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No Vacancy: How beneficial microbes cooperate with immunity to provide colonization resistance to pathogens[#]

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Abstract

The mammalian intestine harbors a community of trillions of microbes, collectively known as the gut microbiota, which co-evolved with the host in a mutually beneficial relationship. Among the numerous gut microbial species, certain commensal bacteria are known to provide health benefits to the host when administered in adequate amounts, and as such are labeled “probiotics”. Here we review some of the mechanisms by which probiotics and other beneficial commensals provide colonization resistance to pathogens. The battle for similar nutrients and the bacterial secretion of antimicrobials provide a direct means of competition between beneficial and harmful microbes. Beneficial microbes can also indirectly diminish pathogen colonization by stimulating the development of innate and adaptive immunity as well as the function of the mucosal barrier. Altogether, we gather and present evidence that beneficial microbes cooperate with host immunity in an effort to shut out pathogens.

Introduction

The mammalian gastrointestinal (GI) tract is home to a community of trillions of microorganisms commonly known as the microbiota. The long co-existence of the microbiota and the host intestinal mucosa has established a mutual beneficial relationship: On the one hand, the microbiota protects the host from infection with pathogenic microorganisms and contributes to both nutrient metabolism as well as to the development and function of the GI immune system; on the other hand, the host provides nutrient-rich niches to ensure the survival of its resident bacterial communities [1].

In the past decade, studies in germ-free (GF) mice and the advent of metagenomics have tremendously contributed to elucidate the complexity of the intestinal microbiota and its contribution to health and disease [2, 3]. In healthy subjects, at least 1,000 different bacterial species contribute to intestinal homeostasis, with Firmicutes and Bacteroidetes representing

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the most common intestinal phyla, followed by Actinobacteria and Proteobacteria [4, 5]. Within these phyla, some bacterial species including Gram-positive *Lactobacillus* spp. (phylum Firmicutes) and *Bifidobacterium* spp. (phylum Actinobacteria) as well as certain Gram-negative bacteria such as *Escherichia coli* Nissle 1917 (phylum Proteobacteria) have been shown to benefit the host by blocking harmful microorganisms; for this reason, they are referred to as "probiotics."

The first observation that certain commensal bacteria have beneficial properties dates back to 1907, when Elie Mechnikoff proposed that lactic acid-producing strains are beneficial to the host by inhibiting the growth of other species within the colon [6]. Today, probiotics are defined by the World Health Organization as "live bacterial species that confer a health benefit when administered in adequate amounts" [7]. In addition to the few known probiotics, the microbiota in general has beneficial effects on the host; for example, the absence of the microbiota renders GF mice more susceptible to infection in comparison to conventionally raised mice [1, 8]. Moreover, the use of antibiotics has been shown to enhance intestinal colonization of enteric pathogens, as alterations to the composition of the gut flora track with increased susceptibility to infection with pathogens such as *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) and *Clostridium difficile* [9–12].

More recently, several studies have begun to elucidate the molecular mechanisms behind the beneficial role of commensal and probiotic strains. It is now becoming clear that beneficial bacteria provide colonization resistance to pathogens by two major mechanisms [13, 14]. The first mechanism involves the direct competition between certain commensals and pathogens for nutrients or niche establishment. The second mechanism comprises indirect effects on pathogen colonization, deriving from the stimulation of the innate and adaptive immune system by commensal bacteria. In this review, we will summarize some of the mechanisms by which commensal bacteria, including certain probiotic species, contribute to colonization resistance against pathogens, both by direct competition with pathogenic bacteria and by stimulation of host immunity.

Direct competition with pathogens

One of the mechanisms by which commensal and probiotic bacteria provide colonization resistance to pathogens is by directly competing for the same niche. Some beneficial microbes acquire similar nutrients as pathogens, often more efficiently, thus hindering the replication and colonization of infectious agents. In addition, some microbes produce antimicrobial proteins that can target pathogens. Below, we will discuss these two aspects of direct competition between beneficial and harmful bacteria (Figure 1).

Competition for nutrients

Numerous studies on the battle between commensals and pathogens belonging to the Enterobacteriaceae family have put forward the idea that competition for nutrients in the gut appears to occur primarily between metabolically related bacteria. For example, in GF mice, certain commensal *Escherichia coli* strains reduce cecal colonization of the enteric pathogen enterohemorrhagic *E. coli* (EHEC), a leading cause of bloody diarrhea in humans, by competing for the amino acid proline [15]. Similarly, pre-colonization of streptomycin-

treated mice with specific human commensal *E. coli* strains prevented the growth of EHEC [16]. This latter effect was later reported to be linked to the capacity of the human commensal *E. coli* strain HS and of the probiotic strain *E. coli* Nissle 1917 (discussed below) to occupy a unique nutritional niche in the mouse gut. Indeed, utilization of multiple sugar molecules (6 by *E. coli* HS and 7 by *E. coli* Nissle 1917) limited the nutrient availability to pathogenic strains of *E. coli*, thereby impeding their successful colonization of the gut [17]. It is worth noting that the competition for nutrients is not limited to bacteria within the same species. For instance, a commensal strain of *E. coli* was able to delay the intestinal colonization and translocation of the pathogen *Salmonella* Typhimurium in GF mice, possibly due to competition for nutrients [18]. More recently, it has been shown that the intestinal microbiota provides colonization resistance to infection with *Citrobacter rodentium*, a mouse pathogen used to model infection with diarrheagenic *E. coli* strains including enteropathogenic *E. coli* (EPEC) and EHEC, by competing for similar carbohydrates [19]. Similarly, consumption of fucose during infection supports the growth of the microbiota and protects mice from infection with *C. rodentium* [20].

As competition for nutrients is one of the mechanisms by which commensals provide colonization resistance, it is not surprising that certain pathogens have evolved to evade this mechanism. This is, for instance, the case for certain pathogenic *E. coli* strains, which can utilize sugars that are not used by commensal *E. coli* [21]. Moreover, pathogens like EHEC, *Salmonella* Typhimurium, and *C. difficile* can catabolize sugars liberated by the microbiota [22, 23]. Additionally, the types of nutrient sources available to both commensals and pathogens can vary if the intestinal environment is altered; e.g. because of inflammation or antibiotic treatment [12, 22, 24]. In such conditions, changes in nutrient type and availability enhance the growth of specific strains capable of exploiting the new nutrient sources; for example, the pathogen *Salmonella* Typhimurium, but not the microbiota, can utilize ethanolamine and fructose-asparagine in the inflamed intestine [25]. Similarly, antibiotic treatment of mice induces substantial changes in the gut metabolome, resulting in an increase of specific carbon sources that support the germination and growth of *C. difficile* [12]. Taken together, these observations indicate that commensal bacteria are best equipped to provide colonization resistance to pathogens in the healthy, unperturbed intestine. Nevertheless, it may be feasible to design probiotics by engineering commensal strains to better compete with pathogens for nutrients in more hostile environments like the inflamed gut. Of note, there is at least one example of an *E. coli* strain that is able to compete with pathogenic Enterobacteriaceae in such conditions: The widely studied and utilized probiotic *E. coli* Nissle 1917 [26].

E. coli Nissle 1917 (hereafter referred to as *E. coli* Nissle) was isolated in 1917 by Dr. Alfred Nissle from the stool sample of a soldier who did not develop diarrhea during an outbreak of shigellosis [27]. Today, *E. coli* Nissle is the active component of a probiotic preparation used for the treatment of both infectious diarrheal diseases and inflammatory bowel disease (IBD) [26]. Although the mechanisms by which *E. coli* Nissle exerts its beneficial effects are not completely understood, new pieces of evidence suggest that this probiotic strain competes with related bacteria and modulates the immune system (reviewed in [28]).

E. coli Nissle possesses multiple features that might contribute to its ability to colonize the intestine, including Curli, Type 1 and F1C fimbriae, which increase its adherence to the mucosa and can prevent the adhesion of other pathogens [29]. In addition, the *E. coli* Nissle genome encodes several redundant mechanisms that might contribute to its fitness, one of which is a large number of iron acquisition systems, including genes for the synthesis and uptake of small iron chelators known as siderophores [30–32]. Such systems allow for growth in environments where the essential metal ion iron is limited, including the intestinal lumen during inflammation [33]. As reported by our lab in 2013, the presence of multiple iron acquisition systems allows *E. coli* Nissle to successfully compete with the pathogen *Salmonella* Typhimurium for colonization of the inflamed gut [34]. As other commensal *E. coli* strains were unable to reduce *Salmonella* Typhimurium gut colonization [34, 35], our work suggests that *E. coli* Nissle exhibits the uncommon ability to compete with *Salmonella* Typhimurium, and possibly with other enteric pathogens, for iron. Nevertheless, it is feasible that other *E. coli* strains which colonize the gut but cause extraintestinal infections, such as those which cause urinary tract infections, may also compete with *Salmonella* Typhimurium and/or with other bacteria via similar mechanisms. Overall, *E. coli* Nissle is a prime example of how a commensal strain can provide colonization resistance against pathogens by better competing for the limited nutritional resources available in the inflamed intestine.

Production of antimicrobial peptides and toxins

Another means of direct competition between commensals and pathogens within the gut involves the secretion of toxins and antimicrobial peptides. Though originally described as a virulence trait of pathogens to kill their commensal competitors [36], new evidence suggests that the secretion of antibacterial toxins via phage-like machinery known as a “type VI secretion system” (T6SS) is also employed by commensals to attack competitors vying for the same ecological niche [37]. To this end, it has been postulated that the abundance of Bacteroidetes in the intestine could be attributed to their T6SSs [38].

Other mechanisms employed by bacteria to deliver toxins to their competitors (for instance, the contact-dependent growth inhibition system [39]) could also promote inter and intra-species competition as well as colonization resistance to pathogens. Moreover, some commensal Enterobacteriaceae, including the probiotic *E. coli* Nissle, secrete small antimicrobial peptides called bacteriocins or microcins, which specifically target and kill related competitors, including pathogenic organisms [40, 41]. Some microcins target competitors expressing the same nutrient receptors as the microcin producers, and are internalized by hijacking these transporters via a so-called “Trojan horse” mechanism [41, 42]. Other probiotic strains such as *Bifidobacterium* spp. also secrete bacteriocins, which can exhibit either a narrow or broad spectrum of activity [43]. As most of the evidence for the activity of bacteriocins and toxins derives from *in vitro* studies, future studies should address the role of antimicrobial peptides and toxins secreted by commensal and probiotic bacteria to compete with pathogens in the host. Moreover, as most studies on human commensals and probiotics have analyzed their effects on the murine gut microbiota, their relevance in the human host is largely unknown. To this end, broader utilization of humanized gnotobiotic mice (i.e., gnotobiotic mice colonized with human microbiota [3]) could begin

to unravel mechanisms by which human commensals and probiotics compete with the human microbiota.

Indirect effects against pathogens

Studies of GF mice have unequivocally shown the importance of the microbiota for the development of a normal gut mucosa and gut-associated lymphoid tissue (GALT) [44]. It thus follows that the ability of the microbiota and of certain probiotics to provide colonization resistance to pathogens is also mediated by their enhancement of the gut mucosal barrier and of the innate and adaptive immune systems. Examples of such mechanisms are described in detail below (Figure 2).

Enhancement of epithelial barrier function

An important first step for gut colonization by pathogens is the adherence to mucosal surfaces. This step, however, is hindered by the thick mucus layer that covers the intestinal epithelium, providing a first layer of defense against bacterial colonization [45]. The optimal development of the mucus layer is dependent on the microbiota; indeed, GF mice develop a much thinner epithelial mucus layer in comparison to conventionally-raised mice. In line with this, the administration of bacterial components such as LPS or peptidoglycan to GF mice restores their mucus layer formation and reduces their susceptibility to bacterial infections [46]. Of particular importance is the highly glycosylated mucin protein MUC2, which is densely packed and insoluble in the inner mucus layer, but loose and soluble in the outer layer, thereby providing a barrier to the colonization and translocation of both commensal and pathogenic bacteria [47, 48].

Although the development of the mucosal barrier primarily results from the generally cooperative interactions between host and microbiota, some specific bacterial species likely contribute to this process. For instance, certain commensal bacteria can enhance the epithelial barrier function through the production of specific metabolites. One example is *Bifidobacterium longum* subspecies Infantis (a probiotic bacterium that constitutes up to 90% of the microbiota of healthy infants [49]), which secretes peptides that normalize intestinal permeability and reduce intestinal pathology in a mouse model of colitis [50]. Additionally, production of the short-chain fatty acid (SCFA) acetate by *Bifidobacterium* improved intestinal defenses mediated by epithelial cells as well as induced protection against lethal infection with EHEC [51]. Tissue culture studies have also shown that certain probiotics modulate the immune response through a direct interaction with intestinal epithelial cells. One such example is *E. coli* Nissle, whose K5 capsule was shown to stimulate the production of chemokines from Caco-2 cells and from *ex vivo* mouse intestine [52], although it is not known whether the K5 capsule plays an immunomodulatory role *in vivo*.

Enhancement of innate immunity

Commensal organisms also employ several mechanisms to boost the immune response against pathogens at epithelial mucosal surfaces. Although pathogens and commensals can exhibit similarity in terms of surface molecules and antigens, mucosal secretion of pro-

inflammatory cytokines is typically dependent on whether the host encounters commensal or pathogenic microbes. In general, macrophages and dendritic cells in the lamina propria of the intestine are hypo-responsive to commensal microbial ligands, whereas their interaction with a pathogen like *Salmonella* Typhimurium results in the secretion of mature IL-1β and in the recruitment of neutrophils to the site of infection [53]. Nevertheless, during infection with certain pathogens, the intestinal microbiota is also able to promote immune defenses by triggering specific responses, which ultimately lead to secretion of host pro-inflammatory and antimicrobial proteins. To this end, Hasegawa et al. have shown that commensal bacteria are able to stimulate the innate immune system, and thus protect the host from infection [54]. Mice lacking the adaptor protein ASC, an essential mediator of IL-1β and IL-18 processing, are highly susceptible to *C. difficile* infection, mostly because of impaired recruitment of neutrophils to the intestine [54]. Surprisingly, translocation of commensal bacteria was essential to promote IL-1β secretion and protection against *C. difficile* intestinal colonization, indicating that stimulation of pro-inflammatory responses by commensal organisms can have a protective function [54]. Nevertheless, the particular mechanisms by which commensals induce IL-1β processing and secretion remain to be elucidated.

An additional mechanism by which commensals enhance the host immune response to pathogens is by triggering the secretion of host antimicrobial peptides. In the small intestine, Paneth cells are the main source of these peptides (predominantly C-type lectins and α-defensins), whose primary function is to protect the host against enteric pathogens [55]. Paneth cell secretion of the C-type lectins REG3γ and REG3β requires the stimulation of the MyD88 pathway, underlining the role of Toll-like receptor (TLR)-mediated recognition of the microbiota for this process [56, 57]. The importance of REG3γ and REG3β is highlighted by studies showing that these two antimicrobial peptides provide protection to infection with some bacterial pathogens, including *Enterococcus faecalis*, *Yersinia pseudotuberculosis*, and *Listeria monocytogenes* [58–60].

The α-defensins are also key components for the control of enteric pathogens [61]. Similar to C-type lectins, the microbiota plays an important role in the induction of α-defensin expression, which in turn controls pathogen growth and contains commensals within the intestine [62–65]. In particular, both mice lacking the MHC class I-related protein CD1D as well as Crohn's disease patients with mutations in the peptidoglycan sensor NOD2 show a reduced secretion of α-defensins, suggesting that recognition of commensal ligands enhances α-defensin expression [66, 67]. However, *Nod2* mutations in mice do not affect the expression of α-defensins or the composition of the microbiota, which could possibly reflect differences between humans and mice [68, 69]. With regard to β-defensins, some bacteria such as the probiotic strain *E. coli* Nissle 1917 were shown to induce β-defensin expression in cell culture through TLR5-mediated recognition of flagellin [70]. Still, *in vivo* studies are required to determine if the ability of *E. coli* Nissle to induce β-defensins subsequently reduces the colonization of enteropathogens and perhaps controls gut homeostasis by inhibiting bacterial translocation.

Another example of commensal-mediated gut immunity enhancement is the induction of IL-22, a cytokine that enhances the mucosal barrier against pathogens by inducing the secretion of chemokines and antimicrobials by epithelial cells [71]. Mice that lack IL-22

were shown to be more susceptible to gut infection with *C. rodentium*, [72, 73]. Secretors of IL-22 include innate lymphoid cells (ILCs), in which the production of this cytokine is enhanced by activation of the aryl hydrocarbon receptor (Ahr) via specific bacterially-derived molecules [74, 75]. Recently, Zelante et al. showed that a subset of *Lactobacillus* species (specifically, *L. reuteri* in the gastrointestinal tract), utilize tryptophan as an energy source and produce a metabolite, indole-3-aldehyde (IAld), which in turns activates Ahr on ILCs. Once activated, ILCs secrete IL-22, which protects the host against the opportunistic pathogen *Candida albicans* by reducing its colonization [76]. This host-commensal interplay is a prime example of how beneficial bacteria might enhance the immune response in order to protect the host from pathogens. In addition, the observation that only a subset of Lactobacilli produces IAld and activates Ahr-mediated secretion of IL-22 highlights the importance of how differences in genomic content and gene expression, even within the same genus, might account for significant differences in host immune regulation.

Enhancement of adaptive immunity

Commensal and probiotic bacteria are not only implicated in the activation of innate immune responses, but are also able to promote adaptive immune responses. In recent years, a few studies have provided evidence that specific commensal bacteria are able to preferentially drive the differentiation of individual T cell subsets. A seminal study by Ivanov et al. demonstrated that bacteria of the Clostridiales family, Segmented Filamentous Bacteria (SFB), specifically induce the differentiation of mucosal T helper 17 (Th17) cells in the lamina propria of the intestine, which in turn secrete the pro-inflammatory cytokines IL-17 and IL-22 [77]. As IL-22 induces the production of antimicrobial proteins, mice administered with SFB were more resistant to infection with *C. rodentium* [77].

In addition to Th17 cells, the differentiation of the T regulatory (Treg) cell lineage is also impacted by the microbiota. Atarashi et al. demonstrated that colonization of GF mice with *Clostridium* spp. generates an environment rich in TGF-β and colonic Treg cells [78]. Moreover, oral administration of a mixture of 17 *Clostridia* strains attenuated the pathogenesis of colitis by shaping an anti-inflammatory environment rich in Treg cells and IL-10 [79]. In some cases, a single component of a commensal bacterium is sufficient to induce a specific cell subset. For example, the protective effect of *Bacteroides fragilis* against experimental colitis induced by *Helicobacter hepaticus* was shown to be dependent on the expression of a specific capsule, termed polysaccharide A (PSA). In this model, administration of PSA alone was able to protect mice from colitis by inducing anti-inflammatory, IL-10-producing CD4+ T cells [80]. In other cases, certain metabolites produced by the microbiota can impact Treg lineage homeostasis. For instance, SCFAs generated by the intestinal microbiota contribute to the regulation of Treg cell size and function by interacting with the G-protein-coupled free fatty acid receptor 43 (GPR43) [81].

Another important arm of the adaptive immune response modulated by the microbiota involves the differentiation and activation of B cells, along with mucosal secretory IgA (sIgA) [82, 83]. Because sIgA plays an essential role in promoting intestinal barrier function and in determining the composition of the gut microbiota, the presence of certain bacterial species is essential to regulate this important aspect of gut homeostasis [84]. In particular,

dendritic cells which phagocytize small numbers of commensal microbes in the intestine can selectively induce sIgA and protect the host from its own commensal bacteria [85, 86]. Akin to the development of T cell subsets, specific commensal microbes can contribute to the development of B cells. For instance, SFB but not commensal *E. coli* were shown to shown to contribute to the development of intestinal lymphoid tissue, including Peyer's patches, and to the development of intestinal sIgA [87]. Some human *in vivo* studies have also reported the importance of the microbiota for B cell maturation, such as with early life colonization by *E. coli* and *Bifidobacterium longum* subspecies Infantis being associated with higher numbers of mature CD20+ B cells [88]. As most of these studies were aimed at investigating the development of the immune response rather than how the microbiota contributes to colonization resistance to pathogens, future studies should address whether the development of specific immune responses are linked to colonization resistance.

Conclusions

Beneficial microbes provide colonization resistance against harmful microorganisms by stimulating the immune response and by directly inhibiting pathogen growth. Although some such mechanisms have now been described, future studies are needed to identify and/or characterize probiotics and their modes of action against specific pathogens. For instance, IL-22-mediated induction of antimicrobial responses, stimulated by *Lactobacillus reuteri*, could be advantageous against pathogens that are susceptible to these effects, such as *C. albicans* [76]. However, the same probiotic bacterium would likely be ineffective against pathogens like *Salmonella* Typhimurium, which evades and exploits IL-22-mediated antimicrobial responses [35]. In contrast, as *Salmonella* requires iron to grow in the inflamed intestine, administration of *E. coli* Nissle, which can compete with the pathogen for iron, would be a better therapeutic and preventive strategy [34]. In light of this, the identification of vulnerabilities within target pathogens is an essential first step to discover and design probiotics.

Another promising strategy is to screen the intestinal microbiota for natural competitors to specific pathogens. This approach has recently proven successful in a seminal study by Buffie et al, who discovered that a specific intestinal commensal bacterium, *Clostridium scindens*, provides colonization resistance to *C. difficile* infection by producing inhibitory metabolites derived from bile salts [89]. With the constant emergence of antibiotic resistance, as well as new knowledge concerning the detrimental effects antibiotics have on the microbiota, the discovery of new probiotics and their mechanisms of action could provide a strong foundation for developing novel therapeutics against infection.

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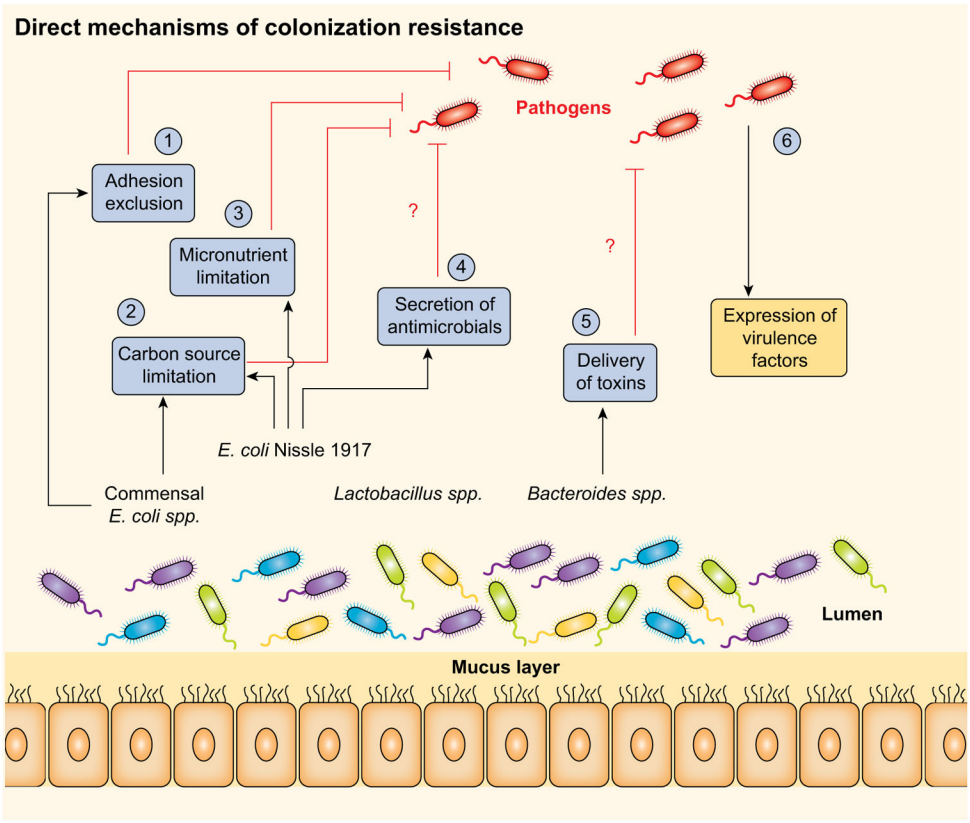

Figure 1. Direct mechanisms of colonization resistance against enteropathogens

The microbiota provide a barrier against incoming enteric pathogens via multiple mechanisms. (1) Adhesion exclusion: Certain commensals reduce pathogen adherence to the intestinal mucosa. (2) Carbon source limitation: Human commensal *Escherichia coli* strain HS and probiotic strain *Escherichia coli* Nissle 1917 both metabolize multiple sugar molecules, which limits the availability of nutrients in the gut to certain pathogens. (3) Micronutrient limitation: The probiotic strain *E. coli* Nissle 1917 can uptake iron via several mechanisms, limiting its availability to pathogens such as *Salmonella* Typhimurium. (4) Secretion of antimicrobials: Commensals act against pathogens not only by limiting nutrients, but also by the production antimicrobial compounds, such as bacteriocins and microcins. (5) Direct delivery of toxins: Although not yet demonstrated *in vivo*, commensals can express type 6-secretion systems (T6SSs) and contact-dependent inhibition (CDI) systems, means by which to deliver growth-inhibiting toxins to close competitors. (6) Circumventing colonization resistance: Pathogens employ a variety of virulence factors to colonize the host and cause disease.

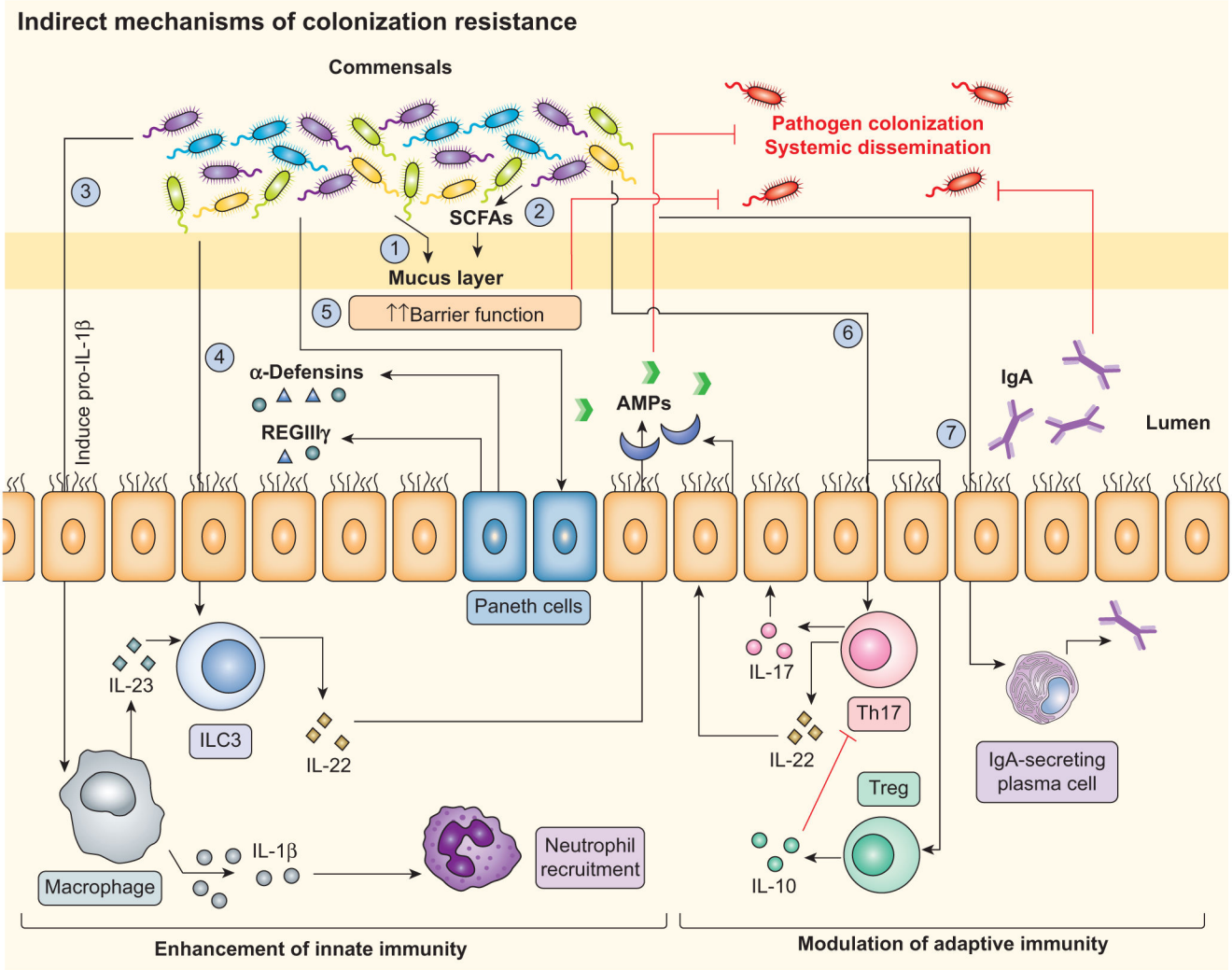

Figure 2. Microbiota-stimulated host immunity provides colonization resistance

Commensal bacteria can indirectly control pathogen colonization by a variety of means. (1) Barrier function: The commensal microbiota up-regulates host barrier function by contributing to the development of the mucus layer. (2) Short-chain fatty acid (SCFA) production: Members of the microbiota such as *Bifidobacterium* spp. can enhance epithelial barrier function by producing SCFAs such as acetate. (3) IL-1β-mediated neutrophil recruitment: Commensals can promote protection against certain pathogens by stimulating IL-1β processing and secretion, resulting in the recruitment of neutrophils to the site of the infection. (4) IL-22-dependent release of antimicrobials: Certain commensals (such as *Lactobacillus reuteri*) induce the secretion of IL-22 by innate lymphoid cells (ILCs), which can in turn protect against some pathogens via the induction of antimicrobial release by epithelial cells. (5) Direct stimulation of antimicrobial production by the host: Secretion of antimicrobial proteins (AMPs), including α-defensins and REG3γ, is a key component in controlling pathogen growth, and is in part mediated by commensal-dependent mechanisms. (6) Induction of T cell differentiation: Commensals can promote adaptive immunity by inducing the differentiation of T cells, such as by stimulating Th17 and Treg cell

differentiation and activation. (7) Secretory IgA (sIgA): Commensals can facilitate host-barrier function by inducing B cells and by regulating the secretion of IgA.