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The microbiome and regulation of mucosal immunity

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Summary

The gastrointestinal tract is a mucosal surface constantly exposed to foreign antigens and microbes, and is protected by a vast array of immunologically active structures and cells. Epithelial cells directly participate in immunological surveillance and direction of host responses in the gut and can express numerous pattern recognition receptors, including Toll-like receptor 5 (TLR5), TLR1, TLR2, TLR3, TLR9, and nucleotide oligomerization domain 2, as well as produce chemotactic factors for both myeloid and lymphoid cells following inflammatory stimulation. Within the epithelium and in the underlying lamina propria resides a population of innate lymphoid cells that, following stimulation, can become activated and produce effector cytokines and exert both protective and pathogenic roles during inflammation. Lamina propria dendritic cells play a large role in determining whether the response to a particular antigen will be inflammatory or anti-inflammatory. It is becoming clear that the composition and metabolic activity of the intestinal microbiome, as a whole community, exerts a profound influence on mucosal immune regulation. The microbiome produces short-chain fatty acids, polysaccharide A, α-galactosylceramide and tryptophan metabolites, which can induce interleukin-22, Reg3γ, IgA and interleukin-17 responses. However, much of what is known about microbiome–host immune interactions has come from the study of single bacterial members of the gastrointestinal microbiome and their impact on intestinal mucosal immunity. Additionally, evidence continues to accumulate that alterations of the intestinal microbiome can impact not only gastrointestinal immunity but also immune regulation at distal mucosal sites.

Keywords: immunity; inflammation; intestinal; microbiome; mucosal.

Introduction

The gastrointestinal tract is the largest environment-exposed surface area in the body, and is in direct contact with a large and varied microbial community.[1] Fortunately, the gastrointestinal tract is also home to a large variety of immune cells and structures that help maintain intestinal homeostasis in the face of microbial challenge.[2–4] Intestinal epithelial cells physically separate underlying tissues from the intestinal lumen,[5,6] while goblet cells maintain a mucus layer to prevent microbial contact with epithelial cells.[7,8] Leucocytes beneath the epithelial cell layer can both promote and inhibit inflammatory responses,[9–12] and are efficiently organized into effector and inductive sites.[13–15] This organization largely prevents unwanted inflammation while retaining the ability to respond rapidly to a wide array of perturbations.

The gastrointestinal tract is also the home of the intestinal microbiome, defined as all of the microbial inhabitants (microbial community) and their collective genomes.[16] While the microbiome provides numerous nutritional benefits to the host, including synthesizing vitamins[17] and short chain-fatty acids (SCFAs),[18] the presence of the microbiome is also vitally important for

Abbreviations: FAE, follicle-associated epithelium; GF, germ-free; IELs, intraepithelial lymphocytes; IFN-γ, interferon-γ; IL-17, interleukin-17; ILCs, innate lymphoid cells; LPDC, lamina propria dendritic cell; SCFA, short chain fatty acids; SFB, segmented filamentous bacteria; Th17, T helper type 17; TLR, toll-like receptor

the development and functionality of the intestinal immune system.[19,20] Animals devoid of intestinal microbial stimulation exhibit large defects in the organization and activity of immune structures in the gut, and proper activity can be restored via microbial stimulation.[19,20] Individually, members of the microbiome can also have profound effects on host mucosal homeostasis, and specific microbes have been demonstrated to promote inflammatory[21,22] or anti-inflammatory[23,24] responses in the gut. Hence, cross-talk between the microbiome and the intestinal immune system is critical in the maintenance of mucosal homeostasis.

The small intestine and the large intestine are physiologically distinct sites. While nutrient absorption occurs in the small intestine, absorption of water occurs in the large intestine.[1,25] Consistent with their varied physiological roles, the structure and organization of the small and large intestines are different. For example, Peyer's patches and isolated lymphoid follicles are both found within the small intestine,[26] but only isolated lymphoid follicles have been described in the large intestine.[27] Hence, we will first discuss the structural and cellular composition of the small and large intestines, establishing the proper context for our subsequent discussion of microbiome modulation of mucosal immunity (Fig. 1a,b).

Structure and cellular composition of the small intestine

The small intestinal epithelium is actually a single layer of cells, all of which are derived from multipotent stem cells located within the intestinal crypts.[2,6] Collectively, these cells are responsible for nutrient absorption, physical exclusion of luminal contents from underlying tissues, antimicrobial peptide production and maintenance of the intestinal mucus layer.[2,6]

Columnar epithelial cells constitute the majority of cells present in the intestinal epithelium.[6,28] Enterocytes provide a physical barrier separating the luminal contents of the gastrointestinal tract from underlying tissues, as well as participating in the absorption of materials from lumen.[5,6] Epithelial cells directly participate in immunological surveillance and direction of host responses in the gut. Epithelial cells can express numerous pattern recognition receptors, including Toll-like receptor 5 (TLR5),[29] TLR1, TLR2, TLR3, TLR9,[2] and nucleotide oligomerization domain 2,[5] and can produce chemotactic factors for both myeloid and lymphoid cells following inflammatory stimulation.[30] Interleukin-17 (IL-17) stimulation of intestinal epithelial cells can drive the expression of neutrophil chemokines.[31] Epithelial cells can produce anti-microbial peptides, such as cathelicidin-related antimicrobial peptide, to directly influence microbial populations in the lumen of the gut.[32] Additionally, epithelial cells can interact with leucocyte populations through the expression of

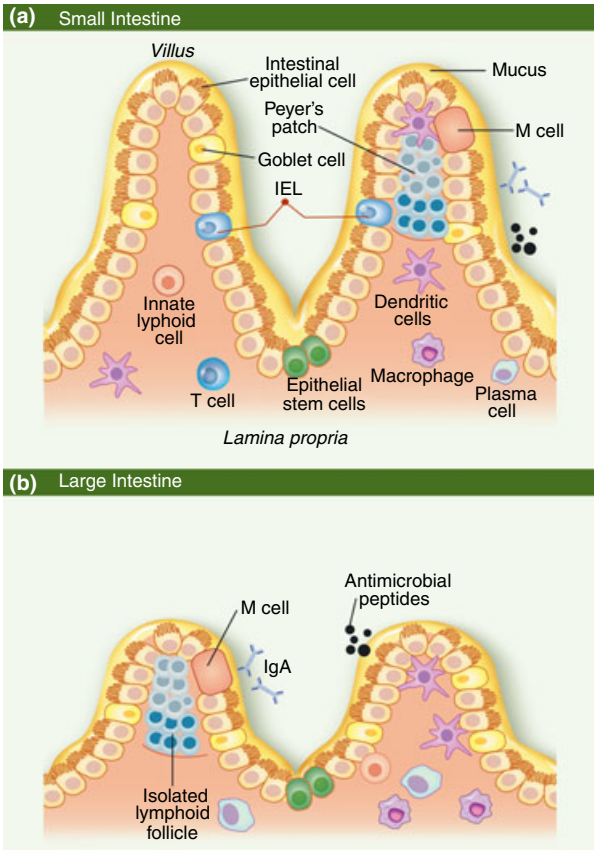

Figure 1. The cellular and structural composition of the small and large intestinal epithelium. (a) Organization of the small intestinal epithelium. Intestinal epithelial cells and a mucus layer separate the intestinal lumen from the underlying tissue. Lymphocytes beneath the intestinal epithelium are found in either inductive and effector sites. Inductive sites, such as Peyer's patches, generate mature lymphocytes that then migrate to effector sites, such as the lamina propria, to respond to microbial stimulation. (b) Organization of the large intestinal epithelium. The organization of the large intestinal epithelium is very similar to that of the small intestine, excepting the lack of Peyer's patches and a predominance of B cells instead of T cells in the underlying lamina propria. IEL, intraepithelial lymphocyte.

both MHCII[33] and MHCI.[34] Therefore, enterocytes play a key role in not only preventing microbes and microbial products from penetrating to underlying tissues, but also initiating and directing inflammatory responses.

Within the epithelium resides a population of lymphocytes referred to as intraepithelial lymphocytes (IELs).[35] Almost all IELs are T cells, with both $\alpha\beta^+$ and $\gamma\delta^+$ populations represented.[35,36] Adherence of IELs to epithelial cells is mediated by interactions between CD103 expressed on IELS, and E-cadherin expressed on epithelial cells.[37] Many IELs at baseline display a mixed phenotype, with expression of some activation markers but not others.[38] However, following stimulation, IELs become activated and express effector cytokines including interferon-γ (IFN-γ) and keratinocyte growth factor.[38–40] The IELs

can exert both protective and pathogenic roles during inflammation: whereas IEL-derived keratinocyte growth factor is believed to protect the epithelium from damage during chemically induced colitis,[41] IELs producing IFN-γ and tumour necrosis factor-α have been associated with the development of inflammatory bowel disease.[42] The proximity of IELs to the lumen of the gut, and their ability to rapidly produce both inflammatory and epithelial-protective signals, make them key "first-line" defenders in the intestinal tract.

Underlying the intestinal epithelium is the lamina propria, an area rich in B and T lymphocytes.[2] In contrast to Peyer's patches, which are inductive sites for the priming of lymphocytes, the lamina propria is an effector site where activated lymphocytes respond to appropriate stimulation.[13–15] αβ T-cell receptor-positive T cells are the most common lymphocyte within the small intestinal lamina propria.[36] In keeping with the effector function of the lamina propria, T cells found within the lamina propria express markers indicative of activation, including high levels of CD69 and CD25,[43] as well as spontaneously secreting IL-4 and IFN-γ.[44] Subsets within this population have drastically different activities: while $CD4^+$ $CD25^+$ regulatory T cells in the lamina propria can inhibit T-cell proliferation, cytokine production and the development of colitis,[10,11] lamina propria $CD4^+$ T cells can secrete both IL-17 and IL-22 and are associated with the development of intestinal inflammation.[9,12] Therefore, lamina propria T cells have the ability to rapidly react to signals received from the luminal environment and initiate both inflammatory and anti-inflammatory responses.

Lamina propria dendritic cells (LPDCs) play a large role in determining whether the response to a particular antigen will be inflammatory or anti-inflammatory. LPDCs capture luminal antigen by extending their processes through the epithelial cell layer, a process dependent on CX3CR1.[45] There are two broad classifications of LPDCs to consider: $CD103^+$ and $CD103^-$. The $CD103^+$ LPDCS promote the generation of $Foxp3^+$ regulatory T cells through the secretion of retinoic acid and in combination with transforming growth factor-β.[3,4] In contrast, $CD103^-$ LPDCs support the development of inflammation, and increase expression of inflammatory mediators such as tumour necrosis factor-α and IL-6 following stimulation with TLR ligands.[46] The presence of $CD103^+$ LPDCs is particularly important in preventing unnecessary inflammation, as the absence of $CD103^+$ $CX3CR1^-$ LPDCs enhances epithelial damage during colitis.[47]

Innate lymphoid cells (ILCs) are another cellular population found in the lamina propria.[48,49] ILCs morphologically resemble lymphocytes, but do not possess recombination activating gene-dependent antigen receptors.[50] They can be broken down into three broad groups.[50] The defining characteristic of group 1 ILCs, such as natural killer cells, is the production of IFN-γ.[50] Many group 1 ILCs are also $T\text{-}bet^+$,[50,51] and group 1 ILCs can be found at sites of mucosal inflammation.[51] In contrast, generation of group 2 ILCs requires GATA3 and RORα,[50] and IL-5 and IL-13 are the signature cytokines of this group.[50] Group 2 ILCs are important in the response to nematode infections,[50] and will be discussed no further in this review.

Particularly relevant to the intestinal tract are group 3 ILCs, which are primarily defined by their ability to produce IL-22 and IL-17.[50] Additionally, the generation and activity of group 3 ILCs is dependent on RORγt.[50] Recent evidence has strongly suggested that $IL\text{-}17^+$ group 3 ILCs drive colonic inflammation during *Helicobacter hepaticus* infection.[49] In contrast, during *Citrobacter rodentium* colitis, group 3 ILCs are known to produce IL-22.[48] The IL-22 drives antimicrobial peptide expression and is required to prevent severe intestinal pathology and mortality during *C. rodentium* colitis.[52] Hence, group 3 ILCs are important intestinal sources of IL-17 and IL-22, and can both promote and protect against intestinal pathology during insult.[49,50,52]

Peyer's patches are one of the most recognizable immune structures present in the small intestine (Fig. 1a). They are primarily a lymphoid structure, containing both germinal centres and a T-cell zone, as well as a subepithelial dome containing dendritic cells separated from the lumen of the gut by the follicle-associated epithelium (FAE).[26,53] The FAE is functionally distinct from other sites in the epithelium: it contains fewer secretory cells, and IgA cannot be secreted across the FAE.[54] A feature of the FAE is the presence of M cells, specialized epithelial cells that facilitate the uptake of antigen and microbes from the lumen of the gut and its delivery to underlying lymphoid tissue.[54,55] Luminal antigens collected through the FAE are the primary antigens available in Peyer's patches because Peyer's patches have no afferent lymphatics.[26] IgA^+ B cells are prevalent in the Peyer's patch germinal centres,[56] and the Peyer's patch dendritic cells promote IgA production from B cells.[57] Additionally, isolated lymphoid follicles are structurally and functionally similar to Peyer's patches, but are smaller in size and can be found in both the small and large intestine.[26,27,58–60] The presence of germinal centres within Peyer's patches and isolated lymphoid follicles, combined with their constant exposure to luminal antigen, make them an ideal site for the induction of adaptive responses along the intestinal tract.

Structure and cellular composition of the large intestine

In contrast to the small intestine, B cells are the predominant lymphocyte present in the lamina propria of the large intestine.[36] Lamina propria B cells secrete dimeric

IgA, which is transcytosed through epithelial cells to the lumen of the gut through the action of the polymeric immunoglobulin receptor.[61,62] Although antigen-specific IgA can be generated during intestinal infection,[63] intestinal IgA secretion also plays a key role at baseline by inhibiting the penetration of commensal microbes through the epithelium and enhancing the uptake of luminal bacterial by M cells.[61] Intestinal IgA can also directly modulate the composition of the intestinal microbiome,[64] highlighting the key role of IgA and lamina propria B cells in shaping both the membership and location of the microbiome.

Goblet cells are another class of specialized epithelial cells found in the intestinal epithelium.[28,65] Goblet cells can be found in both the small and large intestines, but they represent approximately 15% of the cells found in the large intestinal epithelium.[28,65] Goblet cells contain large mucus-laden vacuoles,[65] and express high levels of the MUC2 gene.[7] MUC2 is the major structural component of both intestinal mucus layers.[66] The lower mucus layer makes direct contact with the intestinal epithelium and is rarely contaminated with bacteria, whereas the outer layer contacts the intestinal lumen and the intestinal microbiome.[8] Goblet cells have also recently been found to produce the antimicrobial peptides Ang4, RegIIIγ and RegIIIβ.[67,68] RegIIIγ activity is especially important in preventing microbial contact with the underlying epithelium.[69] Goblet cells may also transfer antigens acquired in the intestinal lumen to dendritic cells in the lamina propria.[70] These studies have demonstrated a potential role for goblet cells beyond mucus production by participating directly in the uptake of antigen and modulating the intestinal microbiome.

The influence of the microbiome on intestinal mucosal homeostasis

The mammalian gastrointestinal tract is home to a large community of bacteria, reaching a density of 10^{11} colony forming units/ml of colonic content in the large intestine, that provide an array of benefits to the host.[71] The importance of the gastrointestinal tract microbiome in the generation of mucosal immune responses has been demonstrated using germ-free (GF) mice.[19,20] The intestinal immune system is largely underdeveloped in the absence of microbial stimulation.[19,20] Germ-free animals produce lower levels of antimicrobial peptides and have smaller numbers of IELs present than conventional animals.[19,72] Additionally, the Peyer's patches of GF animals are less active and contain small germinal zones,[20] and IgA^+ plasma cell levels are also greatly reduced in these animals.[73] The induction of oral tolerance is also deficient in GF mice.[74–76] However, intestinal microbial stimulation in GF animals can restore the proper organization of the intestinal immune system.[19,77]

It is becoming clear that the composition and metabolic activity of the intestinal microbiome, as a whole community, exerts the greatest influence on mucosal immune regulation. However, much of what is known about microbiome–host immune interactions has come from the study of single bacterial members of the host microbiome. For example, *Bacteroides fragilis* produces a polysaccharide (polysaccharide A) with anti-inflammatory properties.[23] Polysaccharide A, in a TLR2-dependent manner, mediates the conversion of $CD4^+$ T cells into $Foxp3^+$ regulatory T cells that produce IL-10, suppress IL-17 production and protect against numerous inflammatory insults.[23,24,78,79] *Bacteroides fragilis* releases polysaccharide A in outer membrane vesicles that are detected by dendritic cells,[79] whereas purified polysaccharide A can also prevent inflammation *in vivo*.[23,24] Additionally, *B. fragilis* can also produce α-galactosylceramide (α-Gal-Cer_{Bf}), a glycosphingolipid which is capable of binding CD1d and activating invariant natural killer T cells.[80]

In another example, monocolonization of GF mice with *Bacteroides thetaiotamicron* can cause changes in the expression of genes involved in intestinal nutrient absorption, mucosal barrier function and angiogenesis.[81] Interestingly, while colonization by a complex microbiome is associated with high-level epithelial expression of RegIIIγ, a secreted C-type lectin that limits microbial contact with the epithelium,[69,82] monocolonization of mice with *B. thetaiotamicron* is not.[82] This failure to induce RegIIIγ expression is probably dependent on IgA, which presumably limits bacterial adhesion to and stimulation of the epithelium, as GF animals deficient in IgA express high levels of RegIIIγ following exposure to *B. thetaiotamicron*.[82] Similarly, in mice lacking RegIIIγ there is increased bacterial colonization of intestinal epithelial surfaces and activation of intestinal adaptive immune responses, including increased levels of IgA^+ cells.[69] Recent work has also demonstrated that the microbiome produces signals that preferentially promote IL-22 transcription, which is required for RegIIIγ expression.[52,83] Therefore, spatial separation of the microbiome and intestinal epithelium is maintained by a complex interplay between both microbiome and host-derived factors.

Another widely studied example is the role of segmented filamentous bacteria (SFB) in promoting intestinal T helper type 17 (Th17) responses.[21,22] SFB associate closely with epithelial cells of the small intestine, and the presence of SFB in the terminal ileum is associated with an increase in the number of Th17 cells capable of expressing both IL-17 and IL-22 in the intestinal lamina propria.[21,22] This enhanced inflammatory state appears to be protective for the host, as animals colonized with SFB are resistant to infection by the large intestine pathogen, *C. rodentium*.[21] Monoassociation of GF mice with SFB promotes high levels of IgA, though only a small fraction of the total IgA produced is SFB-specific.[84] IgA

production, however, is critical for containing the SFB population; mice with deficient IgA levels (due to deficiency of activation-induced cytidine deaminase) have a marked expansion of SFB within the small intestine, which is reversed upon restoration of lamina propria IgA production.[64] Hence, colonization of the small intestines of mice with SFB is a potent immunomodulatory signal for the mucosa, which in turn modulates the intestinal microbiome.

Recent studies have identified numerous species of *Clostridia* capable of inducing the development Foxp3$^+$ regulatory T cells in the large intestine.[85,86] Large intestine colonization with *Clostridia* from clusters IV and XIVa enhances transforming growth factor-β_1 levels and promotes the development of IL-10-expressing Foxp3$^+$ regulatory T cells in GF mice to levels comparable to those seen in conventionally reared animals.[85,86] Colonization of conventionally reared animals with these *Clostridia* strains is also capable of reducing the severity of intestinal inflammation during chemically induced colitis.[85,86] Since clostridial species are a major producer of SCFAs,[87] one likely mechanism is the production of SCFA.

Short-chain fatty acids, such as butyric acid/butyrate, are by-products of fermentation by the microbiome and are detectable in the gastrointestinal tract.[18] Butyrate also possesses potent anti-inflammatory activity on myeloid and lymphoid cells in a variety of *in vitro* culture systems.[88–91] Butyrate has also been used to treat colitis and can reverse the increased mucosal permeability and intestinal ulceration seen in dextran sodium sulphate colitis.[92,93] Conversely, in the absence of G-protein coupled receptor 43, one of the host receptors for SCFAs, mice are more susceptible to experimentally induced intestinal inflammation.[94] Butyrate can also act directly on leucocytes, and inhibits IL-12 production, decreases co-stimulatory molecule expression, and blocks nuclear factor-κB translocation in human monocyte marrow-derived dendritic cells and macrophages.[91,95]

An anti-inflammatory role has been ascribed to members of the genus *Lactobacillus*.[96,97] There are many reviews that discuss this field, so we will not discuss this topic in great detail. However, relevant to our previous point, though lactobacilli are poor producers of butyrate, they can produce ample quantities of lactic acid, which in turn can be rapidly converted to butyrate by other members of the microbiome,[98,99] potentially accounting for one mechanism of their immunomodulatory activity. Taken together, these data clearly demonstrate that individual, non-pathogenic members of the intestinal microbiome can markedly alter the inflammatory state of the intestinal immune system to the benefit of the host (Fig. 2).

Exogenous tryptophan metabolites play an important role in mammalian gut immune homeostasis via aryl hydrocarbon receptor signalling.[83] The aryl hydrocarbon receptor promotes Th17 cell differentiation *in vitro*,[100] as well as the homeostasis and function of Group 3 ILCs *in vivo*.[101,102] Mice that are deficient for aryl hydrocarbon receptor have a significant deficiency in Group 3 ILCs, thereby resulting in much lower IL-22 production and increased susceptibility to intestinal infection.[101] However, intestinal Th17 cells are increased in these mice, rather than decreased, because the reduction in IL-22 permits the expansion of SFB, thereby promoting Th17 cells.[102] As recently demonstrated, the microbiome is one source of tryptophan metabolites, and tryptophan metabolism by *Lactobacillus* populations in mice produces indole-3-aldehyde, an aryl hydrocarbon receptor ligand that can drive IL-22 expression.[83] This is yet another proposed

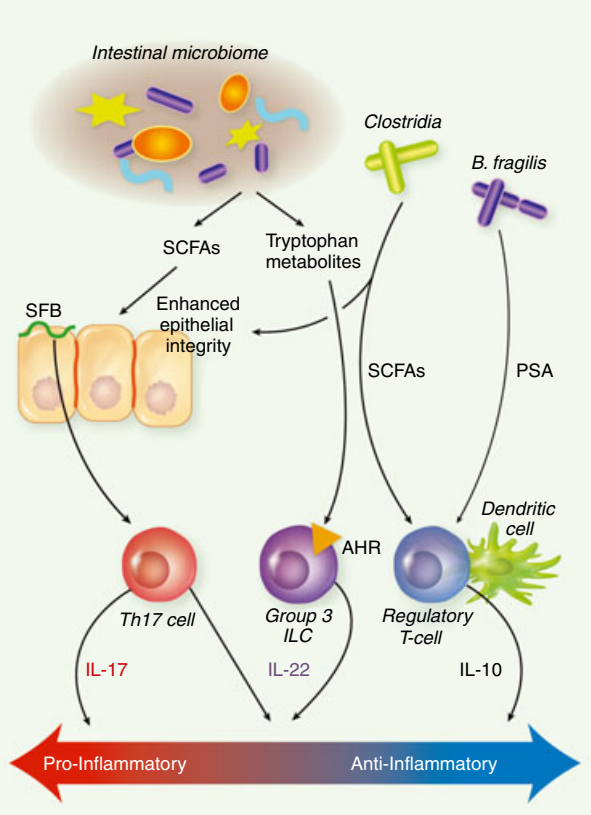

Figure 2. Microbial modulation of mucosal immunity. The intestinal microbiome generates numerous signals, which impact the regulation of intestinal mucosal immunity. The microbiome produces metabolic by-products, such as butyrate and tryptophan catabolites, which can enhance intestinal integrity and stimulate IL-22 production by group 3 ILCs, respectively. Certain members of the microbiome are known to activate specific arms of intestinal immunity. SFB colonization of the small bowel enhances Th17-mediated immunity, while colonization by *Clostridia* from clusters IV and XIVa promotes the development of regulatory T cells. Polysaccharide A, generated by *Bacteroides fragilis*, is also capable of enhancing regulatory T-cell activity in the gut. SFB, segmented filamentous bacteria; PSA, polysaccharide A; SCFAs, short-chain fatty acids; AHR, aryl hydrocarbon receptor; ILC, innate lymphoid cell; IL-17, interleukin-17.

mechanism of cross-talk between the microbiome and the host that promotes a balance between inflammatory and anti-inflammatory signalling and the overall maintenance of mucosal homeostasis.

Gastrointestinal microbiome and regulation of distal mucosal immunity

There is also increasing evidence that the gastrointestinal mucosa, the predominant site of microbiome–host interaction, can also play a role in the development of immune responses at distal mucosal sites. How might the gastrointestinal tract regulate responses to inhaled allergens or other antigens? The mucociliary architecture of the nasopharyngeal cavity and upper airways naturally sweeps all inhaled micro-particulates that stick to the mucus lining into the gastrointestinal tract. Shortly after intranasal inoculation or aerosol delivery, fluids, particles and microbes introduced into the nasal cavity are largely found in the gastrointestinal tract.[103–105] In mice, intranasal inoculation of a volume as small as 2·5 ml still largely ends up in the gastrointestinal tract.[104] Therefore, inhaled micro-particulates and aerosols (which comprise the vast majority of aeroallergens) are also swallowed. Using an animal model of allergic airway disease, it has been reported that 2 days after intranasal administration of antigenic peptide, corresponding antigen-specific CD4 T-cell division had not only occurred in the lymph nodes draining the lungs and nasopharyngeal cavity, but also in the mesenteric lymph nodes.[106] No division was seen in peripheral non-draining nodes. In studies from our laboratory, we have been able to demonstrate that perturbation of the normal microbiome in mice can promote the development of allergic airway disease following allergen challenge.[107,108] In other studies, oral delivery of various *Lactobacillus* strains can modulate pulmonary inflammation in murine systems.[109–111] Other studies have shown that the microbiome composition can regulate the generation of virus-specific CD4 and CD8 T cells and antibody responses following respiratory influenza virus infection.[112] These observations, combined with our knowledge of the mechanisms underlying the gastrointestinal afferent and systemic efferent mechanisms of oral tolerance, support the concept that the gastrointestinal and pulmonary mucosa both also respond to inhaled antigens and generate cross-regulatory immunity. It remains to be determined how these distal mucosal sites interact in generating mucosal immunity.

Concluding remarks

The maintenance of mucosal homeostasis is a delicate balance between the host and the intestinal microbiome. The host employs numerous mechanisms to contain the intestinal microbiome and prevent the development of inappropriate inflammation. At the same time, however, the intestinal immune system requires microbial stimulation for its proper development. Conversely, while certain members of the microbiome can activate specific arms of host mucosal immunity, these host responses often prevent inappropriate overgrowth or translocation of members of the microbiome. Additionally, microbiome-driven host responses can also prevent the development of inappropriate inflammation. The end result is an equilibrium state, where microbial stimulation promotes normal immune function in the intestine, which in turn allows the intestinal microbiome to flourish in the absence of unnecessary inflammation. Further investigation of these complex host–microbiome interactions will undoubtedly reveal new mechanisms underlying the maintenance of mucosal homeostasis and the development of inflammation in the intestines and distal mucosal sites.

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Disclosure

The authors declare no conflict of interest.

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