT [12,13]. The gut microbiota supplements the intestinal barrier by forming additional barriers in the mucus layer that separate pathogens, particles, and pollutants from the internal milieu [75,93,104]. Notably, the gut microbiome has established a symbiotic relationship with our immune system and intestinal barrier [2,4,6,8,19,75]. As an example of the host-microbiome symbiosis, some microbiomes such as *Akkermansia muciniphila* feeds on the mucin layer, while commensal microbiotas are required for fucosylation of the mucus layer and its full intestinal barrier functions [107–109]. The gut microbiotas also participate in the maturation and functions of the innate immune system, including secretion of antimicrobial peptides (α -defensin, interactions with TLRs on Paneth cells; β-defensin, interactions with TLRs on epithelial cells) and production of IL-22, IL-17, and IL-10 [110]. Thus, the gut microbiome-host interactions play crucial roles in the development, maturation, and maintenance of the immune system [105,106] (Fig. 4).

Gut microbiota diversity refers to the number of different commensal microbial species present in an individual. Gut dysbiosis is typically characterized by reduced microbial diversity and substantial shifts in the resident microbial species [5,106]. Dysbiotic gut microbiota increases the susceptibility to infection and leads to impaired vaccine response, as observed in the setting of malnutrition [5,111,112]. A prototypic example of dysbiosis is *Clostridioides difficile* infection, which causes rapid disruption of gut microbial communities [113,114]. This leads to an enrichment of primary bile acids and simple carbon compounds, leading to the germination and growth of *C. difficile* [115]. In addition, IBD patients have dysbiotic gut microbiome [4,6,20], disrupted intestinal barriers [8,19], and dysregulated mucosal immune responses [4,6,20]. Other pathologic conditions, including multiple sclerosis

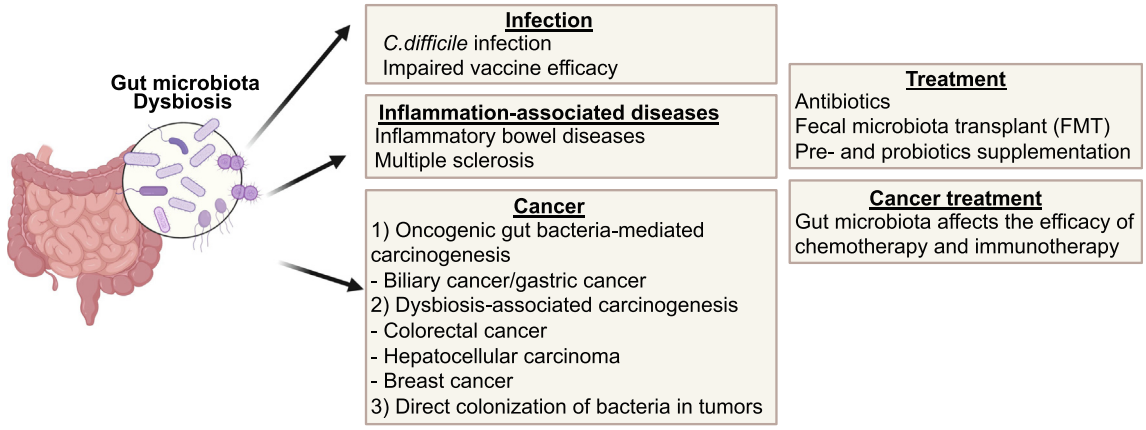

Fig. 4. The role of the gut microbiota in various diseases. Dysbiotic gut microbiota is associated with various local and systemic diseases, including infection, inflammation-associated diseases, and cancer. Created by BioRender.com.

(MS), are also associated with dysbiotic gut microbiota [23,116]. MS is characterized by immune-mediated destruction of myelin in the central nervous system, and the gut microbiota is known to influence this process in genetically susceptible individuals. Depletion of gut commensal bacteria by antibiotics-treatment ameliorated the development of experimental autoimmune encephalomyelitis (EAE) in mice [116]. In contrast, intraperitoneal antibiotic-treatment had minimal effects on the gut microbiota and induction of EAE, suggesting that induction of EAE was affected by the gut microbiome [116]. Furthermore, epidemiological studies of patients with MS have indicated shifts in specific bacterial taxa among MS patients [23]. Taken together, these studies have shown the link between dysregulated gut microbiota and various GIT diseases; thus, modulation of dysregulated microbiota via the use of antibiotics, fecal microbiota transplantation (FMT), or supplementation of probiotics (i.e., a mixture of beneficial commensal microbes) may serve as preventive measures or potential treatments against *C. difficile* infection [117,118], IBD [119–123], and MS [124–126].

The gut microbiome is also increasingly recognized for its role in carcinogenesis. Known oncogenic gut bacteria include *Salmonella enterica* subspecies *enterica* serovar Typhi [127] and *Helicobacter spp.* [128] in biliary cancer and *Helicobacter pylori* in gastric cancer [129,130]. Oncogenic gut bacteria contribute to carcinogenesis by trigger local chronic inflammation, and some bacteria, such as *H. pylori*, have direct genotoxic effects on mucosal cells [129]. Furthermore, preclinical and clinical studies have indicated dysbiosis as an oncogenic driver in colorectal cancer (CRC) [131,132]. Various microbial species, such as *Bacteroides fragilis* [133–135], *Fusobacterium nucleatum* [131,136,137], *Escherichia coli* [138], and *Campylobacter jejuni* [139], are associated with CRC carcinogenesis and metastasis. Dysregulated gut microbiota also has been implicated in hepatocellular carcinoma (HCC) [140] and breast cancer [141]. Intestinal bacterial components, metabolites, and byproducts could be transported to the liver through the portal venous system, causing inflammatory changes and hepatotoxicity. For example, N-nitroso compounds generated in the gut are hydroxylated to their toxic intermediates by the isozyme system in the liver [142]. Similarly, microbial derivatives of bile acids are implicated in carcinogenesis. As unabsorbed bile acids are antimicrobial, bacteria transform bile acids (cholic acid and chenodeoxycholic acid) to secondary bile acids (deoxycholic acid and lithocholic acid) with carcinogenic properties [24,143]. The dysregulated gut microbiota may also promote breast cancer formation by gut microbiota-mediated effects on estrogen metabolism, energy metabolism, and obesity [144,145].

Colonization of bacteria in the tumor microenvironment can also directly impact the growth and immune response of cancer cells, either by direct interactions with the cancer cells or by inducing inflammation or immunosuppression [146,147]. Nejman, *et al.* recently profiled the microbiota associated with tumors from cancer patients and reported distinct organ-specific compositions of microbiome in breast, lung, ovary, pancreas, melanoma, and brain tumors [148]. Notably, there was a strong correlation between the intratumor bacteria and the gut bacterial population. Compromised integrity of the gastric epithelial layer may contribute to bacterial translocation to distal tumors, as recently demonstrated in pancreatic adenocarcinoma. Interestingly, tumor-colonized bacteria may also contribute to resistance to chemotherapy [149]. For example, *Mycoplasma* in pancreatic tumors mediates resistance to gemcitabine by metabolizing it into an inactive form [150]. These studies have shown pro-tumoral properties of the gut microbiome.

In contrast, other studies have reported anti-tumoral effects of "beneficial" gut microbiota [151]. Disruption of the gut microbiota by broad-spectrum antibiotics negatively impacted the patient outcomes in immune checkpoint blockade therapies [152,153], thus highlighting the importance of the commensal microbiota in regulating immune response during cancer immunotherapy. Furthermore, preclinical studies have shown that administration and subsequent gut colonization of commensal bacteria, such as *Akkermansia muciniphila* [154], *Bacteroides fragilis* [155], and *Bifidobacterium* spp. [156], enhanced the anti-tumor efficacy of immune checkpoint blockade therapies. In addition, anti-tumor effects of cyclophosphamide have been partially attributed to the changes in the gut microbiota [149]. Thus, modulation of the microbiome by removing "harmful" carcinogenic bacteria or transplanting "beneficial" microbes with anti-tumor properties may lead to new therapeutic strategies against cancer.

3. Oral nanomedicine for immune system modulation in the lamina propria

Since effector immune cells are located in the lamina propria, approaches that can modulate lamina propria immune cells are being pursued for the treatment of IBD and other GIT-associated diseases. For example, infiltration of macrophages and neutrophils in the lamina propria is the hallmark of IBD [157,158]. Thus, macrophages and neutrophils in the lamina propria are potential therapeutic targets in IBD [22]. If drugs can be specifically delivered to the target immune cells by NPs, this would maximize the efficacy of drugs while minimizing toxicity [22]. To achieve this goal, NPs should overcome the biological and physicochemical barriers in the GIT. They include the pH variation and proteolytic enzymes along the GIT [31,32]. Drug penetration through the mucosal layer and intestinal epithelium are additional barriers to overcome [10]. Differential residence times in small intestine (3–4 h) and colon (1–2 days) are additional factors to consider [36]. Notably, due to the denuded mucus layer and disrupted intestinal epithelium associated with intestinal inflammation in IBD [8,19], it may be possible to deliver drug-loaded NPs passively to the inflamed site through the leaky epithelium [22] (Table 1).

For the treatment of IBD, NP-based targeting of overexpressed surface receptors on activated macrophages has been explored. For instance, mannose receptors [159] and macrophage galactose-type lectin [160] are highly expressed on activated macrophages in inflammatory conditions. Macrophage-targeted NPs have been developed (Fig. 5). These NPs are composed of mannosylated bioreducible cationic polymer, sodium triphosphate, and TNF-α siRNA [161]. These NPs showed efficient macrophage targeting ability without significant uptake by epithelial cells. This led to strong anti-inflammation activity in a murine model of dextran sodium sulfate (DSS)-induced colitis. Furthermore, galactosylated trimethyl chitosan-cysteine NPs were reported to target macrophages via macrophage galactose-type lectin while carrying siRNA against mitogen-activated protein kinase kinase kinase kinase 4, a key upstream mediator of TNF-α production [162]. Oral administration of these NPs ameliorated DSS-induced colitis. Additionally, TNF-α -siRNA loaded polylactic acid-(polyethylene glycol) (PEG) NPs grafted with the Fab' portion of F4/80 antibody exhibited macrophage-targeting ability *in vivo* with promising therapeutic efficacy in the DSS-induced colitis model [163]. However, there is a possibility of degradation of the grafted ligand anti-F4/80 antibody during GIT transit; thus, loading of polylactic acid-PEG NPs into a colon-specific biodegradable hydrogel (chitosan/alginate) protected the Fab' fragments on NPs, leading to improved colon-specific delivery of NPs and therapeutic efficacy [163].

$Ly6C^+$ inflammatory leukocytes in inflamed intestines in IBD also may serve as a target for nanomedicine [164]. Lipid-based NPs carrying IL-10 mRNA achieved a selective expression of IL-10 among $Ly6C^+$ inflammatory leukocytes via targeting with anti-Ly6C antibody, resulting in a favorable therapeutic efficacy in a

Table 1
Key design criteria to consider for GIT-targeted oral NPs.

	Oral vaccine	Oral tolerance	Modulation of lamina propria immune cells	Intestinal barrier enhancement	Modulation of gut microbiota
Harsh GIT conditions (pH variation, proteolytic enzymes)	Should consider (antigens should be protected by NPs encapsulation)		Could consider when loaded drugs or targeting ligands are vulnerable (drugs should be protected by NPs encapsulation, and targeting ligands should be protected, for example by embedding NPs in hydrogels)		
Short residence time in small intestine	Should consider (antigens should be delivered to APCs in the inductive sites of small intestines)		Should consider if targeting the small intestine		
Penetration through the intestinal barrier (mucus layer, epithelium)	Should consider (Both M−cells and epithelium are located underneath the mucus layer)		Should consider (The lamina propria is located underneath the epithelium) when the intestinal barrier structure is intact Not applicable for certain diseases such as IBD with denuded mucus layer and disrupted intestinal epithelium	Should consider penetrating through the mucus layer for epithelium targeting when the intestinal barrier structure is intact	
Targeting strategy	M−cell targeting, Epithelium targeting	M−cell targeting, $CD103^+$ DC targeting	Targeting innate immune cells (e.g., macrophages, neutrophils, $Ly6C^+$ mononuclear phagocytes)	Mucus targeting, Epithelium targeting	Mucus targeting, Large intestine lumen targeting
Other considerations	Should consider using immunostimulatory adjuvants	Should consider using immunomodulatory agents			

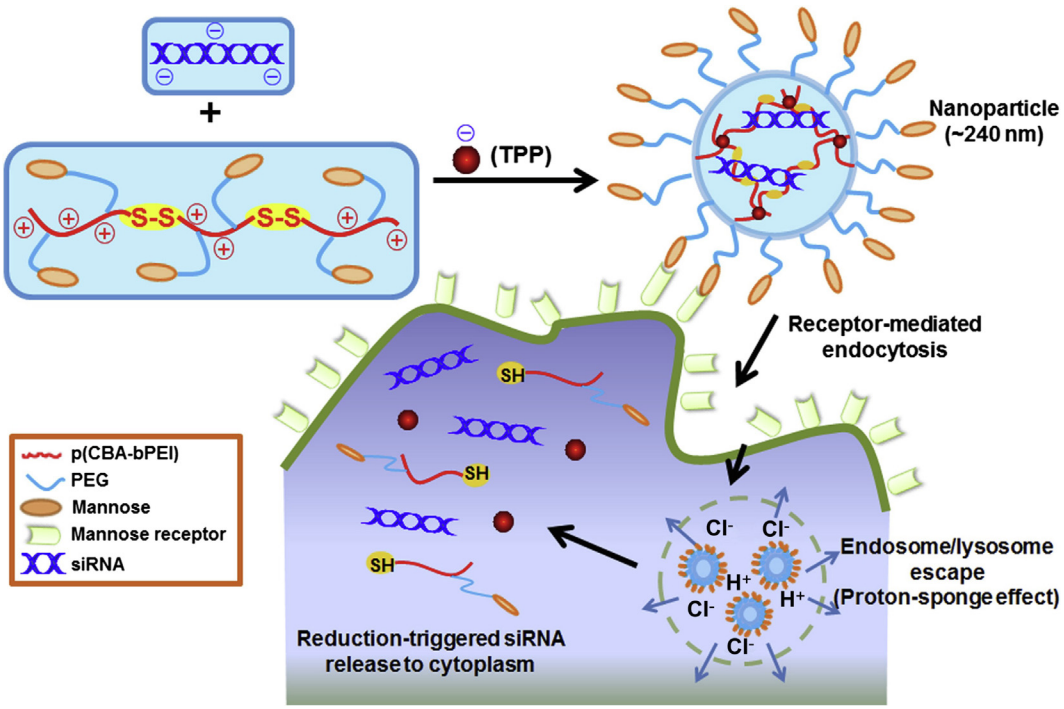

Fig. 5. Oral nanomedicine for targeting the lamina propria. Schematic illustration of TPP(sodium triphosphate)-PPM(a mannosylated bioreducible cationic polymer)/siRNA NP (NP) formation and macrophage-targeting delivery, and the release of siRNAs to the cytoplasm. Reproduced with permission from [161].

DSS colitis model [165]. Other siRNA-loaded NPs have been reported to target inflammatory leukocytes. Epithelial cells and immune cells increase the expression of cyclin D1 (CyD1) in IBD [166]. NPs carrying siRNA CyD1 and targeted to inflamed leukocytes inhibited CyD1 mRNA and inflammatory responses in the DSS colitis model [167]. Similarly, anti-Ly6C antibody-grafted lipid-based NPs carrying siRNA against Interferon Regulatory Factor-8, an immunomodulatory protein, targeted to inflammatory $Ly6C^+$ leukocytes blocked Interferon Regulatory Factor-8 mRNA and significantly decreased the differentiation, polarization, and activation of mononuclear phagocytic cells [168].

Another emerging area in the cell-specific active targeting approaches is to explore naturally occurring extracellular vesicles, such as exosomes derived from edible plants [169,170]. For example, phosphatidylethanolamine and phosphatidylcholine-enriched grapefruit-derived edible nanovesicles were taken up by intestinal macrophages via the clathrin-dependent pathway and micropinocytosis due to the enrichment of phosphatidylethanolamine and phosphatidylcholine on the outer layer [171]. Thus, nanovesicles loaded with methotrexate exhibited a macrophage-targeting ability and ameliorated DSS-induced murine colitis [171]. Furthermore, TGF-β1 gene-modified DC-derived

exosomes induced $CD4^+$ $Foxp3^+$ Tregs while decreasing helper T-cells (Th17) at inflammatory sites in GIT, exerting efficacy against DSS-induced colitis in mice [172,173].

In addition to IBD, GIT-targeted NPs, antigen-carrying NPs with immunomodulatory drugs, such as vitamin D and rapamycin [174,175], have been shown to induce tolerogenic immune responses. These tolerance-inducing NPs may prevent anti-protein drug antibody responses [176] and could be used to treat various diseases, including arthritis [177], allergy [178,179] and diabetes [180]. For more detailed information, readers are referred to section 6.2. Oral nanomedicines for immune tolerance.

4. Oral nanomedicine for modulation of the intestinal barrier

Disrupted intestinal barrier is associated with inflammation and systemic infection, and this may lead to the leakage of bacteria or their byproducts into the underlying tissues and systemic circulation [8]. Thus, restoring the integrity of the disrupted intestinal barrier may have beneficial effects against GIT-associated diseases [8]. For example, in a mouse model of T cell-mediated acute diarrhea, pharmacological agents for controlling actomyosin contractility or endocytosis reduced the symptoms of acute diarrhea [181]. Furthermore, stabilization of C1orf106, which modulates the tight junction proteins [99,102,103], may offer a therapeutic strategy for improving intestinal barrier functions in IBD. In addition, repairing the epithelial layer can strengthen the intestinal barrier functions and may serve as a new approach for the prevention and treatment of IBD [8]. Also, differentiation of intestinal cells fortifies the intestinal crypts, thus providing another potential target for improving the intestinal barrier functions [8].

Oral nanomedicine designed to overcome the biological and physicochemical barriers of the GIT may offer new ways to deliver drugs to the intestinal epithelium and lamina propria (Table 1). Various NP-based strategies have been explored for regulating the intestinal barrier functions as the potential treatments against IBD. Muco-adhesive NPs can deliver drugs to the mucus layer of small or large intestines [182]. Since mucins are composed of hydrophilic components (single-chain amino acid backbone with branched oligosaccharide side chains), NPs should contain hydrophilic functional groups, such as carboxyl or hydroxyl, to facilitate the formation of hydrogen bonds between mucins and NPs [183,184]. Furthermore, mucus can be targeted by exploiting the charge interactions between the anionic charge of mucus and cationic polymers, such as chitosan [185]. Synthetic polymers (e.g., acrylic acid derivative/polyacrylate) and natural polymers (e.g., hyaluronic acid, cellulose derivative, chitosan, alginates, and pectin) that have been shown to non-specifically adhere to mucins could be explored for mucus-targeted therapies [22]. In addition, tomato lectins and bacterial adhesins have been examined for constructing mucin-binding NPs [186].

Notably, as the mucus layer is thin in the inflamed regions in IBD patients [18,187,188], this should be taken into account when designing mucus-targeting NPs for the treatment of IBD. Size and charge of the NP platforms influence their targeting to inflamed epithelium [22,31,189]. Several studies have demonstrated that NPs with less than 200 nm in diameter and a negative surface charge showed better tissue-penetrating activity for IBD treatment as these characteristics allowed for interactions with positively charged proteins expressed on the damaged epithelium of IBD [22,31,189]. To specifically target the inflamed epithelium, active targeting strategies against receptors/molecules upregulated on the inflamed epithelium have been explored. For example, Peptide transporter 1, an oligopeptide transporter, is overexpressed in the colonic epithelium of IBD patients [190–192]. KPV (Lys-Pro-Val) peptide has a high affinity to Peptide transporter 1 and exerts anti-inflammatory effects by restoring inflamed epithelium functions [193]. KPV-based NPs alleviated inflammation by accelerating mucosal healing [194]. Also, Intercellular Adhesion Molecule 1 (ICAM-1) is significantly upregulated in inflamed intestinal mucosal tissues and microvasculature of colon in IBD, colon adenomas, and colon adenocarcinoma [195–197]. Thus, ICAM-1 may serve as a target for oral nanomedicine. For example, anti-ICAM-1 antibody-coated polystyrene NPs have been reported [198]. However, after oral administration, these NPs were mainly deposited in the stomach and duodenum, and approximately 60% of anti-ICAM-1 antibody on NPs was rapidly degraded by GIT enzymes, thus presenting a challenge for successful oral delivery. Transferrin receptor (TfR) is another target that is overexpressed in both the basolateral and apical membranes of enterocytes in inflamed colon [199]. Orally administered anti-TfR-antibody-conjugated NPs targeted the inflamed colon [199]. Thus, ICAM-1 and TfR targeting strategies have the potential to target drugs to inflamed tissues and restore intestinal barrier functions although more research is required to protect targeting ligands from degradation in the GIT.

Since restoration of the intestinal barrier functions and modulation of activated immune cells are both important in the management of IBD, targeting molecules overexpressed on both colonic epithelium and activated immune cells may achieve synergistic therapeutic activity. For instance, both CD98 and CD44 are overexpressed on the colonic epithelium and activated immune cells, thus serving as potential targets [200–202]. An oral hydrogel has been developed for delivering CD98 siRNA-loaded NPs decorated with single-chain CD98 antibody on their surface [203] (Fig. 6). In murine colitis models, orally administered hydrogels targeted CD98-overexpressing macrophages and inflamed epithelium, beneficially modulating their functions. Furthermore, hyaluronic acid has also been used as an active targeting ligand on NPs. Hyaluronic acid is a natural polysaccharide composed of N-acetylglucosamine and D-glucuronic acid units and is generally considered nontoxic and biodegradable. Hyaluronic acid binds to its receptor CD44, which is overexpressed on inflamed epithelium and activated inflammatory cells (e.g., macrophages) [204–206]. Hyaluronic acid-functionalized polymeric NPs carrying CD98 siRNA and anti-inflammatory curcumin protected the mucosal layer and modulated the inflammatory functions of activated macrophages, alleviating mucosal inflammation [207]. Also, polymeric NPs functionalized with hyaluronic acid and loaded with KPV accelerated mucosal healing and alleviated inflammation in a murine model of DSS-induced colitis [189].

The intestinal mucosa of IBD patients is characterized by overproduction of reactive oxygen species and imbalance of antioxidants, which lead to oxidative mucosal injury [208–210]. To address these issues, MeO-PEG-b-PMOT amphiphilic block copolymer-based micelles with stable nitroxide radicals in a hydrophobic segment have been developed [211]. These micelles significantly accumulated in the colonic mucosa area, especially in inflammatory sites, protected epithelial regions from oxidative damages, and exhibited potent therapeutic efficiency in a murine model of DSS colitis.

Overall, these studies have shown that oral nanomedicine may modulate the induction, development, and severity of local GIT diseases and provide a new pathway for treating other systemic diseases.

5. Oral nanomedicine for gut microbiome manipulation

Gut microbiome is intricately linked to immune activation and tolerance as well as various pathologies. Thus, strategies that can modulate the gut microbiome, especially via gut-targeted NPs and microparticles, are being explored [12,212]. Gut microbiome-

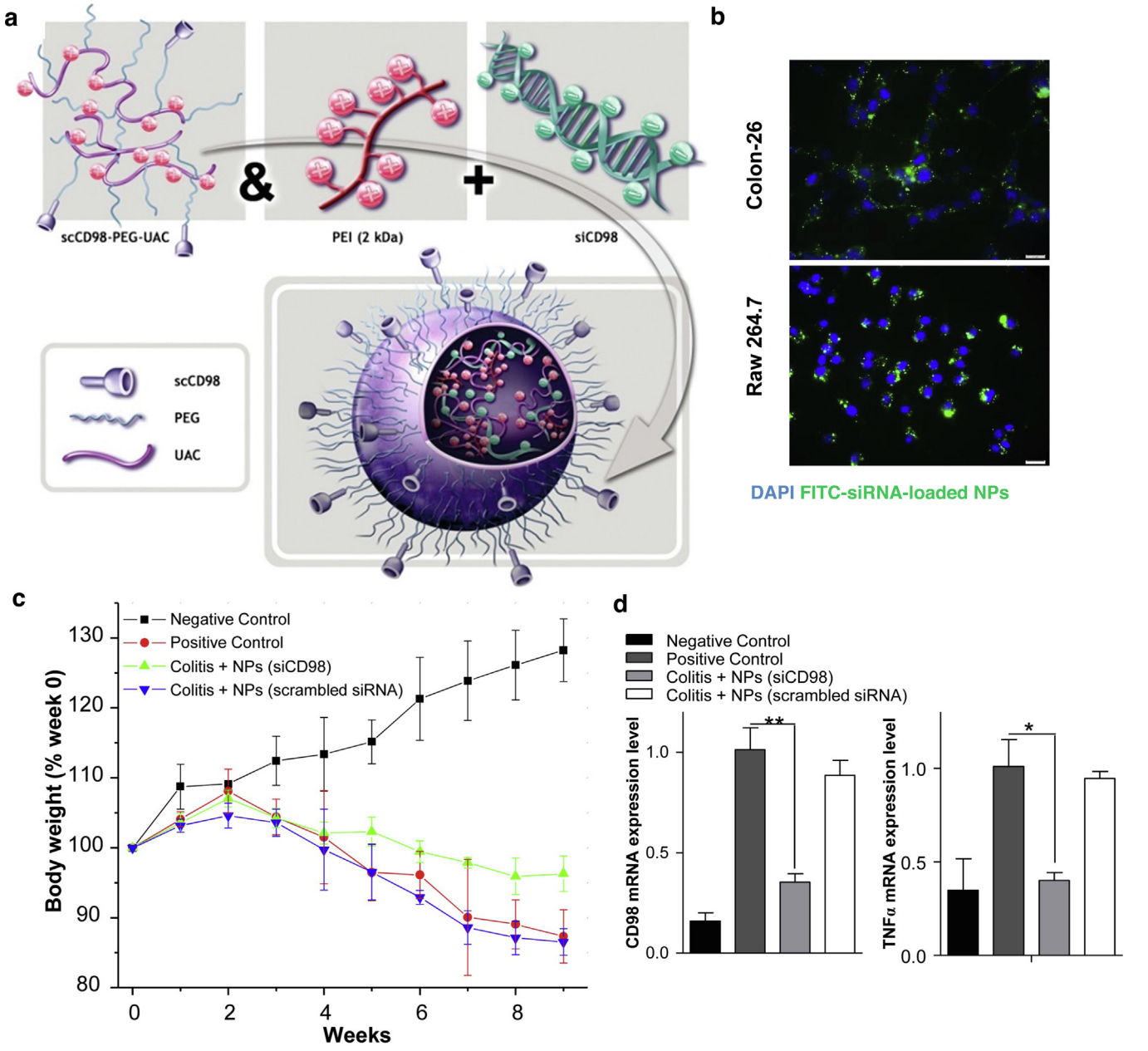

Fig. 6. Oral nanomedicine for improving the intestinal barrier functions. a, Self-assembly procedure of single-chain CD98 antibody-functionalized siRNA-loaded NPs. **b,** Specificity of scCD98-functionalized FITC-siRNA–loaded NPs (green) against *in vitro* colonic epithelial cells (Colon-26 cells; **top**), macrophages (RAW 267.4; **bottom**). **c-d,** *In vivo* therapeutic efficacy of siCD98 NPs as measured by body weight changes (**c**), CD98 mRNA levels (**d; left**), and TNF-alpha mRNA levels (**d; right**) in the colon of DSS-induced colitis mice. . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) Reproduced with permission from [203]

targeted NPs should also overcome the biological and physicochemical barriers of GIT [31,32]. Notably, unlike other systems mentioned above, these NPs would target commensal microbes residing in the gut lumen and thus do not need to penetrate through the gut epithelium (Table 1).

Various nanomedicine systems have been reported for their ability to modulate the gut microbiota as anti-cancer platforms. For example, Fe@Fe3O4 NPs conjugated with ginsenoside Rg3 (NpRg3) have been developed for the treatment of HCC. Ginsenoside Rg3 was used as an autophagy inhibitor and sensitizer of doxorubicin-mediated anti-cancer activity against HCC [213]. Notably, this nanocomposite altered the gut microbiome composition, elevating the relative abundances of *Bacteroidetes* and *Verrucomicrobia* while decreasing *Firmicutes*. Also, NpRg3 significantly decreased 3-indolepropionic acid and urea, which are important metabolites during HCC development, while increasing free fatty acids. Thus, NpRg3 inhibited HCC growth and lung metastasis by remodeling the unbalanced gut microbiota and metabolism. In addition, gut microbe-targeting strategies have been explored for the treatment of CRC. A phage-guided irinotecan-dextran hybrid nanosystem was designed to target a carcinogenic bacterium *F. nucleatum* and a probiotic bacterium *C. butyricum* as a potential therapy against CRC [214]. In this system, a phage was utilized to specifically lyse *F. nucleatum,* which is known to induce chemoresistance and immunosuppression. In addition, dextran was introduced in the nanosystem to promote the proliferation of *C. butyricum,* which suppresses the growth of colorectal carcinoma and induces anti-tumor immune responses via production of

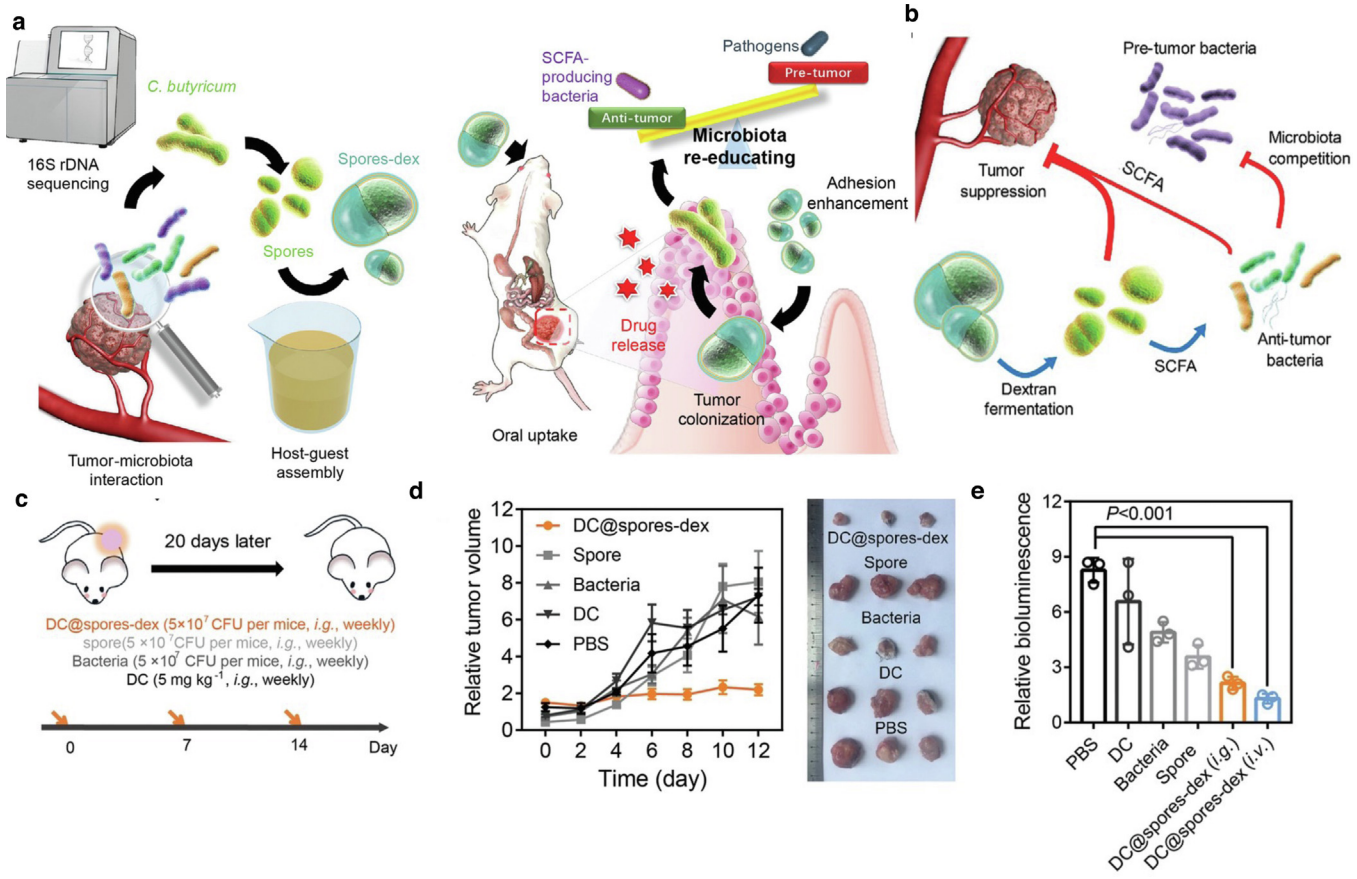

Fig. 7. Oral nanomedicine for modulating the gut microbiome for anti-cancer therapy. a, Dextran-encapsulated probiotics (*C. butyricum*) (Spores-dex) regulate gut microbiota and suppress colon cancer. **b,** Short-chain fatty acids, one of the microbial metabolites, regulate gut microbiota and suppress tumor growth. **c-e,** *In vivo* therapeutic efficacy of diclofenac-loaded spores-dex (DC@spores-dex) in mice bearing subcutaneous (**c, d**) or orthotopic CT26 tumors (**c, e**). . Reproduced with permission from [216]

short-chain fatty acids [215]. This combination strategy showed synergistic anti-cancer activity against CRC. Furthermore, dextran capsules co-loaded with *C. butyricium* (used as a probiotic) and diclofenac (used as a chemotherapeutic agent) have been shown to exert synergistic anti-cancer activity against CRC [216] (Fig. 7). As an alternative strategy, NPs with intrinsic antimicrobial properties have been employed to deplete harmful bacteria as a new form anti-cancer therapy. Silver NPs with antimicrobial properties have been shown to reduce intratumoral bacteria associated with resistance to cancer therapy and exert anti-tumor effects against pancreatic cancer in mice [217]. Interestingly, there is a case report of a patient with refractory metastatic head and neck squamous cell cancer who had sustained radiographic resolution of cancer after consuming home-made silver NPs daily for 3 months [218]. Based on this anecdotal example and prior preclinical evidence, the authors suggested that silver NPs should be explored further for their safety and efficacy against head and neck cancer.

Platform technologies other than nanomedicine are also reported to modulate the gut microbiota for a potential therapy against cancer. We have recently screened the FDA's list of ingredients generally recognized as safe and found that oral administration of inulin, a polysaccharide dietary fiber found in chicory root and Jerusalem artichoke, improved the anti-tumor efficacy of immune checkpoint blocker therapy [219]. Based on this, we engineered "colon-retentive" inulin gel to target "beneficial" commensal microbes prevalent in colon. Oral inulin gel treatments in tumor-bearing mice increased the relative abundances of key commensal microbes known for their "beneficial" roles in T cell immunity (e.g., *Akkermansia, Lactobacillus*, *Roseburia*) and their short-chain fatty acids as metabolites. This led to enhanced memory recall response for IFN-γ^+CD8$^+$ T-cells and establishment of stem-like Tcf1$^+$PD-1$^+$CD8$^+$ T-cells within the tumor microenvironment. Orally administered inulin gel achieved synergy with systemic immune checkpoint blocker therapy in multiple murine tumor models, thus highlighting the potential benefits of targeting the gut microbiome for improving cancer immunotherapy.

Intestinal inflammation is generally associated with significant decreases in the population of *Verrucomicrobia, Bacteroidetes,* and *Firmicutes,* especially in bacterial species of *Akkermansia muciniphila, Clostridium XIV α, Lactobacillus, Clostridium coccoides,* and *Clostridium leptum* [220–225]. Intestinal inflammation is also associated with substantial increases in the communities of *Actinobacteria* and *Proteobacteria,* especially *Enterobacteriaceae* [220,221]. Thus, various probiotic strategies have been explored as a potential therapy against IBD. For example, oral administration of a probiotic strain *Lactococcus lactis* engineered to express anti-inflammatory interleukin-10 (IL-10) restored intestinal homeostasis functions and prevented mice from DSS-induced colitis and the onset of colitis in IL-10$^{-/-}$ mice [226]. Furthermore, other probiotic strategies using engineered *Lactobacillus casei* [227,228], *Lactococcus plantarum* [229,230], and *Streptococcus gordonii* [231], have been reported for the treatment of IBD. These engineered probiotics are thought to colonize the gut and produce recombinant proteins with beneficial roles, thus affecting the microbial ecosystem and reshaping the microbiota structure.

Plant-derived NPs based on edible prebiotics could be used to target microbes. Orally administered ginger-derived lipid NPs (GDLPs) were found to target the *Lactobacillus rhamnosus* GG

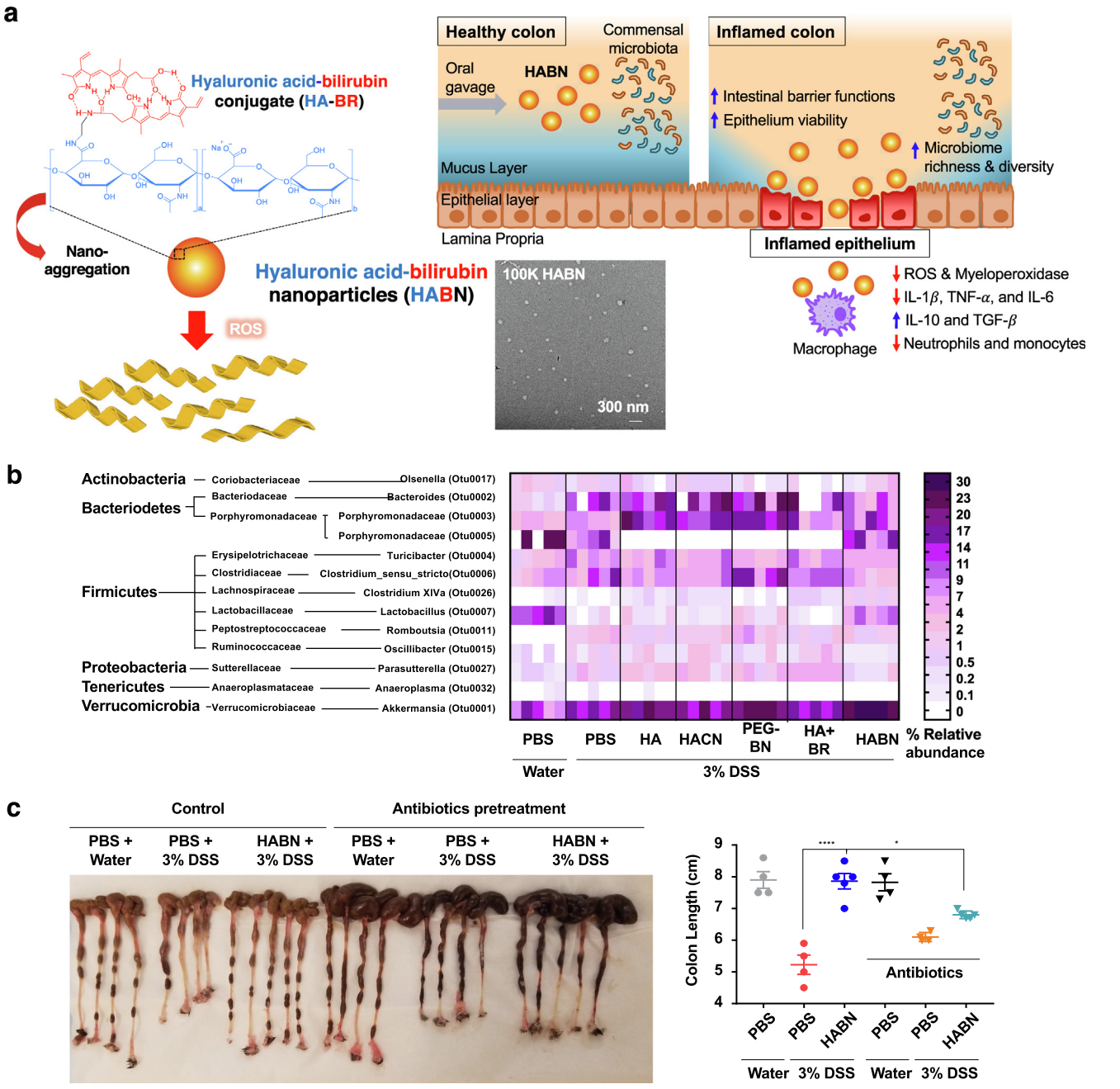

Fig. 8. Oral nanomedicine for altering the gut microbiome for IBD therapy. a, Schematic of hyaluronic acid-bilirubin NPs (HABN) self-assembled from hyaluronic acid-bilirubin conjugate (HA – BR) and their TEM images. HABN accumulates in inflamed colon and exerts therapeutic effects against acute colitis by targeted modulation of immune systems, intestinal barrier, and gut microbiota. **b,** Orally administered HABN modulates the gut microbiome in DSS-colitis mice. Heatmap of the relative abundance of family-level taxa (rows) in each mouse (columns). **c,** *In vivo* therapeutic efficacy of HABN in DSS-colitis mice. Antibiotics partially reduced the efficacy of HABN, showing the importance of HABN-mediated modulation of the gut microbiota. Reproduced with permission from [233].

(LGG) in a lipid-dependent manner [232]. GDLPs carrying mdo-miR7267-3p microRNA mediated targeting of LGG monooxygenase, increased indole-3-carboxaldehyde, and induced IL-22 production, leading to improved intestinal barrier functions in a murine model of DSS colitis. These findings showed that edible plant-based NPs could be used to target microbes and alleviate inflammation in IBD.

Pathogenesis of IBD is associated with disrupted intestinal barrier functions [8,19], imbalance of the gut microbiome [4,6,20], and subsequent dysregulated mucosal immune responses to the gut commensal bacteria [4,6,20]. Thus, targeting all these factors simultaneously may lead to improved results in IBD management. Recently, we reported the development of hyaluronic acid-bilirubin NPs (HABN) that can modulate the gut microbiome, restore the intestinal barrier functions, and exert anti-inflammation effects in a murine model of DSS colitis [233] (Fig. 8). HABN administered orally altered the gut microbiome, increased the diversity and relative abundance of *Akkermansia muciniphila* (known to induce protective intestinal barrier), *Clostridium XIV* α (known to induce regulatory CD4 T-cells), and *Lactobacillus* (known to have anti-inflammation effects) (Fig. 8**b**). Notably, the anti-inflammatory effects of HABN were abrogated by antibiotic-mediated depletion of the gut microbes, indicating the crucial role of the gut microbiome in HABN-based therapy

(Fig. 8**c**). HABN restored the protective intestinal barrier functions and anti-inflammatory immune responses in the gut epithelium, leading to amelioration of DSS-induced colitis (Fig. 8**c**).

These studies have shown the therapeutic potential of gut microbiome-targeted approaches against cancer and IBD. Furthermore, in the future, gut microbiome-targeted strategies should be examined for treating other metabolic diseases and mental health illnesses, such as obesity and Alzheimer's disease, that have been shown to be associated with dysbiotic gut microbiome.

6. Oral nanomedicine for vaccines and immune tolerance

Oral vaccination strategies can generate both humoral and cellular immunity with innate and adaptive components, leading to successful mucosal and systemic protective immunity [14,234,235]. Similarly, successful oral tolerance strategies would generate antigen-specific tolerance in the local intestinal mucosal regions and the systemic compartments [11,14,21]. Induction of either protective or tolerogenic immune responses requires: (1) successful delivery of the intact and active antigen to the intestines, (2) transport across the mucosal barrier, and (3) subsequent immune modulation (protective immunity for vaccine, but tolerogenic immunity for tolerance) with APCs [30,32,236]. In this regard, oral nanomedicine offers potential solutions to these challenges. Encapsulation or entrapment of antigens within NPs protects the cargo molecules against pH- and enzyme-mediated degradation, while preventing their dilution over the large surface area of the GIT [10,22]. Furthermore, NPs may efficiently deliver an antigenic payload to phagocytic APCs through passive or active targeting [10,21], thereby stimulating antigen-specific cellular and humoral responses (Table 1).

When developing oral vaccine and tolerance strategies, a delicate balance between immune tolerance, anergy/deletion, and protective immune response should be considered. First, the dose of antigens delivered should be considered for successful oral vaccination and tolerance. Generally, a higher dose of antigen is needed to induce protective immune responses in GALT when compared to traditional parenteral immunizations [237]. However, since too high doses are known to induce anergy/deletion instead of protective immune responses, it is necessary to deliver adequate doses of antigens with oral nanomedicine for protective immunity [11,238]. On the other hand, it should be noted that repeated oral administrations of antigens in low doses may trigger a Treg-based tolerogenic response [11,238]. Second, for successful oral vaccination, co-delivery of antigens with immunostimulatory adjuvants is needed to avoid immune tolerance [235]. For oral tolerance, co-delivery antigens with immunomodulatory drugs, such as vitamin D [174,175] or rapamycin [239–241], should be considered for inducing oral tolerance. Another important factor is the target tissue site. Whereas antigen delivery targeted to M cells in GALT is needed for successful oral vaccination [242], antigen delivery to $CD103^+$ DCs in the lamina propria is known to induce immune tolerance [11]. On the other hand, delivery of lower doses of antigens to M cells without danger signals could also induce tolerogenic immune responses [242].

6.1. Oral nanomedicine for vaccine applications

Licensed oral vaccines are currently based on live-attenuated organisms and inactivated vaccines [9,234] that elicit broad and robust immune responses that are characterized by serum (IgG) and mucosal (IgA) antibodies and effector and memory T-cells. In contrast, there is no licensed subunit-based oral vaccine in part due to the delivery challenges presented by the GIT system [10]. To efficiently deliver antigens via the oral route, it is necessary to 1) protect the payload during the transit against the harsh conditions of the GIT tract [31,32]; 2) deliver a sufficient amount of antigens to APCs in the inductive sites through the mucosal and epithelial layers within the short residence time (3–4 hr) in the small intestine [36]; and 3) boost immune responses by incorporating adjuvants or using vaccine delivery vehicles with inherent adjuvant properties [235] (Table 1).

Oral nanomedicine carrying antigens for vaccines should be transported from the intestinal lumen into the GALT via M cells and other enterocytes. M cells efficiently internalize and transport particulate matter (e.g., bacteria, viruses) to the underlying Peyer's Patches and are therefore desirable targets for oral vaccine design [52,243]. M cells express multiple receptors suitable for targeting. They include α -L-fucose resides (interacting with lectins [244], β1 integrin (interacting with cRGD [245], claudin 4 (interacting with claudin 4-targeting peptides [246], and glycoprotein 2 (interacting with FimH [247] or other GP2 ligands [248]. For example, in oral immunization experiments, lectinized liposomes were able to effectively target M cells in Peyer's patches, resulting in mucosal responses with high antibody titers [249] (Fig. 9**a-e**).

While M cells are an attractive target for oral vaccination, M cells comprise less than 5% of the follicle-associated epithelium [250]. Therefore, targeting normal gut epithelial cells is an alternative strategy for oral vaccination. A variety of lectins (N-acetyl-D-glucosamine and sialic acid residues) and PRRs expressed on epithelial cells have been examined for transepithelial transport [10,242,251]. Wheat germ agglutinin, which can bind to N-acetyl-D-glucosamine and sialic acid residues [242,252], as well as Toll-like receptor (TLR) agonists [251] have been used as targeting ligands for epithelial cells. TLR agonists, particularly those for TLR-2 and TLR-4, have been shown to enhance the transport of microparticles across the intestinal lumen into follicle-associated epithelium [253,254] (Fig. 9**f-h**). On the other hand, natural polymers, such as CD44-targeting hyaluronic acid [189,233] and muco-adhesive chitosan [185], can bind to intestinal epithelium; thus, NPs based on natural polymers with intrinsic targeting ability should be explored further.

For prophylactic oral vaccines, it is necessary to boost immune responses with adjuvants. Cholera toxin from *Vibrio Cholerae* and heat-labile enterotoxin from enterotoxigenic *E. coli* are potent mucosal adjuvants, but these native toxins pose safety issues [255]. Molecular adjuvants that are widely examined include TLR agonists, such as lipopolysaccharide or monophosphoryl lipid A for TLR4 activation [256], flagellin for TLR5 [257], and CpG deoxyoligonucleotides for TLR9 [258]. Oral NPs incorporated with both antigens and TLR agonists have been shown to generate immune response [259]. For NPs with intrinsic adjuvanticity, such as polyanhydride materials [260], they could modulate immune response without the requirement of adjuvants.

The novel coronavirus, SARS-CoV-2 causing pneumonia-associated respiratory disorder (COVID-19), has led to an unprecedented international health crisis [261]. Oral immunization is a viable strategy that could prevent respiratory illnesses. For example, an oral influenza vaccine has shown promising results in a phase II clinical trial [262], highlighting the potential of oral vaccination against respiratory pathogens. In addition, orally administered adenovirus-vector based vaccine expressing a SARS-CoV-2 antigen and dsRNA adjuvant, termed VXA-CoV-2–1, yielded positive preliminary data from a phase 1 clinical trial (NCT04563702). Enteric coating was used to protect the active ingredient from the stomach's acidic environment. VXA-CoV-2–1 was generally well-tolerated while triggering immune responses against SARS-CoV-2, as shown by increased $CD8^+$ cytotoxic T-cell response against the viral Spike protein and increased plasma cells and pro-inflammatory Th1 cytokines. VXA-CoV-2–1 also induced serum and nasal IgA responses. More oral nanomedicine

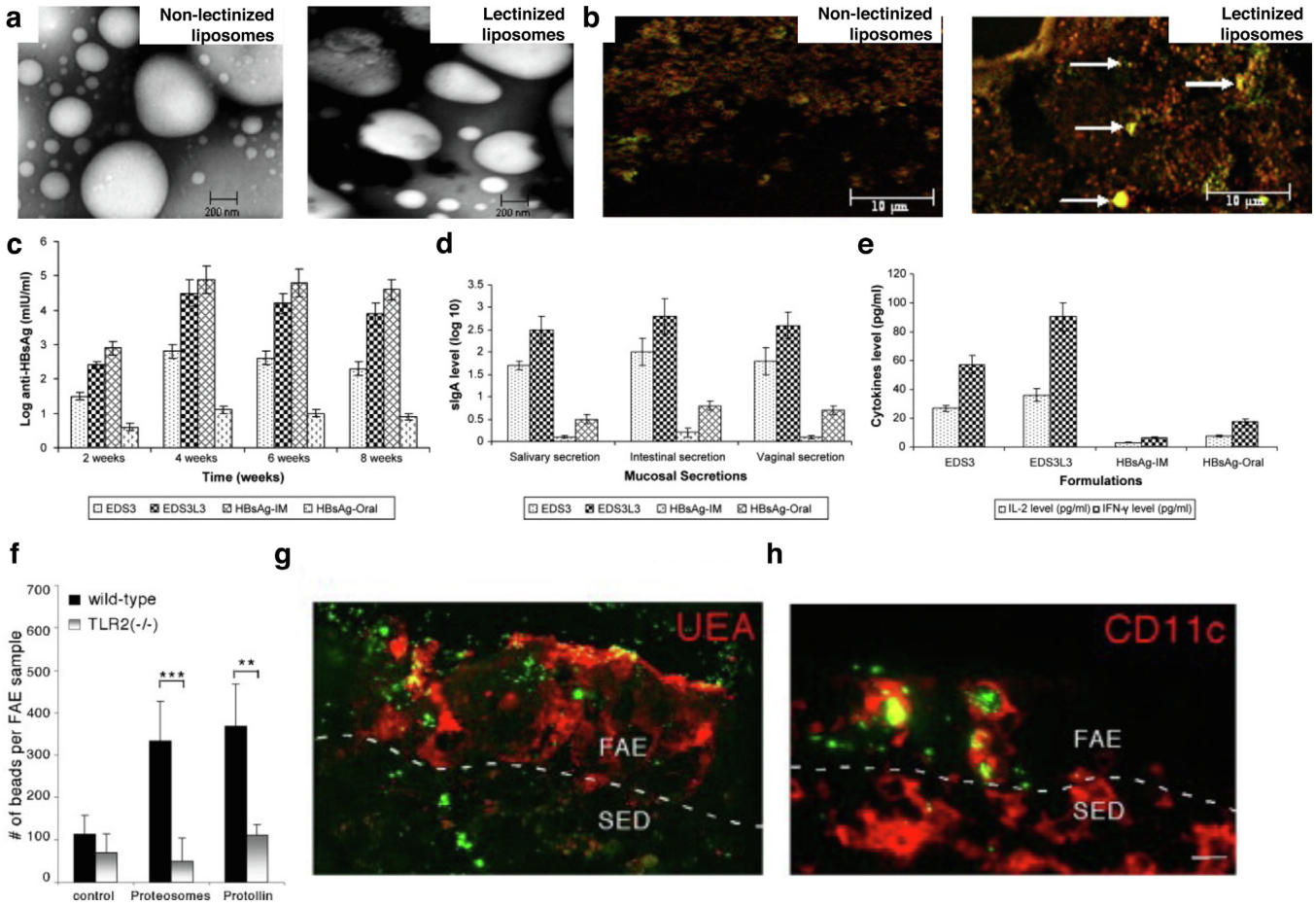

Fig. 9. Oral nanomedicine for vaccine applications. a, TEM images of non-lectinized liposomes and lectinized liposomes. **b,** *In vivo* M cell-targeting ability of lectinized liposomes (shown by arrows) **c-e,** *In vivo* protective immunity induced by orally administered liposomes carrying hepatitis B surface antigen as measured by serum antibody levels (**c**), sIgA levels in mucosal secretion (**d**), and cytokine (IL-2 and IFN-γ) levels in mouse spleen homogenates (**e**). **f-g,** *In vivo* enhancement of TLR2-mediated transepithelial transport of orally administered proteosomes, as measured by counting the number of microspheres in FAE (**f**) and immunofluorescence images with microspheres (green) associated with M cells (red; **g**) and intraepithelial $CD11c^+$ DCs (red; **h**) in the FAE of Peyer's patch. Panels **a-e** and **f-g** are reproduced with permission from [249] and [254], respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vaccines for COVID-19 should be designed, developed, and clinically tested.

6.2. Oral nanomedicine for immune tolerance

While our immune system can detect and eliminate foreign pathogens by generating systemic immune responses, the immune system can inadvertently focus its attack on the host [263,264]. These inappropriate, auto-reactive humoral or cellular immune responses are underlying causes of autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and type 1 diabetes [263,264]. Autoimmune reactions inflict serious damage to cells and organs, sometimes with fatal consequences. In addition, anti-drug antibody responses, caused immune responses directed against therapeutic drugs, are major challenges. Therapeutic protein replacement is a routine treatment for genetic deficiencies, such as hemophilia and lysosomal storage diseases, but many patients develop neutralizing antibodies against the recombinant biologics [265,266]. For example, coagulation factor VIII (F.VIII) and acid α-glucosidase (GAA) have been used as therapeutic protein replacement for hemophilia A and B [265] and Pompe disease (autosomal recessive lysosomal storage disorder) [266], but a subset of patients develops antibodies against the recombinant proteins, presenting a major hurdle for successful treatments [265,266]. Thus, oral tolerance strategies have been explored to induce immunosuppressive immune responses against recombinant protein drugs (for the prevention of anti-drug antibodies) [176,267] as well as autoantigens (for the treatment of autoimmune diseases) [177,268,269].

Even though oral tolerance strategies have shown promising results in preclinical studies, several disadvantages, including the pH variation, protein degradation, requirement for frequent dosing, and high cost, limit their wide applications [21]. Oral NPs may overcome these limitations owing to the stability of autoantigens incorporated into NPs, controlled and prolonged release of autoantigens, and cell- and tissue-specific targeting ability of NPs [21] (Table 1). For example, polylactic-co-glycolic acid NPs delivering collagen type II generated tolerogenic activity against collagen by expanding TGFβ-secreting Th3 regulatory T-cells in a murine model of arthritis [177]. In another study, collagen peptide conjugated to PEG suppressed collagen-induced arthritis by inducing tolerance against collagen [268] (Fig. 10**a-c**). Furthermore, cDNA complexed with chitosan has been employed for oral immune tolerance. Nanoencapsulation through the electrostatic interactions between cDNA and chitosan protected cDNA from digestion. As chitosan binds non-specifically to the intestinal mucus layers, oral administration of cDNA-chitosan complexes led to their accumulation in the intestinal epithelium, and NPs carrying F.VIII cDNA were effective in hemophilia A knockout mice (Fig. 10**d-g**) [267]. Functional F.VIII protein was detected in plasma at a peak level

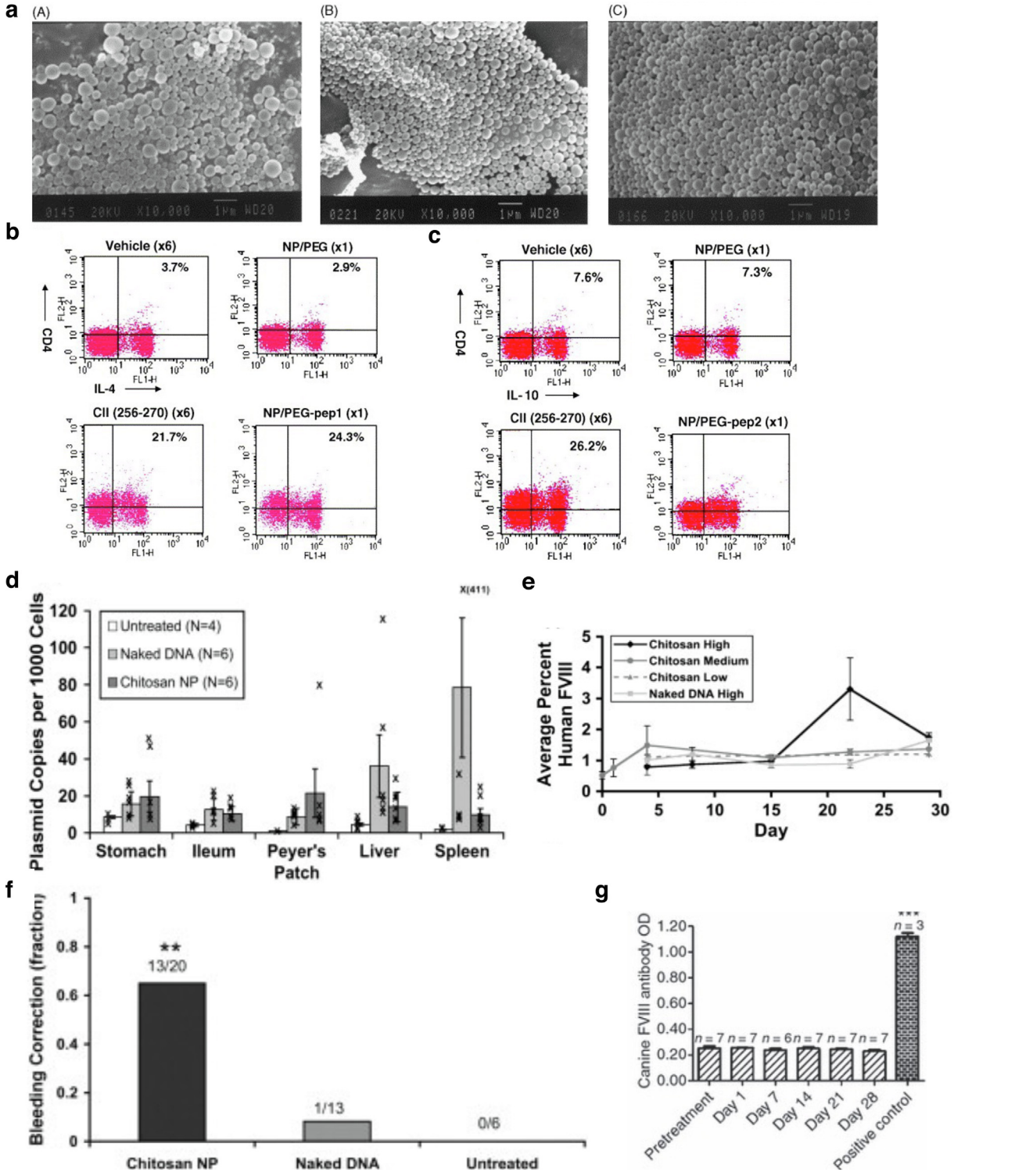

Fig. 10. Oral nanomedicine for inducing immune tolerance. a, SEM images of (A) NP/pep (type II collagen peptide), (B) NP/PEG-pep1 (mPEG-SPDP-peptide), and (C) NP/PEG-pep2 (mPEG-OD-peptide). **b-c,** *In vivo* IL-4 (**b**) and IL-10 (**c**)-producing T-cell induction activity of NP/PEG-pep1 or NP/PEG-pep2 in the Peyer's patches of DBA/1 mice, as analyzed by FACS. **d,** *In vivo* biodistribution of orally administered FVIII DNA-chitosan NPs as measured by quantitative PCR **g-h,** *In vivo* functional FVIII protein production (**g**) and phenotypic correction (**h**) activities of orally administered FVIII DNA-chitosan NPs, measured by a tail-clip assay. **i,** *In vivo* tolerance induction activity against functional FVIII protein of orally administered FVIII DNA-chitosan NPs in hemophilia A mice, as measured by ELISA-mediated detection of plasma FVIIII antibody levels. Panels **a-d**, **e-h**, and **i** are reproduced with permission from [268,267], and [176], respectively.

of 2–4% F.VIII activity, and 13 out of 30 mice showed a phenotypic correction in the bleeding challenge [267] (Fig. 10**e and 10f**). Another study also determined that neutralizing F.VIII antibody was not detected in the blood of mice treated with canine F.VIII cDNA-loaded chitosan, indicating proper induction of oral tolerance [176] (Fig. 10**g**).

Cholera toxin B subunit, CTB, is a promising adjuvant for oral tolerance. CTB binds to GM1 ganglioside expressed on live intestinal epithelial cells and facilitates uptake into the lamina propria via actin- and ATP-dependent processes [270,271]. Biodistribution of CTB-antigen conjugate delivered orally was investigated using CTB-GFP fusion protein [272]. After oral administration of chloroplast transgenic leaf material containing CTB–GFP fusion protein in mice, GFP protein was detected in the ileum, liver, and spleen, especially within macrophages and DCs. As DCs have a crucial role in induction of oral tolerance, CTB may serve as a vehicle to deliver autoantigens to DCs of the lamina propria. Indeed, CTB-coupled autoantigens have been shown to suppress DC activation and induce Foxp3$^+$ Treg. NOD mice administered orally with CTB fused to GAD65$_{531–545}$ peptide exhibited reduced pancreatic inflammation and delayed the onset of hyperglycemia [273]. However, both CTB and antigens may be degraded and hydrolyzed in the harsh conditions of GIT before reaching the target site. Thus, stable co-delivery of CTB and antigens via NPs would be an interesting future direction of the study. In another example, nano-sized recombinant vaccinia virus harboring CTB fused to the proinsulin gene and C-terminal peptide from glutamate decarboxylase has been developed [180]. Oral administration of the system in NOD mice reduced hyperglycemia and insulitis, whereas the control groups developed hyperglycemia. Moreover, plant-based platforms may offer an alternative method for oral tolerance [274]. Oral administration of powdered rice seeds expressing T-cell epitopes induced allergen-specific oral tolerance and improved symptoms against allergies triggered by pollen or mite [178,179].

7. Perspectives, challenges, and future research directions

As discussed in this review article, various GIT factors, including the mucosal immune system, the intestinal barrier, and gut microbiota, affect not only GIT local diseases but also various systemic diseases. Oral nanomedicine allows for new therapeutic approaches that could overcome the physicochemical and biological barriers of GIT and selectively deliver drug cargo to the GIT target sites. Thus, oral nanomedicine may lead to new strategies for oral vaccines, tolerance against autoimmune diseases (e.g., multiple sclerosis, rheumatoid arthritis), and treatment of IBD, cancer, and metabolic diseases (e.g., obesity, diabetes). Compared with other routes of drug administration, the oral administration route has clear advantages, such as limited drug exposure in the systemic compartment, simple self-administration, patient compliance, and ease of distribution. In addition, the gut microbiota plays a crucial role in maintaining the homeostasis of the GIT, and dysbiotic gut microbiota is associated with pathogenesis of various diseases.

Despite the progress made over the last decade in oral nanomedicines, many challenges remain to be addressed for successful their clinical translation. 1) Variations in immune responses generated by oral vaccines has been shown to be dependent on the nutrition and health of the GIT system. For example, tropical enteropathy can cause child undernutrition, intestinal absorption, and inflammatory disorders that diminish the efficacy of oral immunization [275]. 2) Better understanding of the dose and the antigen release kinetics is required for developing effective approaches for inducing protective immunity or immune tolerance. 3) NPs should release their cargo at the target sites without premature release, which can cause systemic absorption-mediated side effects. To prevent premature release, stable drug loading in nanoformulations is required, but the increased stability of the formulation could also lead to a poor drug-release profile at the target sites. Thus, differences (enzyme, pH-, GSH levels, and reactive oxygen species) between non-target and target sites should be exploited further for tissue-specific release of drugs [33]. 4) Many NP systems reported in the literature have complex structures, and large scale manufacturing of NPs and associated quality control issues have hampered clinical translation [33]. Thus, the design of oral nanomedicine systems should be as simple as possible. 5) When developing oral nanomedicine targeting the gut microbiome, it should be noted that the gut microbiota [276] may vary, depending on the age, environmental exposure, health status, genetics, geography, and diet of the population. Also, exercise [277], antibiotic use [152,223], and surgical interventions [278] are known to alter the gut microbiome, thus presenting additional factors to consider for the treatment strategies.

Furthermore, we believe oral nanomedicine should be explored further for treating diseases in the central nervous system (CNS). Notably, the gut-brain axis is an emerging area with intensive research interest [279]. The GIT is considered the "second brain" as there are a half billion neurons innervating the gut [25,280]. Gut microbes and their microbial metabolites affect the development and homeostasis of the CNS [279,280]. Gut bacteria communicate with the brain through receptors expressed on vagal nerves in the mucosal and muscular layers with their ligands, such as cholecystokinin, exogenous peptides, toxins, ghrelin, serotonin, and glucagon-like peptide-1 [281]. Neurodegenerative diseases, such as Alzheimer's disease [282,283] and Parkinson's disease [284], are associated with dysbiotic gut microbiota, their metabolites, and amyloid biofilm. Thus, these are pharmacological targets, and future research effort should focus on developing oral nanomedicine that can target and normalize the gut-brain axis for potential treatment of CNS diseases.

In summary, oral nanomedicine provides a viable strategy for modulating the gut microbiota and microbial metabolites and offers promising therapeutic platforms for various biomedical applications.

Conflicts of Interest

A patent application (WO/2021/061789) for inulin gel-based in situ modulation of the gut microbiome has been filed with J.J. M. as an inventor.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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