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Neonatal gut microbiome and immunity

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Early life is a critical time window for the neonatal gut to be progressively populated with different bacterial species that collectively promote gut maturation. A fully developed and healthy gut microbiome in neonates is an important driver for the development of other aspects of health. Unlike the relatively stable gut microbiome in adults, the developing gut microbiome in neonates exhibits higher plasticity and adaptability. This also underscores the unique window of opportunity for intervention or preventive measures to improve long-term health through modulations of the gut microbiome in early life. Better understanding of the neonatal gut microbiome — how it arises and how it impacts immune cell development — will help us appreciate the underpinnings of immune-related diseases. Here, we examine recent findings on the neonatal gut microbiome and discuss their implications for understanding this important driver of the maturation of the immune system and immunity against infections in early life.

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Introduction

Emerging studies have unraveled important functions of the gut microbiome in supporting the development of the immune system, host metabolism and colonization resistance to enteric pathogens. These functions of the gut microbiome are even more critical during early life [1–3]. Establishment of symbiosis between the immune system and the gut microbiome, as suggested by more recent studies, heavily influences the susceptibility or resistance to various diseases later in life [4,5]. Because of immaturity of the immune system, neonates are more susceptible to infections. Preterm birth and antibiotic use are associated with increased gut inflammation and enteric infection. Furthermore, recent reports uncovered links between altered gut microbiome and metabolome in early life and increased susceptibility to the development of food allergy, asthma and autism [6••,7••,8], all of which are on the rise in recent decades while the underlying causes remain largely undefined. Conflicting results from multiple recent studies have left the notion of *in utero* gut microbiome still unsettled [9,10]. The neonatal gut microenvironment is distinct from the adult gut and only conducive to the colonization of selective bacterial species progressively. In parallel to the dynamic changes of the neonatal gut microbiome and gut environment, the immune cells in the gut are gradually and sequentially matured and cooperate in a concerted effort to promote host-microbe symbiosis while minimizing unwanted immune reaction to gut bacteria. In the meantime, neonatal immunity against enteric pathogens relies heavily on maternal immunoglobulins. Insights into how the gut microbiome is developed in early life and the associated changes of the gut immune cells in parallel in early life can be harnessed in developing gut microbiome-based therapies as preventive measures to improve neonatal health.

In utero microbial stimulation

The maternal gut microbiome can potentially influence the maturation of the fetal epithelium and immune cells without the localization of live bacteria to the fetal gut, as bacterial products, such as agonists of toll-like receptors (TLRs) or metabolites such as short-chain fatty acids (SCFAs), likely circulate through the placenta to the fetus. Interestingly, a study using an engineered *Escherichia coli* demonstrated transient gestational colonization increases intestinal group 3 innate lymphoid cells (ILC3) and F4/80+CD11c+ mononuclear cells in neonates [11]. In addition, pregnant mice given a high-fiber diet resulted changes in the gut microbiome and increased SCFAs; this in utero stimulation resulted in the suppression of allergic airways disease (AAD) in the offspring possibly through induction of regulatory T cells (Tregs) by the increased amounts of SCFAs [5]. A recent study showed that maternal-microbiota-produced SCFAs, propionate in particular, were sensed by embryonic intestinal epithelium cells via GPRs to facilitate the development of enteroendocrine cells in the fetal intestine [12••]. Collectively, these studies demonstrate in utero and postnatal effects of maternal gut microbiome-derived SCFAs that may be important for maintenance of enteric energy homeostasis and prevention of metabolic syndrome postnatally. These studies highlight the role of in utero microbial stimulation, likely through the maternal gut microbiome, in modulating both prenatal and postnatal immune and intestinal cell development.

Albeit still contentious, emerging evidence suggests in utero bacterial colonization as early as the first trimester in humans [13,14••]. A recent study by Susan Lynch's group presented evidence of the seeding of bacteria in the fetal intestine, which were predominantly Micrococcaceae and Lactobacillaceae [14••]. A viable fetal *Micrococcus* isolate was shown to inhibit IFN-γ production by human T cells *ex vivo*, hence suggesting the ability of these fetal bacteria to promote immune tolerance [14••]. A caveat of this study, however, is the lack of clinical information of maternal health; it remains unclear whether the presence of bacteria in the fetal intestine was due to maternal infection or other inflammatory conditions that would permit localization of bacteria to the fetal intestine. *In utero* bacterial colonization of the fetal intestine might be in line with previous reports of the fetal intestine being populated with dendritic cells and memory T cells that were speculated to be induced by microbial antigens [15•]. Another study by Aagard *et al.* also demonstrated detection of bacterial DNAs in human placental tissues [16]. However, most studies supporting the presence of bacteria in utero thus far relied on culture-independent detection of low-abundance bacterial DNA in placental tissues or amniotic fluid with high background noise and false positives [9,17]. More recently, other studies using more rigorous controls suggest that bacterial DNA detected in placental tissues from previous studies might have been a result of contaminations during the DNA purification process, hence casting doubt on the so-called 'in utero colonization' or 'placental microbiome' [18–20]. There might be pre-existing maternal conditions such as gestational diabetes, urinary tract or vaginal infections that increase the likelihood of microbial translocation and colonization in the placenta. While circulating maternal bacterial products can influence fetal development, in utero microbial colonization might be very unlikely in a healthy pregnancy.

Postnatal bacterial colonization of the neonatal gut

Postnatal colonization of bacteria in the neonatal gut is heavily influenced by the mode of delivery and dietary components. Previously, it was shown that the infant microbiome right after birth was dependent on the delivery mode, but that these changes waned after six weeks, suggesting that age and gut maturation are more important factors that drive the progression of the intestinal gut microbiome in neonates [21,22]. However, more recent studies of the infant microbiome in larger cohorts of infants found that vaginally-delivered infants had higher abundance of *Bifidobacterium* species and reduced abundance of potentially pathogenic *Enterococcus, Enterobacter,* and *Klebsiella* species compared to the gut microbiome in infants delivered through Caesarian section (C-section) [23••,24], which was characterized with enrichment with skin and oral commensal bacteria, such as *Staphylococcus, Corynebacterium, Streptococcus,* and *Propionibacterium*

species [25,26••]. In addition, C-section infants displayed delayed or no colonization of fiber-fermenters Bacteroidetes, as well as lower total microbiota diversity throughout the first two years of life [23••,27]. The delayed maturation of the gut microbiome in these infants may delay the development of immune cells such as Tregs and the maturation of the gut epithelium and mucus layer, hence potentially increasing the risk of inflammatory diseases later in life, such as asthma and food allergies.

Additionally, the microbiome differs vastly between formula-fed and breast-fed infants. Breast milk contains proteins, nutrients, immune factors including immunoglobulins A (IgA) and G (IgG) as well as cytokines, and maternal bacteria that collectively promote the growth and development of newborns [28,29]. Maternal IgG and IgA were reported to cooperate synergistically to dampen CD4+ T cells in the neonatal gut [30]. A recent study also reported an important function of maternal gut microbiota-induced IgG to cross-react with pathogens and protect neonates against enteric *E. coli* infection [31]. The gut microbiome of breastfed infants has less diversity and included higher levels of *Bifidobacterium* species and other bacteria that are capable of metabolizing human milk oligosaccharides (HMOs) found in breast milk [26••,32]. In fact, the earliest infant gut microbiome was enriched in genes that facilitate lactate utilization commonly found in *Lactobacilli* [33]. Different strains of *Bifidobacterium* have different sugar-use profiles, suggesting that differences in HMO availability can promote the colonization by specific *Bifidobacterium* species among different neonates [34•]. Breast milk also has its own microbiome, notably, Proteobacteria, *Staphylococcus,* and *Streptococcus* [35,36], which changes during lactation and differs in mothers depending on the mode of delivery. Furthermore, breastfed infants receive about 27% of their gut microbiome from maternal milk and an additional 10% from the areolar skin during the first year of life [37]. The introduction of solid food is associated with higher bacterial loads and diversity, higher abundance of SCFAs, and a shift in dominance to bacteria belonging to fiber-fermenters Bacteroides and Firmicutes [33,38].

Neonatal gut microenvironment

The neonatal gut environment is distinct from a mature adult gut environment. Notably, traces of oxygen can be detected in the neonatal gut right after birth, while the adult gut is almost completely hypoxic [39]. Recent studies suggested luminal oxygen in the neonatal gut decreases over a developmental window to allow the transition from dominance by the first colonizers, typically facultative anaerobes *Lactobacillaceae* and *Enterobacteriaceae*, to obligate anaerobes, including *Bifidobacterium, Bacteroides,* and *Clostridium,* during the next several months [32]. This transition also coincides with the change from simple sugars/HMOs (derived mainly from maternal milk) to more complex fibers as the main

components of the infant's diet. In addition, colon epithelial cells play a critical role in maintaining hypoxia in the gut lumen through cell metabolism to consume oxygen, thus favoring obligate anaerobes such as *Clostridia* that break down dietary fiber and produce SCFAs [40••]. For example, butyrate-producing *Clostridia* decreases epithelial oxygenation in the colon triggered by peroxisome proliferator-activated receptor gamma (PPARγ) signaling [41,42•]. SCFAs are also a critical energy source for gut epithelial cells [43]. The gut is considered mature and 'adult-like' when there is increased diversity and richness of gut bacterial communities as well as a shift from dominance by facultative anaerobes to dominance by obligate anaerobes (see Figure 1) [44••].

Besides oxygen, many other factors shape the neonatal gut environment to select for bacterial species and strains to thrive, including pH, gut motility, availability of digestive enzymes, as well as host metabolism of complex carbohydrates, proteins, and lipids [45]. For example, breast-fed infants were found to have a more acidic intestinal environment due to the higher abundance of

SCFAs as a result of the colonization of *Bifidobacterium* and *Lactobacillus* [46,47]. This acidification allows for the defense of neonatal intestines against common enteric pathogens while the infant immune system matures.

Additionally, the intestinal epithelium in neonates has greater permeability to soluble antigens compared to adults, while lacking small intestinal crypts or antimicrobial peptide-producing Paneth cells, which can provide protection against enteric infections [48,49]. However, this may be compensated by enterocytes that can produce cathelin-related antimicrobial peptides (CRAMPs) [50]. In addition, at birth, neonates lack microfold (M) cells which are found in gut-associated lymphoid tissue (GALT) of Peyer's patches and mucosa-associated lymphoid tissue (MALT) within the intestine [51]. M cells begin to increase at postnatal day 8 in mice and reach adult levels when mice are 2–3 weeks old (Table 1) [51]. While M cells are important for the passage of microbial antigens to be sensed by dendritic cells for induction of T cells in the gut draining mesenteric lymph nodes [52], M cells are also the entry point for enteric pathogens such as

Figure 1
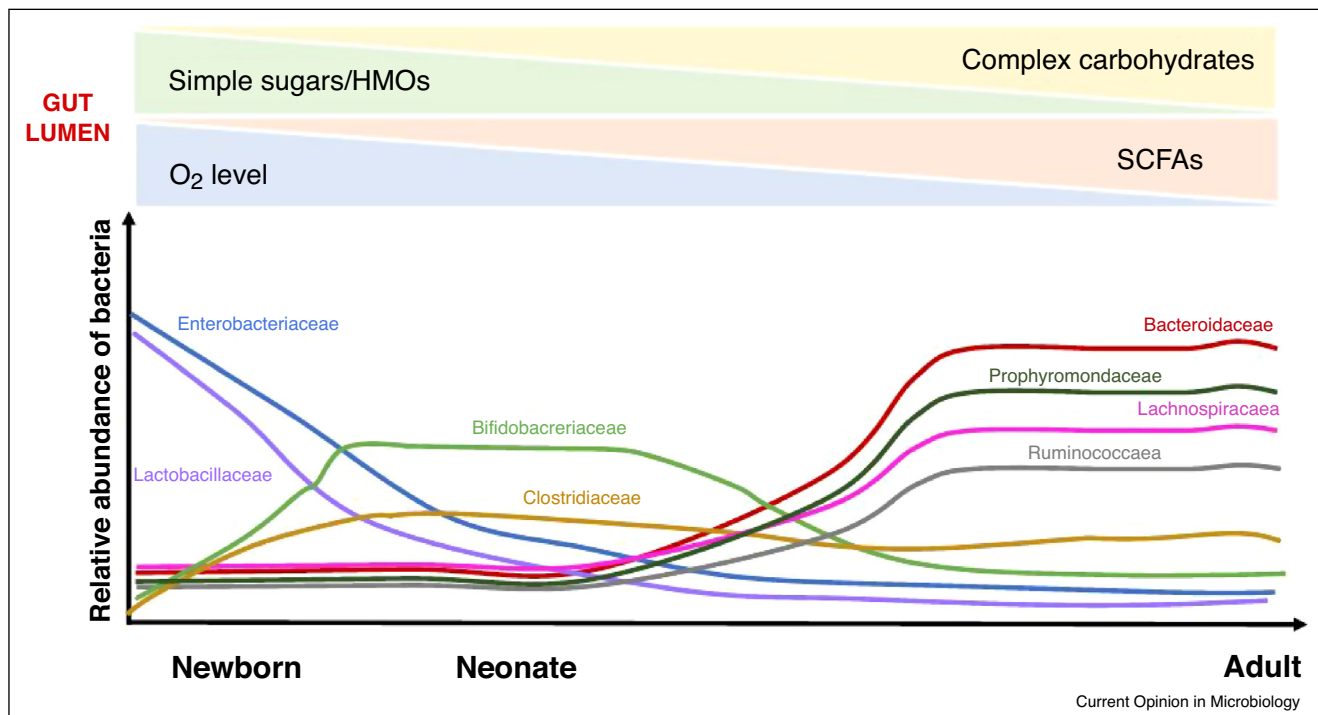
The changing gut lumen environment from early life to adulthood affects the relative abundance of bacteria.

The gut lumen of newborns begins with a higher abundance of oxygen which allows for the colonization of facultative anaerobes Enterobacteriaceae and Lactobacillaceae immediately after birth. Bifidobacteriaceae begin to increase due to the simple sugars and human milk oligosaccharides (HMOs) in the gut lumen from consumption of maternal breast milk. Simultaneously, Clostridiaceae abundance starts increasing which leads to the production of SCFAs like butyrate to induce oxygen metabolism by the intestinal epithelium. This hypoxic gut environment, in addition to the cessation of breast-feeding and switch to solid foods consisting of complex carbohydrates, allows for the colonization of obligate anaerobes Bacteroidaceae, Porphyromondaceae, Lachnospiraceae, and Ruminococcacea which become the most abundant bacteria as adults and collectively establish a stable gut microbiome in the adult gut.

Table 1

Comparison of the roles of the gut microbiome in the development and maintenance of different immune cells between the neonatal and adult intestines

Immune cell type	Neonate	Adult	Role of gut microbiome	Reference(s)
Treg	++	+++	1) SCFAs from Clostridia enhance population and function of Tregs 2) *Bifidobacterium bifidum* induces generation of Tregs	[5,79,61,54••,64,80,81]
CD4 + T cells	+	+++	Segmented filamentous bacteria induce Th17 cells in adult mice	[62,63,82]
B cells	+	+++	1) Peptidoglycan from gram-negative bacteria induces recruitment of B cells to the intestine for maturation 2) In neonates, promotes plasma cell differentiation for induction of IgA and inhibition of IgE production into adulthood	[83••,84,85]
γd T cells	+++	+	1) Promotes gd T cell response in repairing mucosal injury 2) Drives expression of IL-1R and production of IL-17	[86,87•]
DCs	+	+	1) Microbiota are required for DC accumulation 2) Some *Lactobacillus spp.* induce maturation/priming	[75,76]
Macrophages	+	+	1) Recruitment of monocyte precursors to adult gut 2) Transient *E. coli* colonization in maternal gut during pregnancy increases population in neonate gut.	[11,57]
Neutrophils	+	+++	Promotes neutrophil homeostasis in circulation and bone marrow through induction of IL-17 by intestinal ILCs and increasing G-CSF in the plasma in neonates	[88,77••]
NK	+++	+	NK cells are the major mucosal innate lymphoid cells in the human infant intestine but wane over time	[89,90]
iNKT	+	+++	Suppresses accumulation by suppressing chemoattractant CXCL16 in neonates	[58]
Innate lymphoid cells	+	+	1) Influences the transcriptomes and epigenomes of ILC1 and ILC2 cells in adults 2) Transient *E. coli* colonization in maternal gut increases ILC3 in neonates	[11,91]
Microfold (M) cells	+	+++	Unclear role of gut microbiome	[51,92]
Paneth cells	+	+++	1) Increases RegIIIγ expression after weaning 2) Increases cell population	[48,49,93,94]

Salmonella [53]. Hence, the lack of M cells may favor the neonate in two ways – by minimizing both T cell activation and entry of enteric pathogens. Collectively, despite the immaturity of the neonatal immune system, the developing gut appears to be 'designed' in unique ways to protect the neonates from enteric infection while preventing overactivation of gut immune cells.

Effects of gut microbiome on neonatal gut immune cells

The development to gut immune cells (see Table 1) requires microbial signals from the gut microbiome in the neonatal gut [4,5]. A 'weaning reaction,' which is the vigorous immune reaction due to intestinal microbiome expansion during weaning, triggers transient production of high levels of IFNγ and TNFα by T cells which can be mitigated by Tregs that are also induced by the gut microbiome. Inhibition of this process led to increased risk for inflammatory diseases as an adult [54••]. Studies of germ-free mice revealed the requirement for the gut microbiome

in the development of isolated lymphoid follicles (ILFs) and the population of intra-epithelial lymphocytes (IELs) in the gut [55,56]. Additionally, the intestinal macrophages in the neonatal gut are primarily derived from the embryonic yolk sac or fetal liver precursors [57]. Around the weaning period, gut microbiome induces recruitment of monocytes which differentiate into macrophages [57]. Expansion of intestinal ILC3 and F4/80+CD11c+ mononuclear cells as a result of transient colonization of *E. coli* in the maternal gut during pregnancy led to increased expression of epithelial antibacterial peptides as well as metabolism of microbial molecules [11], both critical for compartmentalization of gut commensals within the gut lumen. Furthermore, invariant natural killer T (iNKT) cells exist at low levels in the neonatal colon and start to increase in an age-dependent manner. The gut microbiome suppresses the accumulation iNKT cells in the colon by suppressing the expression of CXCL16 in young mice, an iNKT chemokine [58], thus limiting the potential of iNKT cell-mediated gut inflammation [58].

In the adult gut, the microbiota promotes the generation of colonic Tregs through the generation of SCFAs by predominantly *Clostridium* species [54••,59,60]. Microbiome-induced Tregs are critical to suppress Th2 response in the context of allergies, or Th1/Th17 response in the context of intestinal inflammation such as inflammatory bowel disease [61]. However, it remains still unclear how Tregs are induced in a newborn's gut before colonization of *Clostridium* species. Given the divergent bacterial species that colonize the small intestine and the colon, the role of the gut microbiome in promoting Tregs in the small intestine might be independent of SCFAs. In addition, naïve T and B cells will move to peripheral mucosal body sites from primary lymphoid organs at birth [62]. Interestingly, unlike adults, this homing of lymphocytes to the intestine does not rely on the gut microbiome in neonates [63], but is coincided with a release of thymus-derived regulatory T (tTreg) cells; the latter might be important to promote tolerance towards gut bacteria and dietary antigens during this period [64]. During the postnatal period, neonatal CD4 T cells remain immature due to maternal secretory IgA (SIgA) and Tregs to prevent auto-reactivity [63]. As the mice age, they eventually mature to gain effector functions to maintain the epithelial barrier. This delayed maturation of lymphocytes also occurs in humans, and increased densities of Tregs were found in human fetal lymph nodes compared to adults [65,66]. Taken together, various measures are in place to limit the activation of adaptive immune cells in the neonatal gut to minimize autoimmunity as well as unwanted immune reaction to gut bacteria.

Neonatal immunity

Infection is a major cause of mortality and morbidity in neonates. Early onset sepsis (within 3 days after birth) is predominantly caused by group B *Streptococcus* (GBS) from GBS + mothers. On the other hand, late onset sepsis (between day 7 to day 10 following birth) [67] is commonly caused by bacteria acquired during the birthing process or from the environment, such as GBS and *E. coli*. Of note, in infants with late-onset sepsis, some of the causative bacteria found originated from the gut, most commonly *E. coli* which is a gut opportunistic pathobiont. Maternal milk IgG was recently shown to effectively protect neonatal mice against *E. coli* enteric infection [31], thus highlighting the importance of passive immunity via maternal milk. Enteric infection in neonates can potentially manifest into detrimental sepsis, particularly in more vulnerable infants such as preterm infants [68]. Prematurity of the gut, including alterations of the gut microbiome, in preterm infants also increases the risk of sometimes fatal necrotizing enterocolitis, which is inflammation in the lining of the colon that may lead to perforation of the intestine, translocation of gut bacteria and even sepsis [69]. In fact, Pammi *et al.* found that dysbiosis in preterm infants before the onset of NEC was

characterized by increased Proteobacteria and decreased Firmicutes and Bacteroidetes [70]. Supported by various mouse and human studies, the neonatal gut microbiome has emerged as a critical factor in the resistance or susceptibility to infection.

The gut microbiome is likely an integral source to provide microbial stimulation for priming of innate immune cells in early life (Table 1). Innate immune cells in neonates exhibit characteristics indicative of immaturity due to lack of microbial priming [71]. For example, neutrophils in neonates are impaired in forming neutrophil extracellular traps (NETs), adhesion to the endothelium, phagocytosis, and killing of intracellular pathogens. Antigen presenting cells (APCs) have lower expression levels of MHC-II, adhesion, and co-stimulatory molecules, as well as lower expression of TLRs. In particular, the expression of TLR3, TLR4, and TLR9 are decreased in the epithelium of the neonatal gut [72,73]. The immaturity of these first responders to infection likely contributes to the high vulnerability of neonates to infectious diseases.

In addition, the gut microbiome plays a critical role in conferring colonization resistance against pathogens or opportunistic gut-derived pathobionts. This function of the gut microbiome is even more critical for infants. Group B Streptococcus (GBS) infection in newborns, a common cause of early onset sepsis in neonates, can be prevented by perinatal antibiotic prophylaxis. This antibiotic use however results in transient imbalance of the maternal vaginal microbiome which is important in establishing the gut microbiome in newborns. One study showed perinatal antibiotics induced dysbiosis in the neonatal gut, with a significant reduction in *Lactobacillus* abundance, and that this dysbiosis was correlated with higher risk of earlyctonset sepsis [74]. Some *Lactobacillus* has been shown to help in the priming and maturation of dendritic cells [75] [76], and a decrease in this may cause delayed immune responses in neonates. In addition, studies have shown dysbiosis of the neonatal gut microbiome decreases circulating neutrophils and colonization resistance to gut pathobionts, thus substantially increasing the risk of late-onset sepsis in neonates [77••,78]. Therefore, factors that impede the normal development of the gut microbiome in infants, such as preterm birth, C-section delivery, antibiotic use, and lack of maternal milk-mediated immune protection, will increase the likelihood of potentially fatal enteric or systemic infections in infants.

Conclusions

The developing gut microbiome in neonates is particularly vulnerable to environmental or dietary changes; perturbed gut microbiome in neonates may profoundly increase the risk of neonatal infections, as well as inappropriate activation of immune cells later in life, thus contributing to the development of inflammatory diseases, such as asthma and allergies. The plastic nature

of the neonatal gut microbiome can be leveraged for preventive or intervention measures to promote long-term health by modulations of the gut microbiome, such as utilization of prebiotics and probiotics, or maternal colonization of beneficial bacteria. However, our understanding of the impact of the neonatal gut microbiome on neonatal immune cell development and immunity is still limited. For instance, nutritional immunity has emerged as a critical aspect of host immunity against infection. The gut microbiome likely has a modulatory role in regulating the availability of minerals in the neonate, but up to date our knowledge of nutritional immunity in neonates remains limited. How the neonatal gut microbiome is developed and how it impacts other aspects of health diverges vastly from our understanding of the adult gut microbiome, which has been studied more extensively in various disease contexts. More focused efforts to uncovering all the nuances about the neonatal gut microbiome are needed before innovative and robust gut microbiome-based therapies can be developed to improve neonatal health.

Conflict of interest statement
Nothing declared.

Acknowledgements

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