

EXPERT
REVIEWSA case for antibiotic
perturbation of the
microbiota leading to
allergy development*Expert Rev. Clin. Immunol.* 9(11), 1019–1030 (2013)**Lisa A Reynolds¹ and
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The use of antibiotics to treat pathogenic bacterial infections has been one of the greatest contributions to human health, yet antibiotic use also perturbs the communities of commensal and symbiotic bacteria that reside in the intestine of mammals. The microbiota are critical for normal immune development and for maintaining intestinal homeostasis, and disruption of the microbiota has been linked to the emergence of allergic disease both in humans and in animal models. The evidence and mechanisms for antibiotic-mediated disruptions leading to the onset of allergic disease at mucosal surfaces is discussed, as well as the future challenges for the field. A more complete understanding of the mechanisms by which the intestinal microbiota modulate allergic disease development will allow for interventions to counter the potentially adverse effects of antibiotic treatment on the microbiota.

KEYWORDS: allergy • antibiotics • asthma • bone marrow progenitors • epigenetics • immune development
• metabolites • microbiota • probiotics

Allergies are characterized by exaggerated immune responses to foreign antigens from non-pathogenic substances. The prevalence of atopic individuals in industrialized countries has been steadily increasing over the past 50 years [1], yet surprisingly no single factor is clearly responsible for this phenomenon. Over a similar time frame, there has not been an appreciable increase in allergic disease incidence in developing countries, suggesting that environmental aspects of the industrialized lifestyle are responsible for the allergy pandemic. Air pollution, dietary changes, the absence of helminth colonization and antibiotic use have all been implicated in the rise of allergic disease prevalence [1,2].

The ‘hygiene hypothesis’ was conceived following the observation that a larger family size reduced the risk of developing hay fever [3]. This hypothesis proposed that an absence of infection early in life results in a lack of proper immune system education, leading to aberrant responses when harmless antigens are encountered later in life [1]. More recently, an alternative hypothesis has been proposed to explain

the epidemiological data: the ‘microbiota hypothesis’ [4]. This hypothesis suggests that it is the composition of species within the intestinal microbiota of mammals that drives the development of atopy. Evidence for this hypothesis stems from correlations between microbiota composition and allergic disease development, both in humans and mice [4], and from the wealth of literature describing the contributions of the microbiota toward immune development and immune cell differentiation [5]. Several of the factors implicated in the epidemiological shifts in allergy development, including diet, helminth infections and antibiotic use have been shown to modify the composition of the microbiota [4,6].

In this review, we focus on the ‘microbiota hypothesis’ of allergic disease, and discuss evidence for the use of antibiotics disrupting the microbiota, resulting in the development of allergies at mucosal sites. Current insights into the mechanisms of antibiotic treatment-mediated allergy development, as well as strategies to reduce microbiota perturbations during antibiotic therapy, are discussed.

Immune development & the intestinal microbiota

The intestinal tract of mammals is sterile until birth, after which colonization by microbes begins. Birth route (vaginal vs cesarean section), diet (formula vs breast-feeding), prematurity and antibiotic use (during the first month of life) are key factors which determine the initial microbiota composition of human infants [7–9]. Initial colonizers and early-life modifications of the microbiota likely influence the structure of the microbiota into adult life. The intestinal tract is by far the most densely colonized surface of mammals with a highly diverse microbiota consisting of bacteria, archaea, viruses and fungi. While the intestinal microbiota have received the most attention over the past decade, microbial communities also reside on the skin, in the respiratory tract and vagina of humans [10,11].

The presence of the microbiota during development, and recognition of pathogen-associated molecular patterns (PAMPs) by innate pattern recognition receptors (PRRs) is required for normal immune development and for the maintenance of intestinal homeostasis [12]. Evidence for this has been gained by studying the immunodeficiencies of germ-free (GF) mice, and in mice genetically deficient for PRRs or their adaptor proteins [13,14].

At birth, immune responses are Th2 biased; fetal T cells collected from human umbilical cord blood samples mount Th2-skewed responses toward common environmental antigens [15], and fetal T cells proliferate to a greater extent than adult T cells following exposure to IL-4 [16]. The capacity for Th1 responses in early life appears to be impaired, as circulating mononuclear cells produce less IL-12p70 in response to bacterial stimulation during infancy [17]. GF mice also have reduced IFN- γ -producing CD4⁺ T cells in their spleen compared with conventionally raised mice, which can be normalized by monocolonizing GF mice with the human commensal *Bacteroides fragilis* [18]. Whether this is a property solely of *B. fragilis*, or if this restoration of Th1 responsiveness occurs after colonization with any bacterial species, remains to be determined. Delayed induction of Th1 responses following birth, which could result from antibiotic treatment in early life, has been proposed as a mechanism leading to the development of atopy [19]. In support of this hypothesis, low IFN- γ levels in human cord blood samples correlate with the development of allergic symptoms at 1 year of age [20].

As well as stimulating normal immune system development during early life, the composition of the adult microbiota has been shown in mice to influence the differentiation of intestinal T cells. The observation that C57BL/6 mice purchased from different commercial vendors have different levels of Th17 cells in their small intestinal lamina propria (LP) led to the hypothesis that specific microbes can drive Th17 development [21]. Further work has identified segmented filamentous bacteria (SFB), which are able to penetrate the mucus barrier and directly contact epithelial cells, as key drivers of intestinal Th17 cell development [22,23]. Specific bacterial species can also promote tolerance in the intestine, as colonization with a mix of

Clostridium species derived from murine or human feces promotes Treg induction specifically in the colonic LP of mice [24,25]. Thus, the presence of certain species within the microbiota can dictate local T-cell differentiation. Additionally, the presence of certain species may be critical for limiting the differentiation of certain immune cells, for example, mice deficient in Toll-like receptor (TLR)9 signaling display increased frequencies of Tregs in their small intestinal LP and Peyer's patches (PP), and reduced constitutive IFN- γ and IL-17A production compared with TLR9-sufficient mice [26], indicating that specific microbial signals are important for both promoting and inhibiting immune cell differentiation.

Absence of microbial signals & allergy development

Although GF mice have many developmental immune defects [14], these mice have been extremely valuable to identify mechanisms by which the microbiota can potentially influence immune development and modulate disease susceptibility. GF mice develop more severe allergic disease in the ovalbumin/alum model of allergic airway inflammation than their conventionally housed counterparts [27,28]. The exacerbated airway inflammation is measured through increased airway resistance, total bronchoalveolar lavage fluid (BALF) cell numbers, BALF eosinophilia, serum immunoglobulin (Ig)E levels and lung tissue eosinophil infiltration [27,28]. Several mechanisms for this exacerbated inflammation have been proposed (FIGURE 1). One group has described how GF Swiss-Webster and GF C57BL/6 mice have higher levels of invariant natural killer T (iNKT) cells in their colonic LP and lung tissue than their conventionally housed counterparts [28]. iNKT cells express the chemokine receptor CXCR6, which recognizes the chemokine ligand CXCL16. In GF conditions, the increased recruitment of iNKT cells can be explained by hypermethylation of the *Cxcl16* locus, leading to 5-hydroxymethylcytosine incorporation into DNA, which is associated with higher transcription of *Cxcl16* mRNA in the epithelium of these sites [28]. DNA hypermethylation leading to the incorporation of 5-hydroxymethylcytosine, in contrast to the incorporation of 5-methylcytosine, may be associated with higher levels of gene transcription, rather than repression of transcription [29]. iNKT cells recognize antigen presented by CD1d, and the inflammatory phenotype appears CD1d-dependent, as elimination of the asthmatic response was observed after administration of a CD1d-blocking antibody (19G11). It is not clear whether CD1d was responsible for the exacerbation of inflammation in GF mice, or the development of any inflammation in this model, since 19G11 treatment also blocks disease development in conventionally housed mice [28].

GF mice also have reduced numbers of alveolar macrophages in their lungs compared with conventionally housed mice [27], a cell type implicated in inducing Tregs and promoting tolerance in the lungs [30], although whether it is a lack of alveolar macrophages that is directly responsible for allowing exaggerated inflammation in the airways of GF mice is not yet known. Circulating cells and antibodies may contribute to the increased

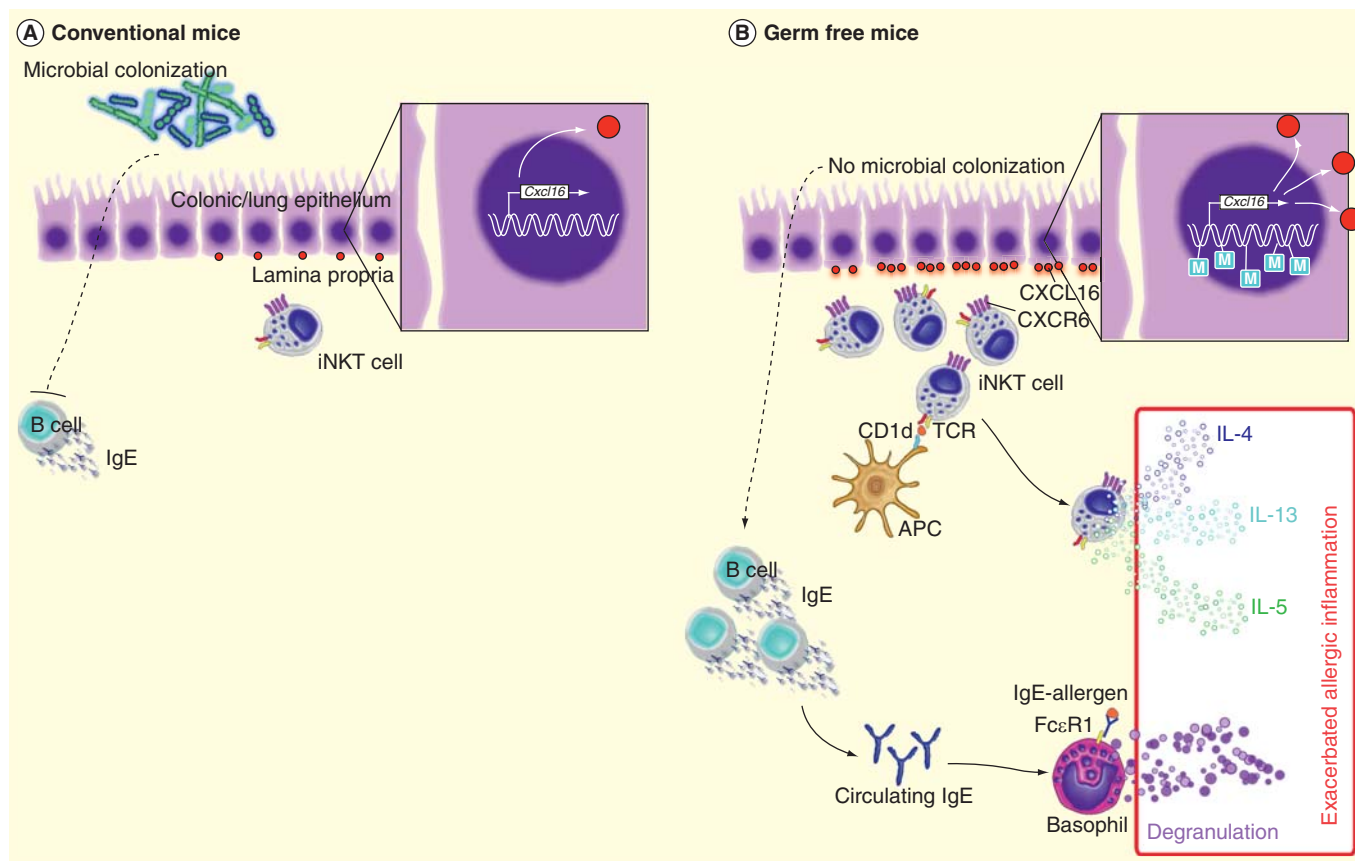


Figure 1. Mechanisms leading to exacerbated inflammation in germ-free mice. (A) In conventionally raised mice, the presence of intestinal microbiota limits circulating IgE levels [31], by as yet undescribed mechanisms. At steady state, the locus for the iNKT chemokine ligand CXCL16 is not hypermethylated, leading to a basal level of transcription and translation of CXCL16 in the epithelium of the lung and colon, causing minimal iNKT cell recruitment to the lamina propria at these sites [28]. (B) In the absence of microbial colonization, in GF mice, no inhibition of IgE production results in high levels of circulating IgE, and basophil surface-bound IgE [27,31]. Additionally, hypermethylation of the *Cxcl16* locus occurs, leading to incorporation of 5-hydroxymethylcytosine into DNA, which is associated with increased transcription and translation of CXCL16 in the colonic and lung epithelium [28]. The chemokine receptor for CXCL16, CXCR6, is expressed on iNKT cells, causing increased recruitment of iNKT cells to the colonic and lung lamina propria [28]. In a manner dependent on CD1d, iNKT cells mediated exacerbated allergic inflammation, likely due to their ability to produce large amounts of Type 2 cytokines following their activation [28].

GF: Germ-free; IgE: Immunoglobulin E; iNKT: Invariant natural killer T.

propensity for allergy of microbiota-deficient mice as in both GF and in mice deficient for the TLR-adaptor protein MyD88, serum IgE levels, circulating basophils numbers and basophil-surface bound IgE levels are elevated compared with conventionally housed and MyD88-sufficient controls [31].

It will be critical to examine whether similar mechanisms lead to an increased propensity toward atopy in antibiotic-treated mice, which will clarify whether the phenotype of GF mice is a result of developmental immune defects, or due to an absence of microbial stimulation at the time of antigen sensitization.

Effects of antibiotic use on the microbiota

Antibiotic treatment generally results in a greater widespread species disturbance than simply the loss of bacterial populations directly targeted by the antibiotic administered, since many microbes are dependent on the presence of other bacterial

species for their persistence in the mammalian host. Thus, antibiotic treatment results in loss of primary bacterial populations, as well as those secondary populations which are dependent on metabolites or waste-removal processes initiated by primary species [32]. Additionally, the sites affected by antibiotic treatment are dependent on the route of administration, and the absorptive properties of the antibiotic.

The persistence of antibiotic-induced perturbations of the microbiota depends on the class of antibiotic being administered. Mice treated with a combination of amoxicillin, metronidazole and bismuth, or with the single antibiotic vancomycin, harbored significant changes in intestinal microbiota composition, although their bacterial communities returned to baseline following withdrawal of antibiotics [33]. By contrast, the diversity of microbes in cefoperazone-treated mice was still quite disrupted even 6 weeks following the end of antibiotic treatment [33].

Human studies have also examined the persistence of antibiotic-mediated disruptions. Following clindamycin treatment in adults there was a reduction in diversity among fecal *Bacteroides* species, accompanied by an elevation in clindamycin-resistance associated genes, which persisted for up to 2 years following antibiotic exposure [34]. A separate study on the long-term effects of ciprofloxacin treatment in adults suggests that the intestinal composition returns to a similar state as before treatment within 4 weeks, yet there is a long-term loss of some individual taxa up to 6 months post-treatment [35]. Taken together, these reports suggest that after antibiotic treatment in adults the microbial communities broadly return to baseline, yet there are long-term effects of antibiotic treatment. In the majority of cases, functional redundancy between individual species, and resiliency of the complex community likely prevents any detrimental effects of antibiotic treatment [35].

Exposure to antibiotic treatment during childhood, however, may have more severe long-term effects on microbial community structure due to the less stable nature of the immature microbiota [36]. The microbiota of human infants receiving cefalexin during the first 4 days of life was less diverse than that of antibiotic-free infants until at least 2 months of age [37]. Longitudinal studies are underway to determine to what extent these early-life perturbations extend to dictate the composition and diversity of the microbiota in adult humans.

Impact of antibiotics on susceptibility to infection

As well as stimulating normal immune development, the intestinal microbiota help prevent enteric infections by bacterial, viral or fungal agents. This is achieved by physically contributing to the mucus layers, by producing and stimulating host-production of antimicrobial peptides, by priming innate immune cells and by competing with invading organisms for nutrients [38–40].

A common result of antibiotic use in humans is the acquisition of opportunistic fungal infections. It has been proposed that disruption of the microbiota leads to an increase in fungal colonization which contributes to increased sensitivity to antigen exposure, leading to allergy development or exacerbation. In a mouse model, treatment with the broad-spectrum antibiotic cefoperazone for 5 days, followed by oral gavage with *Candida albicans*, results in an altered bacterial composition and an increase in intestinal fungal colonization [41]. These mice respond to nasal administration of the mold spore *Aspergillus fumigatus* conidia by production of IL-5, IL-13, IFN- γ , eosinophilia and mast cell expansion in the lungs, and heightened circulating IgE levels [41,42]. Notably, in mice that have not been antibiotic-treated, or received a *C. albicans* gavage, this inflammatory response to *A. fumigatus* is absent [41,42], suggesting that tolerance to intranasal antigen exposure is lost following antibiotic treatment and fungal colonisation. Additionally, following antibiotic treatment, the reassembly of bacterial communities is altered in the presence of fungal colonisation [43], suggesting that if opportunistic infections occur, they can prolong antibiotic-mediated disruption of the bacterial microbiota.

In both GF mice, and in conventionally housed mice treated with a broad-spectrum antibiotic cocktail, impaired immunity to respiratory viral infections is seen, resulting in higher pulmonary viral titres [38,39]. This is of importance, since many asthma exacerbations in humans correlate with viral infections [44]. Additionally, in mice, concurrent viral infections early in life result in attenuated tolerance to administered antigens [45].

Opportunistic infections following antibiotic treatment may be a contributing factor at either the antigen sensitization stage of allergy development, or a cause of asthma exacerbations. It will be important to resolve whether it is the absence of key bacterial species in the intestine, or the presence of opportunistic pathogens such as fungi or viruses following antibiotic treatment which results in a lack of tolerance. Care must be taken when drawing conclusions about the effect of a reduced bacterial burden following antibiotic treatment, when the levels of fungal or viral colonization have not been assessed.

Early-life antibiotic use: microbiota disruption during a critical developmental period

Many human studies have suggested that antibiotic use is associated with the development of allergic asthma and food allergies. Accumulating evidence suggests it is exposure to antibiotics while *in utero* or during the first year of life that is associated with the development of allergy and asthmatic symptoms later in life [46–54]. Moreover, the length and frequency of antibiotic exposure during the first year of life positively correlate with a higher risk of allergy development [49,52,53]. By contrast, some studies have found no association between antibiotic use and allergic asthma development in children where at least one parent is atopic [52,55]. These reports support the hypothesis that only the disruption of a healthy microbiota predisposes children toward allergy development.

Asthma diagnosis in infants is challenging, and in some cases, early-life wheezing can be falsely diagnosed as a respiratory tract infection. The misdiagnosed respiratory tract infection may be treated with antibiotics, thus leading to a potential for protopathic bias in correlative studies [56]. Human epidemiological studies have been revealing, yet due to this potential for protopathic bias, and the many confounding variables, animal studies have been extremely valuable in determining the impact of early-life antibiotics on the development of allergic diseases.

Mice that are exposed to clinically relevant doses of vancomycin *in utero* via their mother's drinking water, and throughout life, have profound shifts in their fecal microbiota communities, which become dominated by Lactobacillaceae family members [57]. These mice exhibit high serum IgE levels, and enhanced disease severity in an ovalbumin-driven model of experimental allergic asthma, evidenced by increased total BALF cell counts, increased eosinophil numbers in BALF, worse lung pathological scores and increased airway hyperresponsiveness (AHR) [57]. The timing of vancomycin exposure is critical for the development of exacerbated airway disease, as

mice given vancomycin as adults do not display any differences in pathology to untreated mice, whereas mice exposed to vancomycin only during their first 3 weeks of life do develop increased airway inflammation [58].

A lack of microbial exposure during early life can have persistent effects on immune cell frequencies later in life (FIGURE 2). Early-life vancomycin treatment also results in reduced proportions of Tregs in the colonic LP, a phenotype which is persistent even following vancomycin withdrawal, which may be a contributing factor to the elevated IgE levels in these mice [58], although the mechanism behind this relationship remains to be defined.

Long-term immune changes are also observed following a lack of microbial exposure during early life. The elevated iNKT cell frequencies seen in adult GF mice persist even when the mice are exposed to conventional conditions at 5 weeks of age [28]. Regions of the *Cxcl16* gene are hypermethylated under GF conditions, and exposing 1-day-old mice, but not 5-week-old mice, born to GF mothers to conventional conditions is sufficient to reverse this hypermethylation, and restore iNKT cell levels to that of conventionally housed mice [28]. The mechanisms by which the absence of a microbiota results in persistent epigenetic modifications are to date unknown, but at least in this example, are MyD88-independent [28]. Enhanced *Cxcl16* mRNA expression in the lung epithelium does not appear to mediate the enhanced airway inflammation seen in early-life vancomycin-treated mice [58], suggesting perhaps that epigenetic modifications of this locus do not occur when the microbiota populations are altered, rather than absent.

It is not clear how persistent the effects of an absence of early-life bacteria are on antibody production later in life. One report suggested that conventionalizing 5- to 6-week-old GF mice by co-housing with conventionally housed mice for 3 weeks returned serum IgE levels to normal levels [27]. By contrast, in a separate report where GF mice were conventionalized by similar methods, elevated serum IgE levels were still seen compared with mice raised in conventional conditions [31]. Additionally, mice treated with vancomycin for the first 3 weeks of life still exhibited elevated IgE levels, and susceptibility to airway inflammation even after vancomycin treatment had ceased [58].

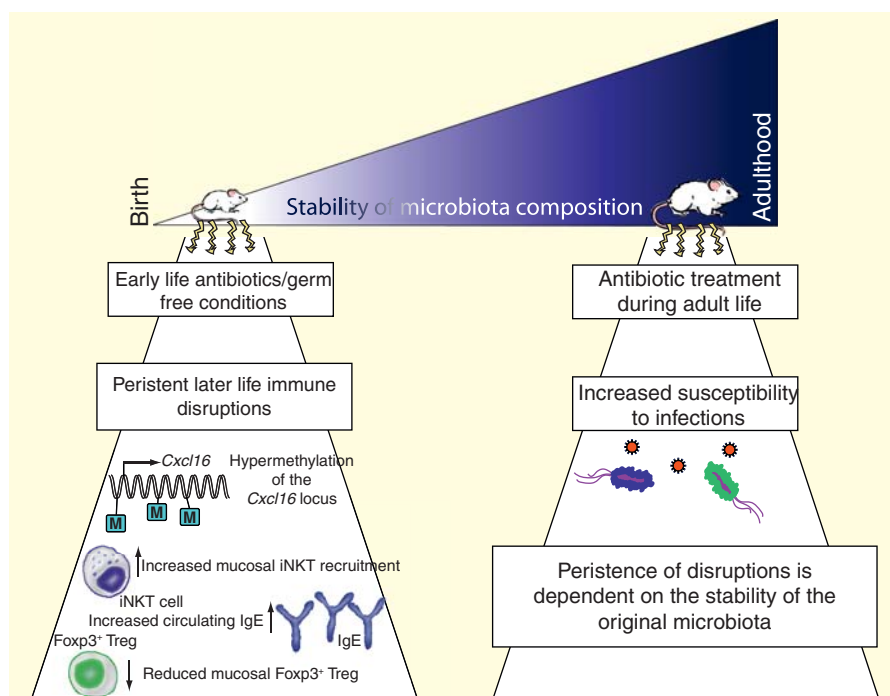


Figure 2. Persistent immune disruptions following absent or reduced microbial signaling during early life.

Several studies have provided evidence for an early window during development, during which time signals from the microbiota are critical for normal immune development. A lack of microbial signaling during early life, or antibiotic-mediated disruption of the microbiota during this time, can have persistent effects on immune cell function into adulthood. Mice born germ-free display elevated incorporation of 5-hydroxymethylcytosine at the *Cxcl16* locus and increased invariant natural killer T cell recruitment to the colonic and lung lamina propria even following conventionalization at 5 weeks of age [28]; circulating immunoglobulin E levels can remain elevated after early-life antibiotic disruption or microbiota absence [31,58], and colonic lamina propria Treg frequencies remain depleted following early-life antibiotic treatment [58]. By contrast, the effects of antibiotic treatment during adult life, after a stable microbiota has developed, may be more limited to the period of treatment. During antibiotic treatment as adults, immune responses to opportunistic pathogens are compromised [38,39,71], leading to increased susceptibility to viral, fungal or bacterial infections, which have been linked to exacerbations in allergic responses [41,42,44]. The persistence of antibiotic-driven microbiota perturbation will be determined by the class of antibiotic used, the timing of administration, host environmental factors and the stability of original microbiota composition.

Antibiotic disruption later in life

Antibiotic treatment in adult mice has also been linked to exacerbations in allergic disease severity. When adult mice (at least 8 weeks old) are treated with a broad-spectrum cocktail of antibiotics, including ampicillin, gentamicin, metronidazole, neomycin and vancomycin for 4 weeks, serum levels of IgE are elevated, along with circulating basophil numbers [31]. IgE appears to regulate basophil numbers in the blood; administration of IgE to Rag1^{-/-} mice (which lack B and T cells) elevates circulating basophil frequencies, and correlations are seen between serum IgE levels and circulating basophil frequencies in human patients with hyperimmunoglobulinemia E syndrome [31]. Antibiotic cocktail treatment also results in increased disease severity to two allergens: after intranasal exposure to extract from the house dust mite *Dermatophagoides*

pteronysinus, antibiotic cocktail-treated mice had greater eosinophilia in BALF and lungs, increased alveolar infiltrates and exaggerated Th2 responses in their mediastinal lymph nodes compared with untreated mice [31]. Additionally, following footpad injection of the cysteine protease papain, antibiotic cocktail-treated mice developed amplified Th2 responses in their popliteal lymph nodes [31], suggesting that the exaggerated allergic responses following this antibiotic treatment regime are not limited to mucosal surfaces.

Following broad-spectrum antibiotic treatment of adult mice, increased trafficking of non-invasive bacteria to the mesenteric lymph nodes by CX₃CR1^{hi} mononuclear phagocytes is seen, suggesting that under normal conditions, the microbiota function to inhibit the migration of these cells [59]. This may be a mechanism by which increased sensitivity to dietary antigens occurs after antibiotic administration, and it will be important for future work to directly test whether this is the case.

Similar to the broad-spectrum antibiotic treated animals, mice lacking MyD88 expression only on B cells also exhibit high serum IgE and basophil levels [31]. Together, these data suggest that signals from the microbiota, mediated by MyD88 on B cells, limit IgE production in adult mice. After broad-spectrum treatment to drastically reduce intestinal bacteria numbers, the inhibition of IgE is reduced, and exaggerated allergic responses ensue. Whether the effects of antibiotic treatment in adults on allergic disease development are persistent after antibiotic administration is stopped, as they appear to be after early-life antibiotic treatment [58], or are limited to the period of antibiotic exposure, perhaps by enhancing susceptibility to infections, has yet to be explored (FIGURE 2).

Quality of bacterial signaling

In many situations, the quality of signals from the microbiota, rather than the total bacterial load, has been shown to influence immune responses in the steady state or in murine disease models. Mice treated with either ampicillin, metronidazole, vancomycin or neomycin all harbored substantial shifts in fecal populations compared with untreated mice [38]. Following infection with A/PR8 influenza virus, however, only neomycin-treated mice had reduced numbers of influenza-specific CD8⁺ T cells [38], suggesting that neomycin-sensitive bacteria are important for priming adaptive immunity to this pathogen. Similarly, vancomycin treatment, yet not streptomycin treatment, during the early period of life results in enhanced airway inflammation in an ovalbumin-driven model of experimental asthma [57]. Human studies have also revealed that different classes of antibiotic present different risks. One study found no association between sulfonamide administration and asthma [49], whereas the use of macrolide [60] or cephalosporins [52,60] has been marked as a predictor of later asthma development, suggesting that shifting the microbiota in specific ways differentially affects the potential for disease development.

Individual species within the microbiota differ in their ability to stimulate immune responses, and the frequency of specific motifs within their DNA correlates with their capacity to

activate LP dendritic cells (DCs) [61]. The composition of the microbiota will dictate the production of metabolites which can alter immune responsiveness [62], and the composition of PAMPs which differentially signal through TLRs. Signaling through TLRs has been shown both to limit the differentiation of Tregs, in the case of TLR9 [26], or promote Treg differentiation, in the case of TLR2 [63]. Thus, a microbiota rich in ligands for a certain TLR could alter the regulatory capacity of the host immune system. Although human data are contradictory, several studies have correlated TLR polymorphisms with serum IgE levels and asthma development [64].

It should be noted that both the absence, as well as the presence of specific bacterial groups within the microbiota dictates the resulting immune system activation status, as specific components of the microbiota are also crucial for limiting arms of the immune response [31,63].

Systemic effects or local microbiota effects?

It has been proposed that immune responses between mucosal surfaces, such as the intestines and lung, are linked, by an as-yet unclear mechanism [65,66]. A major challenge for examining the effects of antibiotic treatment is to distinguish between the systemic effects of the intestinal microbiota, versus local impacts of the respiratory microbiota. The presence of a respiratory microbiota in healthy human lungs has been demonstrated by culture-independent techniques [67,68], although challenges in analyzing these populations include the low density and the potential for contamination from the oral microbiota. It is unclear how stable these communities are in healthy lungs, and whether a respiratory microbiota is present in laboratory mice. Because of the difficulty of analyzing respiratory microbiota populations, few studies have assessed how antibiotics given orally affect these communities, and to what extent these communities can affect allergic airway disease. Oral antibiotics could systemically affect microbial communities if they are well absorbed by the gastrointestinal tract, or if they are administered in the drinking water of mice there is the potential they could be inhaled. Ampicillin, metronidazole, neomycin and vancomycin given in drinking water to mice alter bacterial communities found in nasal washes to different extents [38].

Although evidence at this stage does not determine causality, the abundance and composition of microbes in the upper bronchi of humans has been shown to correlate with the occurrence of asthma [67,69]. Healthy lungs are dominated with Bacteroidetes, particularly *Prevotella* species, in contrast to asthmatic lungs, in which two separate studies have shown Proteobacteria species to be associated with an asthmatic phenotype [67,69]. How these communities are affected by oral antibiotic treatment, and whether their local effects are a contributing factor to asthma development and exacerbations will be an important area for future focus.

There is also ample evidence, both from allergy models and infectious studies, of the intestinal microbiota having system-wide effects on immune responses at systemic mucosal surfaces, including the lung, for which several mechanisms have been

proposed (FIGURE 3). In mice, oral antibiotic treatment results in reduced adaptive immunity to an intranasal influenza virus infection, in terms of reduced frequencies of CD4⁺ and CD8⁺ virus-specific T cells in the lung, and decreased systemic antibody responses [38]. The authors propose that reduced TLR signaling in antibiotic-treated mice lowers the transcription and translation of pro-IL-1 β and pro-IL-18 [38]. Thus, following viral activation of inflammasomes, there is less pro-IL-1 β and pro-IL-18 available for cleavage to their active forms [38]. The reduced proinflammatory cytokine signalling may result in less DC migration to the mediastinal lymph nodes, reducing the priming of adaptive antiviral immunity [38]. It is still unclear, however, whether it is reduced TLR signalling specifically in the intestines, or in the respiratory tract that impacts lung antiviral immunity.

In many of the models whereby the absence of microbiota signaling results in exaggerated allergic symptoms (early-life antibiotic treatment in mice [57,58], adult antibiotic treatment in mice [31], MyD88-deficient mice [31], GF mice [27,31], heightened serum IgE levels are seen. These heightened IgE levels appear to contribute to the development of basophil precursors in the bone marrow, promoting their surface expression of the IL-3R subunit CD123, thereby enhancing their proliferative capacity, resulting in elevated circulating basophil numbers [31]. Additionally, IgE can cause aggregation of the Fc ϵ R1 on mast cells independently of the presence of antigen, which can promote mast cell survival, histamine content and cytokine production [70].

The function of neutrophils extracted from the bone marrow of broad-spectrum antibiotic-treated mice is also altered compared with those from untreated mice; neutrophils from antibiotic-treated mice are impaired in their ability to kill the bacterial pathogens *Streptococcus pneumoniae* and *Staphylococcus aureus* in *in vitro* assays [71]. Peptidoglycan, derived from the microbiota is present systemically in the sera of untreated animals, and following antibiotic treatment, is reduced [71]. Recognition of peptidoglycan by Nod1 is required for normal bacteria killing of neutrophils, and in the absence of the microbiota, administration of Nod1 ligands is sufficient to restore neutrophil function [71]. It is as yet unknown whether it is the translocation of peptidoglycan, or other microbial products or metabolites, across the epithelial mucosa into systemic circulation that are able to inhibit IgE production in conventional mice.

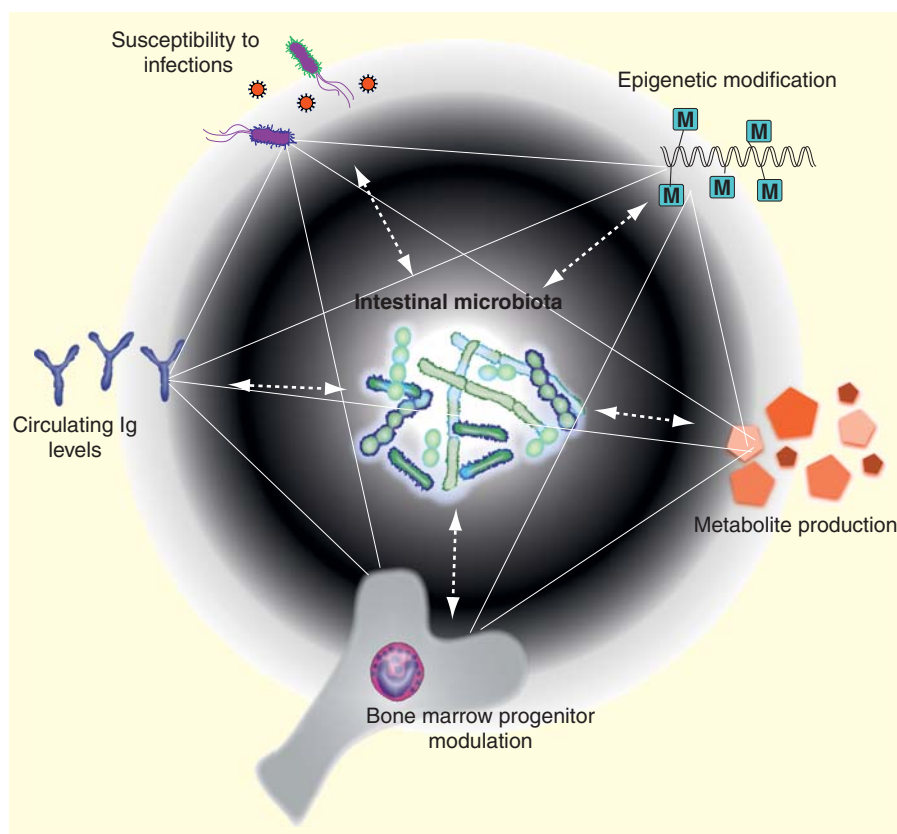


Figure 3. Systemic effects of intestinal microbiota disruption. Several mechanisms have been described by which the intestinal microbiota can regulate immune responses at systemic sites. Signals from the microbiota appear to regulate bone marrow hematopoiesis [31], immunoglobulin secretion [27,31,57,58], susceptibility to infection [38,39,71], epigenetic modifications [28] and metabolite production [62]. Adding to the complexity, each of the listed factors will impact on each other, and on the composition of the microbiota. A multifaceted approach is required to generate a more complete understanding of the immune processes regulated by the microbiota.

The microbiota ferment dietary components to produce a variety of epigenetically active metabolites such as folate and short-chain fatty acids (SCFAs) [62]. It has therefore been proposed that early-life modulation of the microbiota alters the metabolomic profile of the host, causing epigenetic modifications that can alter disease phenotype later in life [72]. Vancomycin-treated mice have a much altered metabolic profile [73], which may be in part responsible for the increased susceptibility to allergic airway inflammation seen following its administration [57,58] through as yet uncharacterized pathways. SCFAs derived from components of the microbiota can stimulate TGF- β production by epithelial cells [24], which may contribute to Treg differentiation at mucosal sites, and reduce inflammatory responses. SCFAs can bind to the receptor Gpr43 (widely expressed on immune cells, epithelial cells and in adipose tissue), and mice deficient in Gpr43 develop exacerbated airway inflammation following ovalbumin-driven experimental asthma induction [74], supporting a role for SCFAs in the prevention of allergic inflammatory responses.

A comparison of histone deacetylase and histone acetyltransferase activity in the peripheral blood mononuclear cells of

asthmatic and non-asthmatic children, revealed higher net histone acetylation in asthmatic subjects, as well as a correlation between cellular acetylation and AHR severity within asthmatic subjects [75]. Although the nature of this study cannot determine causality, the ability of the microbiota to mediate epigenetic modifications early in life will be an important area for future investigations. Taking these studies together, it is clear that the effects of antibiotic treatment on the intestinal microbiota are not limited to the intestine, but have system-wide effects, the mechanisms of which are beginning to be described.

Probiotic strategies for allergy prevention & treatment

Despite the adverse effects of antibiotics on the microbiota, leading to an increased risk of allergy development, antibiotics will continue to be essential tools for controlling bacterial infections. Many studies have therefore investigated whether the use of probiotics during early life can reduce the occurrence of atopy, a strategy that could potentially mitigate the negative effects of antibiotics.

Human studies have failed to demonstrate a significant reduction in asthma onset following probiotic exposure *in utero* or in early life [76], generally consisting of a mixture of *Lactobacillus* and *Bifidobacterium* species [77–80]. A more protective effect of probiotic administration, however, has been seen in murine experiments. When pregnant mice are given freeze-dried *Acinetobacter lwoffii* bacteria, their offspring demonstrate some protection against ovalbumin-driven airway inflammation, with reduced Th2 cell responses compared with untreated mice [81]. Additionally, oral treatment with *Lactobacillus rhamnosus* [82,83], *Lactobacillus reuteri* [84], *Lactobacillus paracasei* [85] or *Bifidobacterium lactis* [82], intranasal administration of *Bifidobacterium longum* [86] and intragastric delivery of *L. rhamnosus* [87] have been associated with protection in murine allergy models. In some cases, this has been demonstrated to be dependent on the administration of live bacteria [84], and there have been associations with induction of regulatory responses following bacteria administration [83,85,88,89], but largely the mechanisms of protection remain unclear.

It is likely that the microbiota act co-operatively to achieve immune modulation, thus administering single species of bacteria to patients may not be the optimal strategy. Whole fecal transfers have been effective in the treatment of recurrent *Clostridium difficile* infections [90]. Approaches that transfer complex functioning communities are likely more stable, and have a greater ability to colonize and have a persistent effect on immune homeostasis [91,92], in contrast to treating with a single species that needs to be repeatedly administered to confer an effect.

In humans, a number of factors, including host genetics, existing microbiota and environment will determine to what extent administered bacteria will be able to colonize, and modulate immune cell phenotypes. It is possible that the effects of probiotic administration will only be revealed in situations when severe microbial dysbiosis has occurred, and it will be interesting to focus on whether probiotics can reduce the risk

of allergy onset in the context of heavy antibiotic use in early life.

Expert commentary & five-year view

Human epidemiology data have been revealing, but it is murine experiments that have solidified the link between antibiotic perturbation of the microbiota and exacerbated airway inflammation. The intestinal microbiota and the mammalian immune system have co-evolved for millions of years, and it is clear that in a healthy host, bi-directional regulation between these components exists [93], and moreover, these interactions depend on a host-specific microbiota [94]. Disrupting this delicate balance disturbs immune homeostasis and in some individuals may result in development of allergies, which appears to be dependent on the timing of microbiota disruption, the extent of the perturbation and the stability of the original microbiota.

In the past decade, our understanding of the microbiota has improved tremendously, due to genomic, transcriptomic, proteomic and metabolomic advances. A flurry of studies have described associations between the composition of the microbiota and susceptibility to allergic and inflammatory diseases [5], and the field is gradually moving toward describing how the functional capacity of the microbiota, rather than simply the presence of individual species, correlates with disease state [95]. An increasing number of studies have moved from describing associations to confirming that the microbiota can have a direct role in altering immune homeostasis, and several mechanisms by which the microbiota can exert its effects have been described. Validating mechanisms of immune modulation by the microbiota will be challenging, as it is likely that multiple complex interactions exist, that will be dependent on host diet, genotype and age. Additionally, care must be taken in generalizing mechanisms which exist in different experimental settings – it should be kept in mind that the absence of a microbiota during development, antibiotic-depleted and antibiotic-shifted mouse models may present similar phenotypes, yet the pathways by which these phenotypes develop may be starkly different.

It appears that certain classes of antibiotic are associated with a heightened risk of disease development [49,52,60], and if this phenomenon can be directly confirmed, then the prescription of these drugs can be avoided where other lower-risk antibiotics are available.

An important area for future focus will be to characterize those immune disruptions following early-life antibiotic treatment that persist into adulthood, and to confirm what the critical window for recovery of these perturbations is. Probiotic treatment in humans has shown little promise to date in reducing the risk of allergy development, however it is possible that a beneficial effect would be seen, if the correct mix of bacteria are administered at the time of antibiotic-mediated microbiota disruption. The characterization of the production and function of metabolites derived from the microbiota is an area of additional therapeutic promise [62].

Antibiotic disruption of the microbiota has been associated with the onset of many inflammatory, infectious and

metabolomic conditions, in addition to allergic disease development and exacerbation [32]. A more thorough understanding of the mechanisms by which microbial species and their metabolites modulate immune cells will likely reveal additional pathways which can be therapeutically targeted during antibiotic disruption, to mitigate their potentially detrimental effects.

Acknowledgements

The authors would like to thank Stephen A Redpath for artwork, as well as Eric M Brown, Henry J McSorley, Shannon L Russell,

Yanet Valdez and Marta Wlodarska for critical revision of this manuscript.

Financial & competing interests disclosure

Work in the authors' laboratory was funded by a Canadian Institutes of Health Research (CIHR) Emerging Team Grant in partnership with Genome BC and the AllerGen NCE. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- The incidence of allergic disease is increasing in industrialized countries.
- Several factors that are implicated in this epidemiological phenomenon, including the use of antibiotics, involve modulation of the intestinal microbiota composition.
- Antibiotic use, particularly early in life, can lead to a long lasting perturbation in microbiota composition.
- Human studies have identified antibiotic use in the first year of life as a risk factor for the development of allergic asthma, and murine studies have confirmed that modulation of the neonatal microbiota composition by antibiotic treatment can exacerbate the severity of airway inflammation in experimental asthma models.
- Murine studies have shown that the absence or manipulation of the microbiota early in life can lead to persistent alterations in immunoglobulin E release, epigenetic modifications, invariant natural killer T cell recruitment, and Treg frequencies.
- Both human and murine studies have found the class of antibiotic used is important in determining the impact on allergic disease, supporting the hypothesis that it is specific members of the microbiota that are responsible for modulating allergy development or exacerbation.
- The microbiota or metabolites derived from the microbiota could modulate allergic disease through affecting epigenetic regulation, by directly altering mature immune cell function, by affecting immune cell progenitor frequencies or by altering the susceptibility to infections.
- The relative contributions of the respiratory or intestinal microbiota toward allergy development have yet to be determined.
- Probiotic strategies to prevent asthma development have had little success in humans, but have shown some promise in murine studies.
- Further characterization of the mechanisms by which specific components of the microbiota alter immune function will be critical, to better design therapeutic microbiota combinations or microbiota-derived products which can be used to mitigate the adverse effects of antibiotics on allergic disease development.

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