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Does the microbiota regulate immune responses outside the gut?

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Perturbations in the gastrointestinal (GI) microbiota composition that occur as a result of antibiotics and diet in 'westernized' countries are strongly associated with allergies and asthma ('hygiene hypothesis'). The microbiota ('microflora') plays a crucial role in the development of mucosal tolerance, including the airways. Significant attention has been focused on the role of the microbiota in GI development, immune adaptation and initiation of GI inflammatory diseases. This review covers the post-developmental functions that the microbiota plays in regulating immunological tolerance to allergen exposure outside the GI tract and proposes the question: is the microbiota a major regulator of the immune system?

The incidence of allergic airway disease in industrialized countries has been increasing for the past 40 years [1]. In the United States, Canada, UK, Ireland, New Zealand and Australia, the incidence among 13–14-year-old children is currently the highest in the world and ranges from 22–32% [2]. However, during the past 40 years, asthma rates have not changed in developing countries. The recent increase in asthma rate, coupled with the dichotomy in the incidence of asthma between industrialized and developing countries, suggests that environmental changes are a major factor in the development of asthma.

These observations have led to the 'hygiene hypothesis' for allergies and asthma, which states that a lack of early microbial stimulation results in aberrant immune responses to innocuous antigens later in life [1]. However, an alternative interpretation of the hygiene hypothesis is that perturbations in gastrointestinal microbiota composition that occur as a result of antibiotic use and dietary differences in 'westernized' countries (Box 1) have disrupted mechanisms that are involved in the development of immunological tolerance. Data supporting this 'altered microbiota' interpretation include the correlation between asthma and/or allergies and antibiotic use in industrialized countries and the correlation between altered fecal microbiota and allergic disease [3–9]. Because germ-free animals display numerous defects in immune response generation [10], are antibiotic-induced changes in the gastrointestinal (GI) microbiota a predisposing factor for asthma?

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Recent reviews have focused on the role of the microbiota in the development of the intestine and how the immune system adapts to the presence of microbes in the gastrointestinal tract [10–12]. Significant attention

Box 1. Effect of antibiotics, diet and infant-feeding regimens on the microbiota

Antibiotics

The downstream effects of antibiotics on microbiota composition are dependent on several pharmacological variables. However, loss of colonization resistance (resistance to colonization by opportunistic pathogens) is a common side effect of treatment with most antibiotics [51]. In humans and animal models, antibiotic treatment often results in long-term decreases in beneficial anaerobic organisms (i.e. *Bifidobacterium*, *Lactobacillus* and *Bacteroides*) and increases in potentially harmful microbes, such as Gram-negative aerobic enteric bacteria, the anaerobic pathogen *Clostridium dificile* and the yeast *Candida albicans*. Antibiotic treatment can also result in reduced levels of short-chain fatty acids and changes in the 16s rRNA microbiota 'fingerprint' patterns long after antibiotic therapy, probably reflecting changes in microbiota composition [52,53].

Diet

The symbiotic nature of the relationship between the host and microbiota is often characterized in terms of nutrient exchange. The host provides a niche and food for the microbiota, and in exchange the microbes produce beneficial breakdown products, such as vitamins and short-chain fatty acids. Scientists in the first half of the 20th century invested much effort into characterizing the effect of diet on the microbiota owing to the popularity of the writings of Eli Metchnikoff, the inventor of probiotic theory. These early experiments demonstrated that the microbiota composition of rodents changes rapidly upon altering the diet (reviewed in Ref. [54]). It was demonstrated that a chow containing enriched bread inhibited the return to pre-antibiotic microbiota composition [55]. More recent studies have focused on the effect of specific dietary carbohydrates. Prebiotic carbohydrates, such as inulin and oligofructose, stimulate growth of *Bifidobacteria*, whereas the simple sugar fructose stimulates growth of coliform and aerobic organisms [56]. Therefore, the composition of the host's diet is responsible for the metabolic activities and species composition of the microbiota, as different species grow better on different substrates.

Infant-feeding regimens

The first observation that significant differences exist in the microbiota composition in breast-fed and bottle-fed infants was made a century ago [57]. These defining studies that examined the stages of colonization of the GI tracts of infants have been confirmed repeatedly. The chief differences between these two feeding regimens are that the microbiota of breast-fed infants is composed mainly of lactic acid bacteria, whereas the microbiota of bottle-fed infants is more diverse, composed of a mixture of anaerobic bacteria and aerobic species (reviewed in Ref. [58]).

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has also been paid to the role of the microbiota as initiators of inflammatory diseases, such as inflammatory bowel disease and arthritis [13,14]. This review will focus on the post-developmental positive regulatory aspects that the microbiota play in maintaining immunological tolerance of mucosa to exogenous antigen exposure.

The normal microbiota: characterization and development

The process of colonization of the GI tract after birth leads to a series of ecological successions with the end result being the establishment of a stable microbiota ('micro-microflora') that is unique to each individual. The stable adult microbiota is composed of autochthonous species (permanent members) and allochthonous species (transient colonizers that are briefly acquired from an external origin). The adult microbiota is composed of 400–1000 species, with as many as 60% that are not culturable outside the GI environment. However, it is estimated that 30–40 species predominate in this ecosystem. Both prokaryotic and eukaryotic microbes are present, with bacterial species dominating. The majority of the bacterial species are strict anaerobes (97%), whereas only 3% are aerobic (facultative anaerobes). The composition of the microbiota differs not only along the length of the GI tract, but also cross-sectionally, with different populations inhabiting the GI mucosa and lumen. The most common anaerobic genera in terms of concentration within the GI tract are *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Fusobacterium*, *Clostridium* and *Lactobacillus*. Among the aerobes are the Gram-negative enteric bacteria (*Escherichia coli* and *Salmonella spp.*) and the Gram-positive cocci (*Enterococcus*, *Staphylococcus* and *Streptococcus*). In addition to aerobic bacteria, aerobic fungal species, such as *Candida albicans*, are also members of the normal microbiota (Box 2).

Altering the microbiota

Environmental pressures, such as antibiotics, diet and microbial inoculation, can cause alterations in an otherwise stable microbiota, both transient and permanent (Box 1). Those microbes that produce only beneficial effects for the host are termed 'probiotic' species and include *Lactobacillus* and *Bifidobacterium spp.* (Box 3). In addition, potentially pathogenic organisms (PPO) make up part of the microbiota and include the aerobic enteric bacteria, *Clostridium spp.* and *Candida albicans* (Box 2). However, these PPO make up a very small percentage of the total microbiota population in healthy individuals. Imbalances in the microbiota are characterized by decreases in beneficial anaerobic bacteria and increases in aerobic bacteria and fungi (many of which are potential pathogens) and harmful anaerobic bacteria. Colonization resistance is a term referring to the inhibitory activity of the obligate anaerobic microbiota on overgrowth of potentially harmful exogenous or indigenous microbes. The end result of a reduction in colonization resistance can either be clinically asymptomatic (leading only to an imbalance in the microbiota) or lead to disseminated infection.

Box 2. The fungal microbiota: *Candida albicans*

The major fungal microbiota species in humans is the yeast *Candida albicans*. It resides in low numbers in the mouth, vagina and gastrointestinal (GI) tract (reviewed in Ref. [59]). The factors affecting *C. albicans* numbers on mucosal surfaces are multi-faceted and include the normal microbiota, hormones, stress, innate immunity and adaptive immunity. Germ-free and antibiotic-treated mice are dramatically more susceptible to *C. albicans* infection. In humans, yeast infection of mucosal sites is one of the most common side effects of antibiotic therapy. Thus, control of *C. albicans* by the normal microbiota is very important.

The ability of the bacterial microbiota to control or prevent *C. albicans* colonization is due in part to competitive exclusion of favored niches. Oral bacteria can inhibit the yeast-to-hyphal phase change in *C. albicans* that is associated with increased epithelial invasion [60]. Lactic acid bacteria are also known to inhibit *C. albicans* colonization of the epithelium of the GI tract in mice and subsequent hyphal invasion and systemic infection [61]. In a mouse model of GI candidiasis, prior inoculation with *Lactobacillus* reduced *C. albicans* levels and invasion [61]. It has also been recently demonstrated that several *Lactobacillus* strains can inhibit *C. albicans* hyphal transformation, which is a key step for epithelial invasion and commensal-to-pathogen switch [62].

Members of the normal microbiota, such as lactic acid bacteria, produce large quantities of biologically active short-chain fatty acids. These fatty acids, which are by-products of anaerobic fermentation, possess an anti-inflammatory function. Butyric acid is the short-chain fatty acid that is most well-known for its immunomodulatory activities [63]. However, butyric acid can also inhibit *C. albicans* hyphal transformation [62,64]. Short-chain fatty acids are produced in large quantities by lactic acid bacteria as a by-product of fermentation [65]. Thus, inhibition of *C. albicans* hyphal transformation is an important probiotic activity of the lactic acid bacteria.

Box 3. Probiotics and prebiotics

The idea that manipulation and/or stabilization of the gastrointestinal (GI) microbiota can be immunotherapeutic is once again gaining in popularity (probiotics). The probiotic theory was first proposed by Eli Metchnikoff to explain the link between the longevity and health of Bulgarian peasants and daily intake of fermented milk products containing non-putrefactive organisms. From these observations, he theorized that maintenance of a healthy gut microbiota (through daily ingesting of beneficial bacteria) was the key to a healthy long life. Probiotics are therefore defined as live microbial supplements that exert a beneficial effect on health and are non-pathogenic. Probiotic supplementation results not only in establishment of the inoculated organism but also can alter the concentrations of other members of the microbiota. Probiotic supplementation is still common in non-westernized areas and parts of Europe in the form of fermented foods. Archeological evidence supports the hypothesis that this practice extends back thousands of years throughout various civilizations [66]. This fact raises the possibility that our GI ecosystem (host and microbiota) has evolved to support and require a daily dose of live lactic acid bacteria [67]. In support of this idea, reduced rates of allergic disorders have been observed in westernized areas among children leading an anthroposophic lifestyle, which includes reduced antibiotic use and daily intake of probiotic organisms [3]. Recent clinical studies have provided evidence that probiotic supplementation can facilitate improved health. Attention is being given not only to development and selection of microbes with specific effects on health, but also to food additives (prebiotics) specifically designed to help promote the growth and/or activities of these microbial supplements. A long-term study investigating the impact of daily ingestion of a milk product containing *Lactobacillus rhamnosus* on fecal microbiota composition demonstrated that probiotic supplementation over a six-month period results in transient establishment of the administered bacteria. However, after termination of probiotic supplementation, *L. rhamnosus* was no longer detected in the majority of subjects during a two-month post-test period [68]. These and other studies indicate that daily intake of probiotics is probably required for maximal efficacy.

The epidemiological connection between altered microbiota and allergies

Antibiotics, diet and infant-feeding regimens are all associated with the development of allergies and asthma [1]. All three of these also have profound effects on microbiota composition (Box 2). Until recently, the evidence to support a role for the microbiota in allergic disease was based on epidemiological studies as opposed to direct testing of this idea. Numerous studies indicate that the composition of the GI microbiota is different in atopic versus non-atopic individuals and in industrialized versus developing countries [5,7–9]. Dietary variation, such as the increased proportion of refined foods in the diet, probably plays a role in GI microbiota differences between industrialized and developing countries [15,16]. Examination of microbiota composition in children from countries with a high (Sweden) and low (Estonia) prevalence of allergic disease revealed that allergic children from either country had similar microbiota composition [5]. Allergic children (as determined using the positive skin-prick test to common allergens) harbored increased levels of aerobic microbes and decreased levels of anaerobic microbes, particularly lactobacilli. Other studies investigated microbiota composition in newborns through the first two years of life and monitored the development of atopy in Sweden and Estonia [8]. Interestingly, infants that developed allergies harbored lower levels of bifidobacteria and enterococci but increased levels of clostridia and *S. aureus*. These studies suggest that microbiota composition might be the underlying factor in allergic disease regardless of other environmental differences that exist in these two countries.

Other groups have also reported decreased levels of bifidobacteria and a reduction in the number of Gram-positive organisms among the aerobic populations in infants with atopic eczema [7]. Among the different bifidobacterial species, a distinct pattern was also observed in allergic versus non-allergic infants, with allergic infants possessing a bifidobacterial composition similar to adults [17]. This suggests that a particular species of probiotic anaerobic bacteria (Box 3) might play a more important role than others in directing healthy immune responses.

Although control of the GI microbiota balance is influenced by several factors, including diet, antibiotics can have the most profound impact on an individual's GI microbiota and antibiotic use in industrialized countries is significantly greater than in developing countries. In a review of the literature, several epidemiological studies have identified a correlation between early antibiotic use in children and the subsequent development of allergies and/or asthma [3,4]. More recent reports continue to support this link. Studies have also compared children of families with an anthroposophic lifestyle (Steiner school children) to children in neighboring schools. Anthroposophic children often abstain from antibiotic use and ingest fermented foods containing probiotic organisms. Rates of allergy among these children are significantly lower than that found in the control children, with an inverse correlation between the number of characteristic features of an anthroposophic lifestyle and risk of atopy [3]. Among anthroposophic children, the use of antibiotics early in life was significantly associated with the development of asthma [4]. In addition, the microbiota of anthroposophic children was examined and found to contain higher levels of lactic acid bacteria than children who had previously been exposed to antibiotics [18]. Among a cohort of 1934 non-anthroposophic subjects, antibiotic treatment during the first two years of life was found to be a predictor of subsequent atopic disease [19]. From these studies, it follows that antibiotic use not only alters microbiota composition but also correlates with the development of allergies and/or asthma.

The immunological consequences of altered microbiota

It has been known for several years that alterations in the GI microbiota can influence mucosal immunity [10]. Germ-free animals have smaller Peyer's patches, fewer intraepithelial lymphocytes, and lower levels of secretory IgA. In the context of allergic responses, germ-free animals are resistant to the induction of oral tolerance that can block IgE production [20,21]. Inoculation of germ-free mice with intestinal bacteria (conventionalization) can restore the ability to generate oral tolerance.

Immunological tolerance, including oral tolerance, is mediated by a diverse group of T cells known as regulatory T cells, which possess anti-inflammatory capabilities [suppress via interleukin (IL)-10 and/or tumor growth factor (TGF)-β] [22]. The development of regulatory T cells is heavily influenced by the type and maturation state of the antigen-presenting cell (APC). Dendritic cells (DCs) are potent APCs that vary in their ability to induce T cells; immature DCs (i.e. have not received inflammatory signals) induce differentiation of regulatory T cells, whereas mature DCs (i.e. have received inflammatory signals) induce differentiation of inflammatory T cells (Th1 and Th2) [22].

Evidence is beginning to accumulate that the GI microbiota might also regulate DC differentiation in the intestinal mucosa [23]. For example, lactobacilli can down-modulate the maturation of DCs and induce the *in vitro* expansion of CD4+ T cells, which resemble regulatory T cells [24,25]. Interestingly, kanamycin treatment of infant mice has been reported to augment basal Th2 versus Th1 responsiveness, including increased circulating IgE, IgG1 and anti-CD3-stimulated splenic IL-4 with decreased splenic IFNγ production. Oral introduction of intestinal bacteria into these antibiotic-treated mice prevented the upregulation of basal Th2 responses in the spleen, indicating that the mechanism occurs via perturbation of the GI microbiota [26]. Overall, pieces of evidence are beginning to accumulate that support a model whereby disruption of the microbiota also disrupts oral tolerance, potentially by interfering with the DCs that promote antigen-specific regulatory T cell responses (Figure 1).

Role of altered microbiota in allergic airway disease

It has been proposed that the lung microenvironment is generally predisposed to Th2 responses [27]. However, repeated intranasal antigen exposure in the lungs leads to decreasing reactivity, a form of tolerance [28,29]. Oral

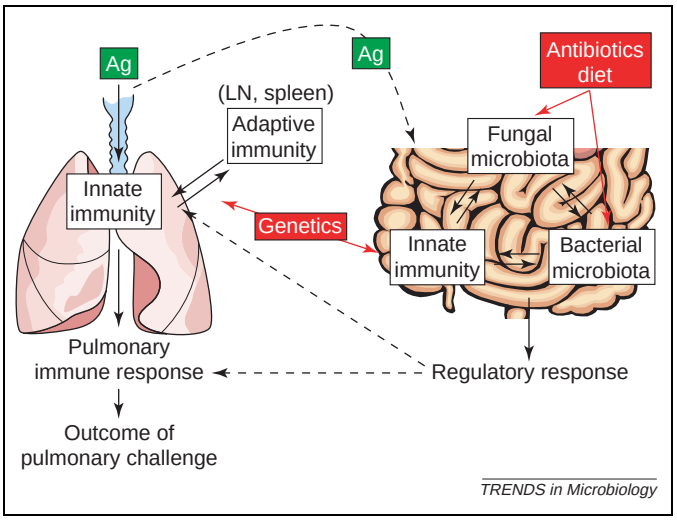

Figure 1. Proposed model for how the gastrointestinal (GI) microbiota might regulate pulmonary immune responses. Antigens (Ags) that are inhaled are captured by cells of the innate immune system, which then stimulate adaptive immune responses in secondary lymphoid organs, such as the lymph nodes (LN) or spleen. Antigen-specific T cells are recruited to the lungs upon subsequent antigen exposure. Regulatory T-cell networks are necessary to prevent the development of over-exuberant Th2 responses in the airways to inhaled antigens that are non-infectious (pollens) or of extremely low infectivity (mold spores). Inhaled antigens are also swallowed because the anatomy of the sinuses and upper airways is designed to trap environmental antigens (aerosols, microparticulates and macro-particulates) in the mucus layer and then 'sweep' them into the throat where they are swallowed. Swallowed Ags are then acquired by dendritic cells (DCs) in the GI tract. Under non-inflammatory conditions, these DCs promote the development of a regulatory T-cell response to the Ag. The precise mechanism by which the GI microflora balance helps to maintain the T-cell regulatory networks that mediate oral tolerance is unknown. However, the GI tract can sample antigens to which the lungs and sinuses are exposed. This results in the development of regulatory T-cell responses to control inflammation in the absence of infection. The link between antibiotic use and dysregulated pulmonary immunity would occur through antibiotic-induced long-term alterations in the bacterial and fungal GI microbiota, which would alter the developmental environment for regulatory T cell responses. Dietary changes could have the same effect as antibiotic use. Host genetics could modulate the response by altering the innate recognition response.

tolerance is mediated by regulatory T-cell responses, which can down-modulate Th2 responses to the same antigens at sites throughout the body, including the lungs [30–32]. Oral tolerance cannot be generated in germ-free mice, indicating that the GI microbiota plays an important role in this process [21].

So what does oral tolerance have to do with tolerance to inhaled allergens? Several studies have demonstrated that fluids, particles and microbes introduced into the nasal cavity are largely found in the GI tract shortly thereafter [33–35]. Even volumes as small as 2.5 μl introduced intranasally into mice will ultimately be swallowed owing to the mucociliary anatomy of the nasopharyngeal cavity [35]. Thus, the GI tract will be exposed to any antigens that the respiratory tract is also exposed to. Because the ingestion of many antigens can induce tolerance to that antigen (a phenomenon known as 'oral tolerance' [30]), the intriguing possibility is raised that the mucosal immune system in the GI tract might act as a 'sensor' for the development of tolerance to inhaled antigens. If this hypothesis is true, then it would logically follow that this 'sensor' system could be modified by genetics (affecting innate immune cells) and to an even greater extent by microbiota perturbations exerted by antibiotics and diet (Figure 1).

We recently reported that *C. albicans* (and many other fungi) secrete prostaglandin-like molecules *de novo* or via conversion of exogenous arachidonic acid [36]. A PGE_2 (prostaglandin E2) cross-reactive compound that is biologically active on mammalian cells with an activity that is comparable to purified PGE_2 can be purified from *C. albicans* [36]. Prostaglandins are potent immunomodulatory molecules that can inhibit Th1-type immune responses, chemokine production, phagocytosis and lymphocyte proliferation, and promote Th2-type responses and tissue eosinophilia (reviewed in Ref. [37]). PGD_2 receptor (DP)-knockout mice do not develop airway Th2 responses to ovalbumin (OVA). Evidence is now accumulating that prostaglandins (especially PGE_2) play a role in overall immune regulation (positive and negative). Microbe-derived PGD_2 can alter DC migration and biology. Fungal cell-wall glucans are also powerful inflammatory stimulants in tissues [38] and might play a role in the immunomodulatory activity of yeast in the GI tract. Thus, increased levels of fungal microbiota, which often occurs during antibiotic therapy, might diminish the ability to generate regulatory T-cell responses to swallowed antigens, possibly by interfering with tolerance-inducing antigen presentation via fungal oxylipins and glucans.

We have recently developed a mouse model of antibiotic-induced microbiota disruption that includes stable increases in gastrointestinal (GI) enteric bacteria and GI *Candida* levels with no introduction of microbes into the lungs. Mice are treated for five days with cefoperazone in the drinking water, followed by a single oral gavage of *C. albicans*. This results in alterations of GI bacterial populations and increased yeast numbers in the GI microbiota for at least 2–3 weeks. If the mice are subsequently exposed intranasally to allergens, this microbiota perturbation will produce an allergic airway response that is mediated by IL-13 and CD4 T cells [39,40]. These animal model studies underscore the concept that alterations in the microbiota can play an important role in regulating immune responses in the lungs to inhaled antigens.

Can the microbiota be manipulated to alter allergic responses?

Numerous studies have indicated that probiotic and prebiotic supplementation (Box 3) can produce positive results in both therapeutic and preventative ways [41]. Although the majority of human trials have focused on the benefits of probiotics in GI diseases (reviewed in Ref. [42]), there is some evidence to suggest that probiotic supplementation can alleviate and/or prevent development of allergic disease. Human trials have targeted neonatal or infant subjects who are at risk (one or more first-degree relatives with allergic disease) or have already developed an allergic disease. Although allergy encompasses a broad range of diseases, atopic eczema is often the earliest manifestation in infants and children. In a randomized placebo-controlled study, oral *Lactobacillus rhamnosus GG* decreased the incidence of atopic eczema in children at high risk [43]. A follow-up of this study two years later demonstrated that the protection against allergic disease

in children who had received previous probiotic supplementation extended beyond infancy [44]. Several studies examining infants who developed atopic eczema during infancy showed that probiotic-supplemented formula decreased severity of disease compared with those infants that were given control formula without probiotic supplementation [45,46]. An examination of fecal microbiota composition in infants with atopic eczema revealed that *E. coli* levels correlated with the extent of allergic disease and that bifidobacteria supplementation subsequently decreased *E. coli* levels [47]. Interestingly, probiotic supplementation of mothers pregnant with at-risk babies also prevents subsequent atopic disease. Pre- or post-natal probiotic supplementation reduced the rates of atopic dermatitis by 50% in a group of at-risk infants [48]. In children who have already developed atopic dermatitis, several studies have shown that *Lactobacillus* supplementation can alleviate clinical symptoms in some patients [49]. In mice, the oral feeding of *L. casei* strain Shirota was effective in inhibiting IgE production to OVA [50]. Altogether, these studies point to modulation of the microbiota in children and infants as a preventative or therapeutic strategy against allergic disease. More studies are needed to determine optimal probiotic strategies and to determine the effectiveness of this approach in adults with allergic airway disease.

Is the microbiota a major regulator of the immune system?

For a century, science has known that diet can dramatically alter the microbial composition of the microbiota. For decades, science has been able to demonstrate in animal models the importance of the microbiota for the development and maintenance of the immune system. However, the impact of these findings on human health has not become apparent until the past 20–40 years. During this period of time, powerful forces have been introduced into everyday society that can significantly alter the microbiota for long periods of time. The widespread use of antibiotics and dramatic change in the diet in industrialized countries has been accompanied by a striking increase in the incidence of allergies and asthma. Thus, we unfortunately appear to be proving that the animal models are correct in that our microbiota plays a significant role in regulating immune responses.

So, is the microbiota a major regulator of the immune system? Adaptive immunity was identified many decades ago, argued possibly using the first vaccination studies. The contribution of innate immunity to host defense and physiology was proposed and accepted within the past decade. Even with these advances, there is still a significant amount to be understood about the basic biology of immune tolerance and chronic disease. Thus, is there another facet of immune regulation that has not been factored into models of immunophysiology? It is important to realize that we peacefully co-exist with ten times the number of microbes in our body compared with our own cells (the ratio is even greater if we consider only the microbe to leukocyte ratio!). Thus, the role of the immune system is not to eliminate microbes, it is to prevent damage to the other systems in physiology (respiratory, gastrointestinal, nervous and endocrine, among others). Sometimes, elimination of a microbe is necessary to prevent damage. The remainder of the time, co-existence is largely sufficient. However, if it was simple co-existence, then it could be argued that germ-free animals would be at least as healthy or healthier than conventional animals; however, they are not. Germ-free animals show numerous defects in immune function. If a knockout mouse was generated that lacked a single gene (*gfa*) and this *gfa* knockout mouse showed the same immunological defects as observed in germ-free mice, it 'would' be argued that the *gfa* gene plays a central role in immune regulation. Thus, although the microbiota can be an initiator of inflammatory diseases, the emerging evidence is that the microbiota is also a major positive regulatory force for immune responses well after development. It is clear that normal microbiota can play a positive role in protecting the host against pathogenic microbial challenge by microbial exclusion from the mucosa (an ecological effect). However, these ecological principles do not explain the protective role of the microbiota during allergen challenge, where the agent is usually a non-pathogenic particulate, such as animal danders or pollens that cannot cause disease in the absence of the microbiota. The challenge for researchers in the future will be to identify the 'crossover points' where microbial signals regulate immune function. As discussed in this review, one possible point is via the production of fatty acid metabolites, such as oxylipins and short-chain fatty acids, by the host and microbiota.

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