

Review

Antibiotics as Instigators of Microbial Dysbiosis: Implications for Asthma and Allergy

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The human body and its resident microbiota form a complex ecosystem, shaped by both inherited and environmental factors. The use of antibiotics represents an extreme example of environmental pressure and can broadly disrupt the microbial landscape. The benefits that antibiotics have brought to modern medicine are unquestionable; however, their overuse comes with consequences, including the potential for secondary infections by opportunistic pathogens and the spread of antibiotic resistance. Here, we discuss the implications of microbial dysbiosis driven by antibiotics, with a focus on potential links with allergy and asthma. We review epidemiological data on humans, as well as mechanistic studies performed in animal models, and highlight gaps in current knowledge, which if addressed, could drive the design of novel therapeutic strategies and improved clinical care.

Risks Associated with Antibiotic Use

The discovery of antibiotics was one of the greatest milestones in the history of medicine. There is no doubt that antibiotics have saved countless lives to date and marked the dawn of a new era in public health (Box 1). However, what could not have been predicted at the time of their discovery is that not only do antibiotics not selectively target pathogens, but they also affect the viability of nonpathogenic species that shape health and physiology [1]. The importance of this has only recently started to be realized, as the impact of the **microbiota** (see Glossary) on host physiology has begun to be unraveled and the associations between antibiotic use and various diseases have been documented. Other unwitting targets of antibiotics are mitochondria, due to their prokaryotic origin; however, the physiological consequences of these interactions are not clear (Box 2).

The reasons why antibiotic treatment may be detrimental are diverse. First, it may result in disruption of multifaceted microbe–microbe, as well as microbe–host, interactions that are key for the maintenance of homeostasis within this ecosystem. This can lead to the expansion of pathogenic microorganisms, otherwise suppressed by the presence of surrounding commensal populations that may compete for similar nutrient sources (a phenomenon known as ‘colonization resistance’). Also, it may promote the expansion of antibiotic-resistant strains and/or lead to the acquisition of antibiotic resistance by otherwise susceptible bacteria, including pathogens (Box 3). These mechanisms can contribute to irreversible alterations of the microbiota composition [2,3], potentially leading to loss of beneficial and/or over-representation of deleterious species. Finally, reduced stimulation of the immune system by commensal bacteria could undermine the appropriate ‘education’ of the immune system, leading to the development of immune responses against self or innocuous antigens and predisposing individuals to autoimmunity or **allergy**.

Highlights

Antibiotics have proven to be powerful weapons against infectious diseases. However, their major downside is a lack of selectivity: in addition to targeting a given pathogen, they also affect viability of commensal species.

Antibiotic-induced changes in microbiota may have several detrimental consequences, including the increased risk of secondary infections by opportunistic pathogens, spread of antibiotic resistance, or higher susceptibility to allergy and asthma.

Novel approaches are being undertaken to substitute the use of broad-spectrum antibiotics with more-selective drugs. However, understanding of antibiotic-induced alterations in microbe–microbe and microbe–host interactions will be key to design better strategies or supplements for antibiotic treatment.

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Box 1. Brief History of Antibiotics

Although antibiotics are commonly associated with the 20th century, traces of their use in a natural form date back to ancient times. For example, trace amounts of tetracycline were discovered in human bones from ancient Sudanese Nubia (A.D. 350) [116] and Egypt (A.D. 400–500) [117]. In addition, active treatments with natural sources of antibiotics, such as application of moldy bread onto the skin in ancient Rome, Greece, Egypt, China, and Serbia [118], or the use of herbs with antimicrobial activities in traditional Chinese medicine, have been documented [119].

Synthetic Antibiotics: From Dyes to ‘Magic Bullets’

The concept of synthesizing chemical compounds that selectively kill bacteria but not mammalian cells is attributed to Paul Ehrlich, a German researcher recognized as one of the fathers of modern immunology. Ehrlich started his scientific career in the field of histology. During this time, he realized that various cell types and microorganisms have different affinities to dyes, which allow their visualization in histological preparations. Building on his subsequent work on toxins, he envisioned the existence of toxic molecules that would specifically target and kill pathogens but not host cells [119–123].

The efforts of Ehrlich and his team to find these molecules (or ‘magic bullets’, as Ehrlich referred to them) led to the discovery of arsphenamine, a drug introduced on the market under the name Salvarsan for the treatment of syphilis in 1910. Many scientists followed this path, which led to the development of various clinically relevant antibiotics, including Prontosil discovered by Gerhard Domagk, the first antibiotic to revolutionize medicine.

Antibiotics Derived from Microorganisms: From a Lucky Accident to ‘Miracle Drugs’

Many of us are familiar with the anecdote of penicillin discovery. According to this, Alexander Fleming returned to his laboratory after a holiday and noticed the inhibition of bacteria growth in one of his Petri dishes [124,125]. The inhibition was caused by accidentally grown mold, later identified by Fleming to be a certain type of *Penicillium* [126]. Despite this remarkable observation, Fleming was unable to extract the active substance (named ‘penicillin’) produced by this fungus. It was not until 1940 when a group of scientists from Oxford University led by Howard Florey succeeded in extracting and purifying a sufficient yield of penicillin to be tested *in vivo*. Remarkably, as predicted by Fleming’s *in vitro* assays, penicillin showed high therapeutic efficacy in mice infected with *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Clostridium septicum* [127].

Soon after this, when sufficient quantities of penicillin could be produced, the first human clinical trials were launched. Their results demonstrated unequivocally the therapeutic usefulness of penicillin in treating infectious diseases. Penicillin saved thousands of soldiers and civilians during and after the WWII and became a true ‘miracle drug’.

The ‘penicillin story’ inspired others to search for bactericidal agents of microbial origin. During the 1940s, Selman Waksman introduced the first platform for antibiotic discovery. It was largely based on Fleming’s zones of growth inhibition but applied a systematic screen. Waksman’s approach led to the discovery of many antibiotics, including streptomycin, the first effective drug treatment for tuberculosis [128].

Loss of Colonization Resistance

The interactions within microbial ecosystems are diverse, and typically geared towards the exclusion of environmental microbes. Most relevant within a mammalian context is protection against invading pathogens. The microbiota has dual effects in conferring this protection: first, it directly inhibits the growth of pathogenic bacteria as part of the competition for ecological niches; and, second, it stimulates the immune system of the host, enhancing its antimicrobial activity.

Direct inhibition of pathogen expansion by commensal species can be achieved via different mechanisms. One such example is competition for nutrients. It has been demonstrated that commensals inhibit the growth of *Citrobacter rodentium*, a mouse pathogen, by competing for structurally similar carbohydrates [4]. Also, the capacity of the microbiota to take up fucose liberated during inflammatory insults improves host tolerance to *C. rodentium* because fucosyltransferase-deficient mice had a more severe inflammation upon *C. rodentium* infection despite equal bacterial loads. The mechanisms behind this phenomenon might involve fucose

Glossary

Allergy: a chronic condition characterized by inflammatory responses against innocuous environmental antigens, called allergens.

Antimicrobial peptides: peptides displaying capacity to kill certain microorganisms.

Atopy: genetic predisposition to develop allergies.

Contact sensitivity (CS): an allergic reaction to an environmental irritant.

Dendritic cells (DCs): a cell type specialized in antigen uptake and its presentation to lymphocytes, leading to induction of adaptive immunity.

Dysbiosis: imbalance in microbiota composition usually associated with illness.

Germ-free (GF) mice: mice with no detectable microorganisms inside or on the surface of their body.

Innate lymphoid cells (ILC): lymphoid cells lacking antigen-specific receptors.

Lipopolysaccharide (LPS): a component of the outer membrane of Gram-negative bacteria, usually triggering an immune response.

Microbiota: the community of microorganisms inhabiting a given ecological niche.

Peptidoglycan: a polymer of amino acids and sugars, an integral component of bacterial cell wall.

RegIII-γ: an antimicrobial peptide targeting Gram-positive bacteria.

Regulatory B cells: a subset of B cells suppressing immune responses, mainly due to secretion of anti-inflammatory cytokine IL-10.

Regulatory T helper cells (Treg): a subset of T helper cells suppressing immune responses.

Secretory immunoglobulin A (sIgA): a dimeric form of IgA actively transported across the epithelial barrier and released into external secretions.

Short-chain fatty acids (SCFA): fatty acids with fewer than six carbon atoms per molecule, mainly products of bacterial fermentation of dietary fibers.

Specific pathogen-free (SPF) mice: laboratory mice free of certain pathogenic species that could interfere with the validity of the experiment.

T cell receptor (TCR): receptors expressed on the surface of T cells

Box 2. The Influence of Antibiotics on Mitochondrial Function

According to the widely accepted endosymbiotic theory, mitochondria are descendants of a bacterial endosymbiont that merged with a eukaryotic host cell more than 1.5 billion years ago [129]. Given the bacterial origin of mitochondria, it appears plausible that they could be targeted by antibiotics. Indeed, many experimental studies have shown that various antibiotics interact with mitochondrial components [130–132] and that this may lead to mitochondrial dysfunction [133–135]. By measuring extracellular lactate concentration, Duewelhenke *et al.* indicated that various antibiotics may alter mitochondrial energetics *in vitro* when used in high concentrations [133]. Kalghati and colleagues went on to demonstrate that quinolones, aminoglycosides, and β -lactams used in clinically relevant doses led to mitochondrial dysfunction, reactive oxygen species overproduction, and oxidative damage *in vitro* and *in vivo* [134]. Finally, Moullan *et al.* demonstrated that doxycycline induces the repression of mitochondrial gene expression, affecting pathways of mitochondrial protein synthesis, mitochondrial transport, mitochondrial membrane potential, electron transport chain, and ATP synthesis. Strikingly, these effects impacted host physiology across several eukaryotic models, including worms, flies, plants, and mice [135]. Unfortunately, the influence of antibiotic-induced changes in mitochondrial function on susceptibility to secondary infections, autoimmunity, or allergy is not known.

metabolism by bacteria, shown to inhibit the expression of bacterial virulence genes [5]. Another direct mechanism involves the production of **antimicrobial peptides** and toxins. For instance, the probiotic strains *Escherichia coli* Nissle 1917 and *Bifidobacterium* spp. were shown to secrete pathogen-targeting bacteriocins [6,7]. Similarly, thuricin CD produced by *Bacteroides thuringiensis* was able to kill many isolates of *Clostridium difficile* while not affecting the viability of bacteria belonging to most other genera [8] (Figure 1A).

An indirect way through which the microbiota sustains protection against pathogens is via stimulation of host defense mechanisms. For example, bacterial products, such as **lipopolysaccharides** (LPS), **peptidoglycans**, or **short-chain fatty acids** (SCFAs), were shown to

Box 3. Acquisition and Spread of Antibiotic Resistance

Clearly, a given antibiotic does not kill all bacteria. Certain species can resist the effect of the administered drug in two major ways: first, the antibiotic is not able to penetrate the bacterial cell wall and, thus, no effect can be triggered; second, the antibiotic can enter the cell, but bacterial defense mechanisms neutralize its harmful influence.

The first scenario applies to many species, especially Gram-negative bacteria, the cell wall of which comprises the outer and the cytoplasmic membranes. Some antibiotics, such as vancomycin, cannot penetrate the former and, thus, are not effective against Gram-negative bacteria [136]. Others, such as daptomycin, are inefficient at crossing their cytoplasmic membrane due to low levels of the anionic phospholipids required for this process [137].

The second scenario reflects the evolution of active mechanisms aimed at counteracting the effects of antibiotics. These involve reduction of membrane permeability (e.g., by downregulating porin expression), antibiotic efflux through pumps, modification of the antibiotic targets (either by mutation or post-translational modification), and finally, neutralization of antibiotics themselves (via hydrolysis or modification). Similar to many antibiotics (see Box 1 in the main text), these mechanisms are evolutionarily ancient. Antibiotic-resistant genes were discovered in the microbiomes of ancient specimens, which could not have been influenced by modern drugs. These include an 11th century A.D. pre-Columbian Andean Mummy [138], 30 000-year-old permafrost sediments [139], and a cave in New Mexico estimated to be isolated for over 4 million years [140]. Existence of these mechanisms underlines the complex interplay between different microorganisms and their competition for the ecological niche. Acquisition of the ability to produce antibiotics gave certain species an evolutionary advantage to be able to colonize their niche but, at the same time, created a selective pressure for susceptible bacteria to develop resistance to them. Nowadays, with the prevalent use of antibiotics, this pressure is amplified and, thus, the frequency of antibiotic resistance acquisition is higher [141–143]. The ancient character of antibiotics and antibiotic resistance implies opportunities to use metagenomics to discover novel pathways and drugs with better specificities for targeted bacterial species.

In some cases, bacteria that acquired antibiotic resistance can transfer it to others, including potentially pathogenic bacteria. This process, called horizontal gene transfer (HGT), is of major concern in clinical practice, because it is a key contributing factor to the emergence of antibiotic-resistant pathogens, including carbapenem-resistant bacteria (e.g., *Acinetobacter baumannii* and *Pseudomonas aeruginosa*), extended-spectrum β -lactamase-producing *Enterobacteriaceae*, vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, or fluoroquinolone-resistant bacteria (e.g., *Campylobacter* spp. and *Salmonellae*).

recognizing specific peptides in a complex with MHC proteins displayed on the surface of antigen-presenting cells.

Toll-like receptors (TLRs):

receptors recognizing conserved microbial patterns and initiating immune responses against microorganisms bearing them.

Type 1 interferons: a group of cytokines produced by various cell types (especially plasmacytoid DCs); usually associated with viral infections.

Type 1 T helper cells (Th1): a subset of T helper cells effective against intracellular bacteria and viruses, characterized by the expression of T-bet and secretion of interferon- γ .

Type 2 T helper cells (Th2): a subset of T helper cells specialized in response against helminthes; characterized by the expression of GATA-3 and secretion of IL-4, 5, and 13. Deregulated induction of Th2 cells is the main driver of allergic reactions.

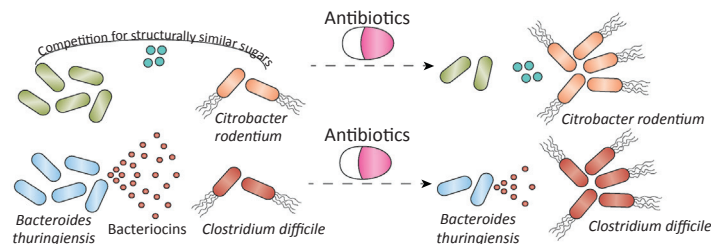
Type 17 T helper cells (Th17): a subset of T helper cells effective against extracellular bacteria and fungi, characterized by the expression of ROR- γ t and secretion of IL-17.

Type 3 innate lymphoid cells

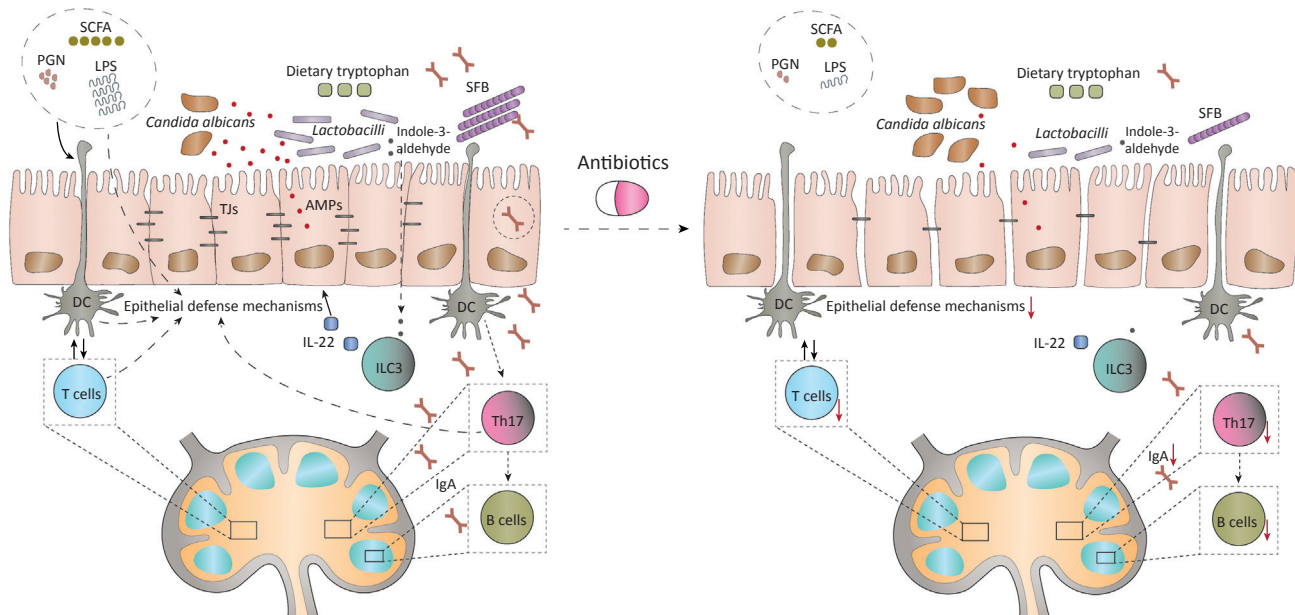
(ILC3): a subset of ILCs secreting IL-17 and IL-22, involved in the initial response to extracellular bacteria and fungi.

$\gamma\delta$ T cells: a subset of T cells with a restricted diversity of TCR recognizing conserved microbial structures.

(A) Direct colonization resistance



(B) Indirect colonization resistance



Trends in Immunology

Figure 1. Effects of Antibiotic Treatment on Colonization Resistance. (A) Different species within microbiota compete for the ecological niche provided by the host directly, such as by competing for structurally similar sugars or by secreting bacteriocins. In some instances, this provides protection of the host against outgrowth of certain species, including pathogens. This phenomenon is known as a 'direct colonization resistance'. Antibiotic treatment may result in interference with these mechanisms, which in some cases leads to expansion of opportunistic pathogens, such as *Citrobacter rodentium* or *Clostridium difficile*. (B) Indirect colonization resistance can be shaped in various ways. These include the increase in epithelial defense mechanisms in response to various bacterial products, stimulation of the innate immune cells (e.g., type 3 innate lymphoid cells; ILC3) by microbiota-derived dietary metabolites and the induction of the adaptive immunity by some commensals (e.g., segmented filamentous bacteria; SFB). Antibiotic treatment may result in the downregulation of these processes and impaired immunity at the mucosal surfaces, leading to expansion of opportunistic pathogens. Abbreviations: AMPs, antimicrobial peptides; DCs, dendritic cells; IgA, immunoglobulin A; IL-22, interleukin-22; LPS, lipopolysaccharide; PGN, peptidoglycan; SCFA, short-chain fatty acid; Th, T helper cell; TJs, tight junctions.

increase epithelial barrier integrity [9,10]. Sensing of the tryptophan metabolite, indole-3-aldehyde, by **innate lymphoid cells** (ILCs) leads to secretion of IL-22, a cytokine orchestrating the induction of epithelial cell defense mechanisms, which suppress the expansion of *Candida albicans*, an opportunistic pathogen [11]. A prime example of commensals influencing adaptive immune responses are segmented filamentous bacteria (SFB), shown to induce **T helper (Th)-17** responses [12] and polyclonal **secretory immunoglobulin A** [13], crucial first-line defense mechanisms of mucosal tissues (Figure 1B).

Loss of these tonic signals observed in **germ-free** (GF) or antibiotic-treated mice may result in impaired capacity to fight invading pathogens. For example, antibiotic-treated mice were

shown to have diminished secretion of **RegIII- γ** , which rendered them susceptible to vancomycin-resistant *Enterococcus* (VRE) infection. Importantly, oral administration of LPS restored the expression of RegIII- γ and enhanced killing of VRE [14]. Also, GF mice failed to clear mouse cytomegalovirus (MCMV). The effect was due to impaired priming of natural killer (NK) cell responses by mononuclear phagocytes, which showed diminished expression of **type I interferons** [15]. Reduced stimulation of innate immune cells may translate into a failure to induce appropriate adaptive immunity. For example, antibiotic treatment resulted in impaired migration of **dendritic cells** (DCs) and IL-1 β /IL-18 secretion, leading to diminished adaptive immunity and reduced clearance of influenza virus in mice. The response was rescued following rectal administration of **Toll-like receptor** (TLR) ligands [16]. Other examples of impaired adaptive immunity in GF or antibiotic-treated mice include: a diminished ability to prime **Th1** cells, a process that can be restored by administration of *Bacteroides fragilis*, whose zwitterionic polysaccharide A (PSA) primes the induction of Th1 cells [17]; reduced priming of Th17 cells in mice whose microbiota lacks segmented filamentous bacteria (SFB) [12]; and, finally, reduced titers of IgA [18] and IgG [19,20].

The local and systemic effects described above have their origins in the reduced sensing of microbial ligands at the interface between the microbiota and the mucosal surface. However, bacterial metabolites may also be distributed systematically and detected in distant organs. For example, the efficacy of *Streptococcus pneumoniae* and *Staphylococcus aureus* killing by neutrophils depends on the recognition of microbiota-derived meso-diaminopimelic acid-containing peptidoglycan in the bone marrow, and antibiotic treatment severely reduces this efficacy [21].

Impaired Education of the Immune System

As previously mentioned, the microbiota has a profound effect on immune responses. Examples described so far point to the role of the resident microbiota in aiding the host immune system to combat infections, predominantly through mobilization of the innate immune system, mounting of effector Th cell responses and antibody production. However, microbiota also has a role in the induction of tolerance towards self and innocuous antigens and its loss may result in the increased risk for the development or exacerbation of autoimmunity or allergy. The rationale behind this notion is that some commensals are potent inducers of **regulatory Th cell responses** (Treg). For example, clusters IV, XIVa, and XVIII of *Clostridium* were shown to promote colonic Treg cell accumulation and their oral administration protected mice from experimental colitis and allergic diarrhea [22]. The ability of *Clostridiales* spp. and *Parabacteroides distasonis* to induce Tregs was also identified by screening of colonic Treg **T cell receptors** (TCRs) [23]. Other species that have been described to induce regulatory T cell subsets include members of the *Lactobacillus*, *Bifidobacterium* and *Bacteroides* genera [24]. Some bacterial ligands that can confer this effect have been identified, such as TLR ligands or SCFAs. For example, GF mice lacking MyD88 and Ticam-1 adapter molecules (and, thus, deficient in all TLR signaling) had impaired development of microbiota-induced Tregs after colonization [25]. However, since MyD88 deficiency also results in the abrogation of IL-1/18 signaling, known to be important in the gut homeostasis, the specificity of TLR ligation in these settings needs to be experimentally confirmed. Later, *Clostridium butyricum*-mediated production of TGF- β , a cytokine that is crucial for colonic Treg induction, was shown to partially depend on TLR2 [26]. Signaling through MyD88 on macrophages was also implicated in microbiota-driven interactions with **type 3 ILC** (ILC3), which resulted in secretion of GM-CSF by the latter and induction of regulatory factors, such as IL-10 and retinoic acid in macrophages and DCs. This, in turn, was important for the induction of colonic Tregs and maintenance of oral tolerance to dietary antigens [27]. Finally, MyD88 expression in Tregs themselves was shown to

be necessary for the maintenance of this subset of cells in the gut mucosa [28]. The importance of SCFAs in driving Treg responses was demonstrated in a series of recent papers. By oral administration of SCFA, three laboratories pointed to their efficacy in Treg induction [29–31] and two of them underlined their protective role in a mouse model of colitis [29,30].

Notably, the protective roles of microbiota-derived ligands, such as TLR agonists or SCFA, were also shown to be Treg independent. Hayashi and colleagues showed that the protective role of *C. butyricum*-derived TLR2 ligands in a mouse model of colitis does not rely on the induction of Foxp3⁺ CD4⁺ T cells but rather on IL-10-producing macrophages [32]. Complementary research underlined that the protective role of propionate (one of the SCFA) in a mouse model of allergic airway inflammation is Treg independent and results from the inability of DCs to drive **Th2** responses [33].

In conclusion, it is well documented that microbiota-derived signals may promote effector immune responses and that loss, or perturbation, of this tonic signaling, for example by antibiotic treatment, may lead to the development of infectious diseases. At the same time, microbiota and their products have the ability to induce anti-inflammatory responses, which holds promise for the development of therapeutic strategies to combat autoimmune diseases and allergies. However, there remains a paucity of mechanistic studies evaluating whether the development and/or exacerbations of these diseases have their basis in loss of these microbiota-derived signals, for example, as a result of antibiotic treatment.

In this review, we further expand on the possible link between antibiotic use and occurrence of atopic diseases. In the first instance, we discuss human epidemiological data, followed by relevant experimental studies on mice.

Antibiotic Use and Epidemiology of Allergic Diseases

Various factors have been proposed to explain the rapid increase in the incidence of allergy in industrialized countries over the past century. These include reduced contact with diverse environmental microorganisms [34,35] or change in dietary habits [36–39]. Regarding the first hypothesis, there are two main facets of exposure to environmental microorganisms: (i) contact with diverse nonpathogenic microbes that do not drive disease or clinical symptoms but modulate the immune system (e.g., through induction of Tregs) and, thus, potentially protect against development of allergy later in life; and (ii) contact with pathogens that cause host infections and drive host inflammatory responses (e.g., Th1/17) that result in clinical symptoms, but at the same time, potentially protect from developing Th2-related diseases (e.g., allergies) later in life.

One of the first reports pointing to the positive correlation between the use of antibiotics and allergy was published in 1998. The authors found a positive association between antibiotic use during the first 2 years of life and occurrence of atopic disorders [40]. This observation was supported by a study published the following year, showing a marked association between antibiotic use and asthma in children. In this work, the odds ratio was higher when antibiotics were used during the first year of life than when used after the first birthday (Table 1). Also, a higher frequency of antibiotic administration resulted in a higher odds ratio for asthma development [41]. Similar conclusions were reached a year later by Droste and colleagues, who showed a positive correlation between antibiotic use during the first year of life and **atopy**. Importantly, after data stratification for the existence of parental hay fever, this association was only significant for children whose parents were atopic [42]. These data implicated a possible role for crosstalk between genetic predisposition and environmental insult in the manifestation of allergy.

Table 1. Summary of the Studies Discussed in the Main Text with a Focus on the Correlation between Antibiotic Use during Early Childhood and the Development of Atopic Diseases

Study	Type of study	Observation	Adjusted odds/hazard ratios (OR/HR)	95% Confidence intervals (CI)
Farooqi <i>et al.</i> [40]	Retrospective	Positive correlation between antibiotic use during the first 2 years of life and atopy	2.07	1.64–2.6
Wickens <i>et al.</i> [41]	Retrospective	Positive correlation between antibiotic use during early childhood and asthma	2.74 (overall)	1.1–6.85
			4.05 (0–12 months)	1.55–10.59
			1.64 (>12 months)	0.6–4.46
			2.27 (1–2 courses)	1.14–4.51
			4.02 (>3 courses)	1.57–10.31
Droste <i>et al.</i> [42]	Retrospective	Positive correlation between antibiotic use and atopy in children atopic parents	2.3 (asthma)	1.1–5.1
			2.8 (hay fever)	1.0–2.6
			1.6 (eczema)	1.0–2.6
von Mutius <i>et al.</i> [43]	Retrospective	Positive correlation between antibiotic use against fever in children and asthma	7.95	6.02–10.50
Illi <i>et al.</i> [46]	Prospective	No association between antibiotic use during early childhood and asthma	1.08	0.59–1.99
McKeever <i>et al.</i> [47]	Prospective	Positive correlation between allergy and extensive antibiotic use (≥ 4 courses) during early childhood	1.99 (asthma)	1.72–2.31
			1.14 (hay fever)	0.88–1.47
			1.04 (eczema)	0.88–1.17
Celedon <i>et al.</i> [48]	Prospective	No association between antibiotic use during the first year of life and having at least one out of three allergic diseases (asthma, hay fever, or eczema)	0.7 (one versus no antibiotic course)	0.4–1.4
			0.9 (two or more versus no antibiotic courses)	0.5–1.4
Celedon <i>et al.</i> [49]	Prospective	No association between antibiotic use during the first year of life and asthma initially diagnosed at the age 2–5 and persisting until the age of 5	1.1 (1–2 courses)	0.8–1.4
			1.3 (3–4 courses)	0.9–1.8
			1.0 (≥ 5 courses)	0.7–1.4
Marra <i>et al.</i> [50]	Meta-analysis (retrospective+prospective)	Positive correlation between antibiotic use during the first year of life and atopy	2.05 (overall)	1.41–2.99
			1.12 (prospective)	0.88–1.42
Kozyrskyj <i>et al.</i> [51]	Prospective	Positive correlation between antibiotic use during early life and asthma	1.86	1.02–3.37
Marra <i>et al.</i> [53]	Prospective	Positive correlation between antibiotic use during childhood and increased risk for asthma	1.11 (1–2 courses)	1.07–1.15
			1.15 (3–4 courses)	1.09–1.22
			1.30 (>4 courses)	1.20–1.41
Ortqvist <i>et al.</i> [57]	Prospective	Positive correlation between antibiotic use early in life (the age of treatment) and asthma	3.97 (0–6 month)	3.66–4.30
			2.74 (6–12)	2.61–2.87
			2.43 (12–18)	2.31–2.55
			2.17 (18–24)	2.04–2.31
			1.9 (≥ 24)	1.77–2.03
Pitter <i>et al.</i> [59]	Prospective	Positive correlation between antibiotic use during the first year of life and asthma development	1.51	1.48–1.54

These are three out of at least six studies [40–45] performed before 2002 that all pointed to the positive correlation between antibiotic use and atopy. Importantly, they were all retrospective studies (i.e., relied on questionnaires). This approach may not be ideal since it could introduce ‘recall bias’ to the study, especially since parents with atopic children may visit medical doctors more frequently and be more likely to report the use of antibiotics. Additionally, a positive correlation between antibiotic use and allergy may be confounded by reverse causation (i.e., when the use of antibiotics is merely the consequence of having allergy, which is misdiagnosed as an infection, due, for example, to similar symptoms, such as cough).

To minimize these issues, prospective birth cohort studies were launched in which children’s medical histories were followed from birth to a certain age. From 2001 to 2004, at least four such studies were performed [46–49], one of which supported [47] and three refuted [46,48,49] a positive association between antibiotic use and atopy. The first meta-analysis of published reports conducted in 2006, taking into account both retrospective and prospective studies, pointed towards an overall positive correlation between antibiotic use and atopy, although the effect in prospective studies was modest [50].

Since 2005, at least 14 prospective cohort studies have been conducted to clarify inconsistencies in the field, ten of which suggested a direct correlation between antibiotic use and atopy or wheezing later in life [51–60], three found no association [61–63], and one found positive association only in the case of macrolides but not cephalosporins, ampicillin, penicillin, trimethoprim, or wide-spectrum antibiotics [64].

One of the problems that may result in failure to identify a statistically significant correlation, is a small sample size. Of note, all prospective studies using large cohort groups (>10 000 subjects) mentioned in this review suggested a positive correlation between antibiotic use and atopy [47,51,53,54,57,59]. Additionally, a recent nation-wide population-based study in Sweden, taking into account 493 785 children, identified a strong positive association between antibiotic use and the development of asthma, with the effects most pronounced during the first 6 months of life and gradually decreasing when antibiotic treatment was initiated later in life [57].

In conclusion, although there is still controversy in the field, most studies point to the positive association between antibiotic use in childhood and the development of atopy later in life, with conclusions firmer in the case of retrospective studies. However, prospective studies using large cohort groups uniformly point to the existence of such a correlation. Future epidemiological studies will be crucial to further define this relationship and delineate the effects of each antibiotic class on the development of allergy later in life. Careful design of these studies, dissociating the role of antibiotics from the viral infections, for which antibiotics are often prescribed, will be important to address this issue. For this, the development of better diagnostic tools (e.g., point-of-care testing) will be a great advantage.

A phenomenon that could be perceived as a counterpart to antibiotic treatment is the exposure to diverse microbial communities, such as those evident in rural areas. Numerous epidemiological studies link exposure to farming environments with protection against allergies [65]. This ‘farm effect’ is in line with the ‘biodiversity hypothesis’, which points to an urbanization-induced decline in the variability of living species, including the microbiota, and, consequently, an increase in allergy [66–68]. Various factors were proposed to be responsible for the observed associations, from which contact with livestock and consumption of unpasteurized cow’s milk appear to be most important [69–73]. Future studies are needed to further delineate these protective effects and unravel the mechanisms they trigger in the host, which will complement

studies on the influence of antibiotics, and contribute to the design of novel strategies to combat allergy and asthma.

Antibiotic Treatment and Allergic Inflammation in Animal Models

It is well documented that GF mice are more prone to mount Th2 responses [17] and are more susceptible to allergic models of diseases [74–76]. These data suggest an ability of microbiota to educate the immune system and prevent the induction of unnecessary responses.

Notably, this appears to be of major importance early in life since recolonization of GF mice during the first 5 weeks after birth was shown to be sufficient to reduce IgE levels to that of **specific pathogen-free** (SPF) mice, while no effect was observed when mice were recolonized after 12 weeks of age [75]. The concept that there is a time frame after birth when microbial colonization is critical is often referred to as the ‘window of opportunity’ and has been extensively covered elsewhere [77–83].

These data match the observations made in epidemiological studies and suggest that antibiotic treatment of mice, especially early in life, exacerbates subsequently induced allergic inflammation. Unfortunately, only a handful of reports have addressed this notion. Bashir and colleagues reported that a 1-week antibiotic treatment of mice beginning at the age of 2 weeks rendered them more susceptible to peanut allergy, with elevated levels of Ara h 1-specific IgE, Th2 cytokines, and the overall systemic anaphylaxis score [84]. In two subsequent studies by Noverr *et al.*, cefoperazone treatment followed by oral gavage with *Candida albicans* resulted in exaggerated *Aspergillus fumigatus* [85] and ovalbumin (OVA)-induced [86] allergic airway inflammation, although no firm conclusion was reached due to the absence of antibiotic-only control groups. Nevertheless, these experiments implicated a deregulated interplay between commensal bacteria and fungi as a possible driver of atopic diseases. This notion was later supported by Kim and colleagues, who observed outgrowth of *Candida parapsilosis* upon antibiotic treatment and correlated its loads with the increased severity of papain-induced airway inflammation [87]. The role of fungal **dysbiosis** in driving exaggerated allergic responses was further confirmed by Wheeler and coworkers, who treated mice with the antifungal drugs, fluconazole or amphotericin-B, followed by the induction of allergic airway inflammation. Interestingly, antifungal treatment did not result in complete eradication of fungal species, but rather in an alteration of their composition, with elevated levels of *Aspergillus amstelodami*, *Epicoccum nigrum*, and *Wallemia sebi*. Mice undergoing the treatment displayed an exacerbated allergic response, with higher levels of eosinophils and conventional DCs in the bronchoalveolar lavage fluid (BALF), increased levels of total IgE and HDM-specific IgG1 in the serum, as well as an elevated frequency of Th2 cells in mediastinal lymph nodes. Importantly, administration of the mix of *A. amstelodami*, *E. nigrum*, and *W. sebi* into naïve mice followed by allergen administration led to the exacerbation of the allergic response, recapitulating the results obtained with the use of antifungal drugs [88].

To further evaluate the influence of antibiotics on allergy, Russel *et al.* initiated vancomycin treatment during pregnancy in mice and continued it for several weeks after birth. This resulted in exaggerated OVA-induced allergic inflammation, characterized by higher numbers of lung eosinophils, elevated concentration of serum OVA-specific IgE, and increased airway hyper-responsiveness and total pathology score. By contrast, treatment of adult mice did not confer this effect [89]. In a follow-up study from this group, the authors observed that perinatal but not prenatal exposure to antibiotics recapitulated this phenomenon, indicating that antibiotic exposure from birth until weaning was crucial in this model. Also, the authors found a positive correlation between antibiotic treatment and the increase in IgE titers in serum and IgE

expression on basophils, and an inverse correlation with the numbers of Tregs, offering plausible mechanisms that could explain the role of vancomycin in the exacerbation of allergic inflammation [90].

Finally, Hill *et al.* showed that antibiotic treatment of adult mice (using a mix of ampicillin, gentamicin, metronidazole, neomycin, and vancomycin) resulted in exaggerated Th2-mediated responses in a papain model of allergic airway inflammation. Interestingly, IgE levels directly correlated with the increase in circulating basophils. MyD88 expression in B cells was important to limit IgE titers and maintain low levels of circulating basophils, indicating a causality behind this correlation. Importantly, depletion of basophils with the use of α -Fc ϵ RI antibodies resulted in the reduced frequency and numbers of Th2 cells, underlining the importance of basophils in driving the allergic inflammation [91]. Overall, these data, together with the work of Russell *et al.* [90], point to the possible role of antibiotic administration in the induction of IgE secretion and the subsequent increase in basophil frequency, which contribute to the exaggerated allergic response [91].

In contrast to the studies described above, a handful of reports indicated the ability of antibiotics belonging to the macrolide class to attenuate allergic airway inflammation. In a study by Beigelman and colleagues, azithromycin administration initiated after sensitization to OVA inhibited inflammatory cell recruitment into the airways, mucus cell metaplasia, and production of Th2 cytokines. Interestingly, azithromycin treatment initiated 1 h after the last challenge conferred similar protection, underlying the potential use of this antibiotic as a therapeutic for asthma [92]. Properties similar to azithromycin were described for clarithromycin [93] and roxithromycin [94]. The results from these studies match observations made in some clinical trials evaluating macrolide therapy for asthma treatment [95–97]. However, its clinical efficacy is controversial and some recent systematic reviews and meta-analyses point to a lack of sufficient evidence to support this hypothesis [98,99].

Finally, a study using a broad-spectrum antibiotic, enrofloxacin, showed its potential to attenuate trinitrophenyl chloride-induced **contact sensitivity** (CS). Enrofloxacin-treated mice developed less pronounced disease, which correlated with the increase in *Clostridium coccoides*, *Clostridium coccoides*–*Eubacterium rectal*, *Bacteroidetes*, and *Bifidobacterium* spp. and with the induction of regulatory subsets of cells, including Tregs, **regulatory B cells**, and IL-10-producing **$\gamma\delta$ T cells**. Administration of microbiota from antibiotic-treated donors into naïve recipients partially recapitulated CS suppression. Similarly, reconstitution of antibiotic-treated mice with microbiota from untreated animals rendered them susceptible to CS [100]. It remains to be investigated whether administration of *C. coccoides* and/or *C. coccoides*–*E. rectal* may have therapeutic potential in this model. Given the ability of *Clostridium* spp. to induce Tregs [22], this appears to be a plausible scenario. Additionally, further delineation of *Bacteroidetes* and *Bifidobacterium* spp. contributing to the observed effect in this model will be of interest. Defining molecular pathways triggered by these beneficial bacteria is the next challenge to undertake.

In conclusion, there are only a few experimental reports evaluating the influence of antibiotics on allergy. With the exception of studies using macrolides, most point to their harmful effect on the severity of this disease. Future studies will be crucial to define the impact of antibiotic use on allergy and to understand the molecular cues behind it. In addition, it will be important to define bacterial species positively or negatively correlating with the severity of allergy in patients and test their immunomodulatory properties *in vivo* using GF animals. Such an approach was recently used by Arietta and colleagues, who identified four bacterial genera (*Lachnospira*,

Rothia, *Faecalibacterium*, and *Veillonella*) that were inversely associated with the occurrence of childhood asthma. Using GF mice, the authors also demonstrated a remarkable effect of oral administration of chosen species belonging to these genera on the reduction of allergen-induced neutrophil influx into the BALF [101]. Using a complimentary approach, Fyhrquist *et al.* identified a positive correlation between the presence of *Acinetobacter* and anti-inflammatory gene expression in healthy but not atopic individuals. Importantly, administration of *Acinetobacter lwoffii* into SPF mice conferred protection in an OVA model of asthma [102]. As our knowledge regarding the characteristics of microbial dysbiosis in allergy and asthma expands [103–106], it will be important to combine bioinformatics analyses of samples from patients with *in vivo* animal models to validate the role of identified candidate species.

Concluding Remarks and Future Perspectives

Discovery of antibiotics was one of the breakthroughs in medicine that marked a modern era in public health. Although there is no doubt that antibiotics have saved countless lives to date, the potential negative consequences of their use have only recently started to be unraveled. These include increased susceptibility to certain infections (due to loss of colonization resistance or impaired education of the immune system) and spread of antibiotic resistance. Potentially, widespread use of antibiotics may also be one of the contributing factors in the rapid increase in autoimmunity/allergy occurrence over the past few decades. In the case of allergy and asthma, the epidemiological evidence indeed supports this notion. Unfortunately, experimental studies assessing this issue are scarce. From the handful of reports that exist, most underline a causative relationship between antibiotic use and allergy. However, caution should be taken when interpreting these studies, because they all have certain limitations. First, studies assessing the impact of individual antibiotic classes are scarce, with cephalosporins (based on cefoperazone [85,86]), glycopeptides (based on vancomycin [89,90]), aminoglycosides (based on streptomycin [89,90]), macrolides (based on azithromycin [92], clarithromycin [93], and roxithromycin [94]) and fluoroquinolones (based on enrofloxacin [100]) as the only ones studied. Systematic evaluation of the properties of each antibiotic class on modulation of allergy will be crucial to bring this knowledge into the clinic (see Outstanding Questions).

In most studies, decreased biomass of microbiota is assumed to confer the observed effect. However, there are alternative explanations. For instance, depletion of only selected bacterial populations might have a predominant role. Conversely, rapid growth of certain strains not targeted by a given antibiotic and having capacity to enhance Th2 immunity might be important. In either case, identification of these strains would be of interest and could be a step towards the design of novel therapeutic strategies to combat allergies.

Also, the dynamics of the microbiota reconstitution following antibiotic treatment and its possible impact on allergy are unknown. In all studies described so far, mice were maintained on antibiotics at least until the day of allergen sensitization. Therefore, microbiota reconstitution could not have occurred before this event. Assessing the efficacy of microbiota recovery and its influence on susceptibility to allergy would indicate whether this is a temporary disturbance of microbiota composition or a long-lasting dysbiosis that promotes allergy later in life.

This approach might better resemble the physiological situation in humans and could contribute to a better understanding of the long-term consequences of antibiotic treatment.

In addition, further research should focus on the development of new generations of antibiotics with a narrowed spectrum of activity. The feasibility of this approach is exemplified by the identification of genus-specific (e.g., *Mycobacterium* [107] and *Staphylococcus* [108,109]) or

Outstanding Questions

What is the influence of each antibiotic class on the susceptibility to allergies? Can differences between classes explain some of the controversies between distinct studies performed in the field? Can these differences be explained by the effect of antibiotics on distinct microbial species?

What is the driving force for the increased susceptibility to allergy upon antibiotic treatment? A temporary reduction of microbial products is generally seen as a driving force of the disease, but how this effect occurs in the long term is unclear. What are the dynamics of microbiota and microbial-derived signals recovery after an antibiotic insult?

Can we use therapies that are safer than antibiotics to treat infectious diseases? Selective compounds displaying bactericidal activity against a narrow spectrum of bacteria are being discovered and alternative therapies (e.g., phage therapy) are being continuously improved and may limit the adverse effects seen with broad-spectrum treatments.

What is the impact of antibiotic-induced changes in mitochondrial function on susceptibility to secondary infections, autoimmunity, or allergy? How can we separate these effects from microbe-dependent effects in future studies?

even species-specific compounds (e.g., colicins and pyocins targeting *E. coli* [110] and *P. aeruginosa* [111], respectively). Using antibiotics displaying such selectivity would reduce the destruction of the commensal microbiota and could protect against adverse effects of antibiotic treatment. In addition, therapies in place of antibiotics should be further developed. A promising example is phage therapy, which has the potential to allow specific targeting of microbial species within a community [112]. At the same time, there is a strong need for the development of better diagnostic tools for viral infections to prevent the prescription of antibiotics for common viral infections during childhood.

Overall, there is evidence that antibiotics may exert adverse effects with potentially negative health consequences. Given the overuse and, sometimes inappropriate, prescription of antibiotics [113–115], more care should be taken by physicians when evaluating the appropriate treatment. Similarly, more research is needed to understand microbiota–host interactions that confer protection against allergies. In particular, combining microbial sequencing data with experimental approaches to study the role of dysbiosis in patients will be of value for the identification of beneficial microbes and the delineation of the pathways they trigger, with the long-term goal to develop new therapeutic strategies to combat allergies.

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