How colonization by microbiota in early life shapes the immune system

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Microbial colonization of mucosal tissues during infancy plays an instrumental role in the development and education of the host mammalian immune system. These early-life events can have long-standing consequences: facilitating tolerance to environmental exposures or contributing to the development of disease in later life, including inflammatory bowel disease, allergy, and asthma. Recent studies have begun to define a critical period during early development in which disruption of optimal host-microbial interactions can lead to persistent and in some cases irreversible defects in the development and training of specific immune subsets. Here, we discuss the role of early-life education of the immune system during this “window of opportunity,” when microbial colonization has a potentially critical impact on human health and disease.

The commensal microbiota, which widely colonize the body, strongly influence the host immune system. Most of the internal and external surfaces of a mammal’s body during adult life—including the gastrointestinal tract, skin, and oral mucosa—are heavily colonized by microbiota, with the largest congregation of organisms contained within the colon. The human body was recently estimated to be composed of $3 \times 10^{13}$ eukaryotic cells and $4 \times 10^{13}$ colonizing bacteria (1). The exposure of the mammal to microbiota begins in utero and expands rapidly after birth. For example, maternal gut bacteria can be detected in the amniotic fluid of pregnant mice (2), and bacteria can be isolated in the meconium from preterm human babies (3). After birth, the composition of microbiota is initially derived from opportunistic colonization by the first types of bacteria to colonize the body, which may be affected by diet, environment, and other factors (4, 5). Maternal and neonatal exposure to gut microbiota can be transmitted through the placenta and amniotic fluid (6) and can have long-term effects (7). The microbiota composition of an adult may therefore in part reflect the history of exposure to microbes and environmental factors in early life. This concept is supported by experiments with mice (5, 8) and familial studies in humans (7) that indicate that the environment in early life may account for at least in part, microbial composition in the adult. These events and their immune consequences are perhaps most important during the first year of life, when the microbial composition is highly variable (8), with progressive stabilization to an adult-like community structure after 3 years of age (9). The instability of the microbiota during early life makes the community structure of microbiota during this time more sensitive to environmental incursions (10). It is now increasingly clear that early-life colonization also coincides with a potentially time-limited period during which the immune system is permissive to microbial instruction.

Further, emerging evidence suggests that the immune influences induced by the microbiota during this early period of life may be durable, creating a “window of opportunity” for proper (or improper) immune education to occur and resistance (or susceptibility) to disease in later life. This Review focuses on these issues and the recent evidence in support of this concept.

Evidence that the microbiota influences the immune system: Lessons from germ-free animal studies

The use of germ-free (GF) animals allowed for development of the concept that the microbiota influences the immune system. In 1885, Louis Pasteur first proposed the generation of animals deprived of common microorganisms to study their role in disease. Pasteur postulated that the body would respond to the lack of commensal bacteria by producing antibodies with greater specificity and intensity, thus representing a potentially critical time in which early-life microbial exposures can have profound influences on the morphological and functional development of the immune system. Neonatal immune cells differ in function from adult immune cells so that they preferentially develop tolerance in response to antigen exposure in a process that still remains to be defined but does not involve an intrinsic property of the T cell but rather the environment in which the T cell develops (26, 27). Neonatal immune cells therefore learn to tolerate the new environment experienced after birth, such as the commensal microbiota. These concepts have been experimentally tested by varying the time of microbiota introduction into GF animals and observing the changes in immune development and/or function.

Although most abnormalities in GF animals are age-independent and can be corrected by introducing commensals at any age (31), the ability to restore a few cellular defects that occur in the absence of microbiota is restricted to a short time interval in early life and thus is
Age-dependent. Consistent with this, GF mice conventionalized during adult life possess a different transcriptional profile in the jejunum and colon in comparison with conventionally raised SPF mice, suggesting that if colonization does not occur during a critical “window of opportunity,” intestinal immune development cannot be fully achieved in the adult (32). This supports the notion that early exposure to microbes may have durable consequences for the host that may extend into adult life. As a corollary, in the absence of appropriate microbial exposures in early life, the immune consequences may elicit irreversible and potentially deleterious implications for the host.

**Age-independent influences**

**T cell subsets**

GF animals exhibit major defects in the development of primary and secondary lymphoid organs—including the GALT, spleen, and thymus—which is associated with a decreased frequency of CD4+ and CD8+ intestinal T cell subsets as well as reduced numbers of intraepithelial lymphocytes that express the αβ T cell receptor (TCR) (33). Normalization of these morphological and cellular defects in αβ T cells can be achieved by reintroducing standard mouse or human microbiota in adult life (33–36). Furthermore, characterization of the TCR repertoire in intestinal tissues at the time of initial commensalization suggests an influx of polyclonal TCR-αβ T cells early in life that lose diversity and revert to an oligoclonal state in the adult (37). To address this, subsequent studies comparing colonized and mono-colonized mice with GF animals have begun to clarify the role of distinct commensal bacterial communities in modulating specific T cell effects. GF mice are typically T helper 2 (Th2)-skewed. A balancing of systemic Th1 and Th17 cells is observed after monocolonization of GF mice with *Bacteroides fragilis* that is due to a single microbial molecule, polysaccharide A (PSA) (38). In another example, segmented filamentous bacteria (SFB) play a key role in intestinal Th17 cell responses and the induction of secretory immunoglobulin A (IgA) (39, 40). Colonization with rat microbiota or monocolonization with SFB, however, only partially restores CD4+ and CD8+ T cell numbers, suggesting that a diverse microbial population is necessary to restore a fully mature immune system in the adult (39). Particular bacterial populations have been linked to the development of specific T effector subsets, such as Th17 cells, a potent source of interleukin-17 (IL-17) cells that plays an important role in the maintenance of mucosal barrier integrity and clearance of pathogens in the tissues. Th17 development is dependent on microbiota and is absent in the small intestine of GF animals. Th17 cells can be restored in GF adult mice through colonization with standard mouse microbiota or SFB but not others (41). Consequently, SFB colonization confers enhanced protection compared with GF animals after infection with the bacterial pathogen *Citrobacter rodentium*, which is a direct result of enrichment of Th17 cells in the small bowel (42).

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**Fig. 1.** iNKT cell colonization is regulated by the microbiota during early life and influences susceptibility to colitis and asthma later in life. (A) iNKT cells migrate from the thymus to the colon during the first weeks of life. Their abnormal accumulation in the colon of GF mice leads to later-life susceptibility to oxazolone-induced colitis. Conventionalization with a standard microbiota or monocolonization with *B. fragilis* (*B. frag*) but not *B. fragilis* deficient in sphingolipids (*B. fragASPT*) during the window of opportunity restores colonic iNKT cell numbers and abrogates the increased susceptibility to colitis. In contrast to conventionalization with a standard microbiota during the first 2 weeks of life that decrease iNKT cell number in a mechanism dependent on CXCL16, monocolonization with *B. fragilis* regulates colonic iNKT cell number in a CXCL16-independent manner that depends on inhibitory sphingolipids from *B. fragilis* that impede iNKT cell proliferation. (B) iNKT cell accumulation in lungs of GF mice lead to later-life susceptibility to asthma. Conventionalization with a standard microbiota but not monocolonization with *B. fragilis* during the window of opportunity restores colonic iNKT cell numbers in a CXCL16-dependent manner and abrogates the increased susceptibility to asthma.
Regulatory T cells (T_{reg} cells) are a major source of IL-10 and capable of recognizing commensal-derived antigens (42), which supports the maintenance of tolerance to intestinal microbes (43). GF mouse colon, but not small intestine, contains decreased T_{reg} cells, which can be normalized through standard conventionalization and monoclonization with certain Clostridium species or a variety of intestinal microbes, regardless of age (44, 45).

**B cells**

B cells are essential to the immune system as a source of the five different isotypes of functionally distinct antibodies: IgA, IgE, IgG, IgD, and IgM. GF mice exhibit normal numbers and phenotypic maturation of B cells. However, they exhibit a general defect in the production of IgA and IgG1 antibodies in mucosal and nonmucosal organs that can be normalized after conventionalization by microbiota (31). Consistent with this, GF mice possess few IgA-expressing B cells in the small intestine. This decrease in IgA is likely due to ILF deficiency. Conventionalization of adult GF mice thus restores ILF numbers and rescues IgA production by B cells in the small intestine (31, 46). This may be through dendritic cells that do not migrate outside of intestinal tissues and retain live microorganisms for prolonged periods of time, which allows them the ability to induce IgA expression in the adult mouse intestine (47).

**Innate lymphoid cells**

Innate lymphoid cells (ILCs) represent lymphoid cells with innate immune function that lack a B or T cell receptor (48). Conflicting results surround our understanding of ILC regulation by microbiota. Natural killer (NK) cells (ILC group I) exhibit reduced phenotypic evidence of activation in nonmucosal organs of GF mice, a process that is orchestrated by mononuclear phagocytes in a Toll-like receptor (TLR)-dependent manner and can be reversed through the colonization of adult mice with a standard microbiota (49). It has also been shown that GF mice display impaired development of Nkp46+ RAR-related orphan receptor–γ (ROreyt)–dependent group 3 ILCs (50) and decreased expression of IL-12 by these cells, an important cytokine in epithelial responses to microbes in the small intestine (50, 51). However, another study has shown that GF mice exhibit no change in the numbers of Nkp46+ ROreyt-dependent ILCs and increased IL-22 expression owing to IL-25 production by IECs (52, 53). In either case, dysregulation of IL-22 expression in GF mice can be normalized by means of standard microbiota conventionalization in adult mice (50, 52). Despite these inconsistencies, it is clear that microbiota regulate ILCs in an age-independent manner.

**Epithelial cells**

IECs are found at the interface between the luminal microbiota and immune system in the mucosa and play an increasingly recognized role in a variety of immune functions (54). One of the functions of IECs is to produce anti-microbial peptides. RegIIIγ is a secreted C-type lectin that regulates the interaction between microbiota and the host through its antibacterial activity on Gram-positive microbes and is mainly expressed by Paneth cells at the base of the crypts of Lieberkühn in the small intestine. In GF mice, IECs exhibit decreased expression of RegIIIγ that can be rescued after colonization with standard microbiota (55) or monoclonization with Bacteroides thetaiotaomicron in small intestine and colon (56). RegIIIγ expression is also regulated by IL-22 derived from Nkp46+ ROreyt-dependent ILC3 (57). Resistin-like molecule β (RELMβ) is a secreted protein highly expressed in the intestinal tract and goblet cells in particular. In GF mice, RELMβ expression by goblet cells is strongly decreased when compared with that in SPF mice but can be rescued after colonization with standard microbiota (58). RELMβ can regulate the expression of inflammatory cytokines through intestinal macrophages influencing host responses to artificially induced colitis (59). Separately, GF mice exhibit a lower expression of major histocompatibility complex (MHC) class II on IECs that can be rescued through colonization with microbiota, an interferon-γ–dependent event (60). In parallel, microbial derived factors such as acetate from Bifidobacterium longum can enhance protective functions of IECs in response to infection (61). Current studies also suggest that conventionalization of GF adult animals leads to substantial changes in gene expression associated with IECs (62). In future studies, it will be interesting to examine the age dependency of these effects and the manner in which they alter epithelial expression of factors well known to play an important role in epithelial cell–immune interactions such as IL-25, transforming growth factor–β (TGF-β), B cell activating factor (BAFF), a proliferator-inducing ligand (APRIL), and thymic stromal lymphopoietin (TSLP).

**Age-dependent influences**

Innate natural killer T (iNKT) cells express an invariant TCR-α chain and a diverse array of TCR-β chains and recognize endogenous and exogenous (bacterial) lipid antigens when presented by CD1d, a MHC class I-like molecule (63). In GF animals, iNKT cells are decreased in peripheral tissues such as the spleen and liver and are hyporesponsive to lipid antigen stimulation, which is normalized by monoclonization with bacteria expressing iNKT antigens in adult mice (64). In distinct contrast, mucosal tissues such as the lung and colon contain substantially increased quantities of iNKT cells when mice are GF in comparison with that observed.
in SPF mice in association with augmented inflammatory responses in experimental models of colitis and airway hyperresponsiveness (AHR) (65, 66). This exaggerated accumulation of iNKT cells and hyperresponsiveness to environmental triggers of iNKT cells that induce colitis can be normalized through colonization of GF mice with standard microbiota, Bacteroides fragilis monocolonization, or treatment with B. fragilis–derived sphingolipid antigens during the first 2 weeks of life but not thereafter (Fig. 1A) (65, 66).

iNKT cell accumulation in the lung and susceptibility to asthma of GF mice can be rescued by colonization with standard microbiota but not by monocolonization with B. fragilis during the first 2 weeks of life (Fig. 1B).

These studies have revealed at least two nonmutually exclusive mechanisms by which the microbiota can modulate iNKT frequency in the colon and lung. Accumulation of iNKT cells in the colon in GF animals is associated with elevated levels of the chemokine ligand CXCL16, whose expression is normalized by conventionalization during this critical 2-week time period in a pathway dependent on microbiota-induced epigenetic changes of the Cxcl16 gene (65). In contrast, normalization of iNKT cell frequency by the human commensal B. fragilis appears to involve an inhibition of iNKT cell proliferation and expansion in early life through B. fragilis–derived sphingolipids that are inhibitory (66). It is particularly interesting that the effects of intestinal B. fragilis colonization is tissue-specific because it is restricted to the colon, strongly supporting the idea that specific microbes and potentially signals can regulate iNKT recruitment and proliferation in distinct organs.

**B cells and IgE**

IgE are antibodies produced by B cells that play a pivotal role in response to allergens and immunity to pathogens. Whereas IgA and IgG1 levels are decreased in GF mice, a recent study showed that GF mice have elevated levels of serum IgE after weaning (~30 days) because of enhanced rates of B cell isotype class switching to IgE in PPs and MLNs (67). Elevated IgE levels in GF animals was associated with exaggerated responses to orally induced systemic anaphylaxis (67). Hyper-IgE levels in GF mice serum are associated with a concomitant increase in IL-4 production in PPs and MLNs and are dependent on CD4+ T cells. Colonization of GF mice with standard microbiota from birth to 4 weeks of age but not thereafter normalizes the IgE levels in adults, which is consistent with a regulation of IgE production by the microbiota that is specific to this time frame (Fig. 2). IgE production in adult mice is dependent on intestinal bacterial diversity in neonates rather than on colonization with specific bacterial species so that a low diversity of microbiota is not sufficient to normalize IgE levels during early life.

**Treg cells in lung and skin**

Exposure to house dust mite (HDM) antigen—which possesses CD1d-restricted antigens for iNKT cells (68), during the first 2 weeks of life, but not thereafter—induces a helios-negative (presumably induced) subset of Treg cells from conventional CD4+ T cells in the lung (69). Consequently, initial HDM exposure during adult life results in increased AHR relative to that observed when HDM exposure first occurs in early life. Mechanistically, the allergen-induced development of the helios-negative Treg cells in early life is dependent on microbiota and involves the expression of the immune checkpoint molecule programmed cell death protein 1 (PD1) on the T cell and PD-L1 on dendritic cells so that early-life blockade of PD-L1 abrogates the expansion of the helios-negative Treg cell and increased asthma.

A similar age-restricted regulation of Treg cells also exists in skin. Colonization of mice with a commensal skin bacterial strain during the first 2 weeks of life, but not thereafter, induces accumulation of activated Treg cells in the skin that are derived from the thymus and which maintain tolerance to commensal-derived antigens in the adult (70). When colonization is initiated in adult mice after the time of weaning, such commensal antigen-specific tolerance is not initiated (Fig. 3). Further, the accumulation of activated Treg cells during this early-life period appears to be specific to the skin and is not observed in the intestinal lamina propria. Antibiotic treatment in neonatal mice can induce long-term modification of microbial composition of the skin and susceptibility to psoriasis development in adulthood, which is associated with increased IL-22–producing γδ T cells (71). Regulation of thymically derived and induced Treg cells by microbiota during early life therefore appears to be tissue-specific, suggesting that even the same cell type (such as Treg) is under the influence of distinct organ-specific mechanisms.

**Epithelial cells**

The establishment of tolerance during the neonatal period can be regulated by non-immune cells. As an example, TLR signaling is specifically down-regulated during the first 2 weeks of life in mouse IECs but not thereafter (72, 73). This renders epithelial cells hyporesponsive to TLR stimuli such as lipopolysaccharide derived from Gram-negative bacteria. This neonatal tolerance is dependent on TLR4 and the mode of birth delivery. Indeed, IECs from cesarean-born neonates do not down-regulate TLR signaling and are more prone to develop epithelial damage, suggesting an important role of specific types of microbial exposure in the development of epithelial tolerance. These studies emphasize the important potential role played by epithelial cells in the tolerance pathways that are established during early life.

**Erythrocytes in spleen**

A small subset of erythrocytes express the transferrin receptor CD71. CD71+ erythrocytes are enriched in spleen from birth to 2 weeks of age and diminished in adult life (74). Their development is independent of microbiota; similar numbers are observed in GF and SPF mice. However, they function in suppressing responses of myeloid cells to pathogens (for example, Listeria monocytogenes) or commensals. In the latter case, CD71 inhibition leads to increased basal expression levels of the inflammatory cytokine tumor necrosis factor in intestinal myeloid cells in...
neonates maintained under SPF conditions. These studies show that not only do microbial exposures regulate immune development during early life, but also that the host maintains critical and specific mechanistic pathways that regulate host response to microbes during this critical period of life.

**Early-life perturbations of microbiota and their relationship to human disease**

Because microbe-immune interactions appear to play an important role in early-life development of the immune system and when disrupted may result in potentially persistent immune abnormalities, it is worth considering whether early-life exposure to microbes may affect later-life susceptibility to disease. There is emerging evidence to support this hypothesis.

Children exposed to farm environments have a decreased risk for the development of allergic disease, possibly through mechanisms that involve the ubiquitin-modifying enzyme A20 (75, 76). Moreover, farming exposure during pregnancy modulates immune responses and protects against asthma in a mother's offspring (77). This protective effect can be correlated with increased Treg cell activity in the infant. Although it is not firmly proven, one of the plausible explanations for the protective effect of early-life farm exposure is the role of microbiota because individuals exposed to a farm environment possess different microbial diversity compared with other lifestyles (78). In an opposite manner, exposure to antibiotics is associated with persistent immune abnormalities that may result in potentially persistent immune abnormalities (79), and their relationship to human disease may play an important role in early-life development during the establishment of the neonatal microbiome (79, 80).

Breast milk contains proteins, maternal bacteria, and nutrients important for the development of the newborn that may also participate in the establishment of the neonatal microenvironment (96, 97). This includes the neonatal immune system given the protective effects of breastfeeding on the development of obesity or IBD in adult life (98, 99). However, the specific maternal factors, including microbes and the mechanisms involved, remain to be identified.

**Concluding remarks**

The mucosal tissues of the host are a highly dynamic system of structures that reside at the interface between the external world and the internal immune and nonimmune tissues. These surfaces are also a critical site of microbial commensalism that plays a critical role in defining the morphology and function of the host and are, like the host that they serve, under the influence of a variety of environmental factors. It is becoming increasingly clear that the ordered establishment of this interface is critically important to the proper development of the immune system and likely the microbiota themselves. Moreover, there has been a recent recognition that a number of the critical immune mechanisms that determine the maintenance of homeostasis and tolerance to environmental exposures at this interface are determined by a set of poorly defined microbial-host interactions that occur during a narrow time frame contained within the earliest days of life. This specifically has led to the notion of a “window of opportunity” in which particular microbial exposures during early life determine specific immune events that are durably imprinted, or not. This time frame correlates with the sucking period of rodents, whose relation to human development remains to be defined. A recent study nicely demonstrates that such early-life influences by the microbiota may even be evident during gestation, in that microbial colonization during pregnancy regulates the number of specific innate immune cells and their activity in the offspring (100). This process is dependent on microbiologically, maternal antibodies specific for microbiota-derived molecules that are present in the milk and transferred to the neonate during the period of suckling. In their totality, these insights have profound implications for human disease by suggesting that disease risk may begin at the earliest days of life, including the antenatal period. Defining the specific details of these events will therefore have great implications for intervention and understanding in the prevention of complex disease mechanisms.

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PERSPECTIVE

Antibiotic use and its consequences for the normal microbiome

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Anti-infectives, including antibiotics, are essentially different from all other drugs; they not only affect the individual to whom they are given but also the entire community, through selection for resistance to their own action. Thus, their use resides at the intersection of personal and public health. Antibiotics can be likened to a four-edged sword against bacteria. The first two edges of the antibiotic sword were identified immediately after their discovery and deployment in that they not only benefit an individual in treating their infection but also benefit the community in preventing the spread of that infectious agent. The third edge was already recognized by Alexander Fleming in 1945 in his Nobel acceptance speech, which warned about the cost to the community of antibiotic resistance that would inevitably evolve and be selected for during clinical practice. We have seen this cost mount up, as resistance curtails or precludes the activities of some of our most effective drugs for clinically important infections. But the fourth edge of the antibiotic sword remained unappreciated until recently, i.e., the cost that an antibiotic exerts on an individual’s own health via the collateral damage of the drug on bacteria that normally live on or in healthy humans: our microbiota. These organisms, their genes, metabolites, and interactions with one another, as well as with their host communities, represent our microbiome. Our relationship with these symbiotic bacteria is especially important during the early years of life, when the adult microbiome has not yet formed.

For 70 years, antibiotics have been a pillar of medicine and are being used worldwide on an enormous scale. In many countries, antibiotic use exceeds one course per capita per year. In 2010, the top seven antibiotic classes were consumed in an estimated 70 billion individual doses, which equates to about 10 pills, capsules, or teaspoons for every man, woman, and child on earth (7), an annual rate that appears to be rising. This magnitude of use is based at least in part on the perception, among both health professionals and the public, that antibiotics are completely safe. We all are aware of mild, self-limited problems, such as rashes and drug reactions, and doctors know about serious but very rare side effects, yet at a functional level, most of us consider these risks so close to zero that they do not usually factor into the equation about use. There also is the cost of antibiotic resistance, but because it predominate affects the community rather than the treated individual, its avoidance does not usually affect clinical judgments about whether or not treatment should be given. Parents would rather have their ill child treated with a drug they see as safe and effective than worry about the impact of that single course on the future of human-kind, and their doctors generally agree.

However, this construct of essentially complete personal safety is illusory. Shortly after antibiotics began to be used to treat ill people and animals, farmers discovered that adding low doses of antibiotics to the food or water of their livestock would promote their growth; the earlier in life the exposure began, the more profound the effect (2). This observation alone provides an important clue that antibiotic exposure affects metabolic development. Antibiotic use has been widespread because it leads to growth promotion and, therefore, increased profit for farmers. But does this massive decades-long worldwide “experiment” on the farm teach us anything about human health? Is it possible that the antibiotics that we give our children early in life to treat their infections—whether severe (uncommon) or mild (very common)—are influencing a critical window in the development of their own metabolism?

In recent years, scientists have been exploring this question, with mostly consistent results. Observational, clinical, and epidemiologic studies focused on young children are providing a growing body of evidence that antibiotic exposure is associated with increased risk for a variety of diseases including obesity, types 1 and 2 diabetes, inflammatory bowel diseases, celiac disease, allergies, and asthma [see (3, 4) for experiments]. Experimental models are providing increasing evidence that these associations are not just correlative but are causal. Studies in mice have found that antibiotic exposure, by disrupting the development of the early-life microbiome, which often causes loss of species and strain diversity (i.e., biodiversity loss), leads to metabolic perturbations that affect adiposity and bone growth and alter normal immunologic development (5, 6).

A variety of evidence indicates that the risks appear greatest for young children (7). Paradoxically,
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Thomas Gensollen, Shankar S. Iyer, Dennis L. Kasper and Richard S. Blumberg (April 28, 2016)
Science 352 (6285), 539-544. [doi: 10.1126/science.aad9378]