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# Spatial and temporal key steps in early-life intestinal immune system development and education

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## Keywords

antigen sampling; breast milk; maternal factors; microbiota; neonatal immunity; weaning

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Education of our intestinal immune system early in life strongly influences adult health. This education strongly relies on series of events that must occur in well-defined time windows. From initial colonization by maternal-derived microbiota during delivery to dietary changes from mother's milk to solid foods at weaning, these early-life events have indeed long-standing consequences on our immunity, facilitating tolerance to environmental exposures or, on the contrary, increasing the risk of developing noncommunicable diseases such as allergies, asthma, obesity, and inflammatory bowel diseases. In this review, we provide an outline of the recent advances in our understanding of these events and how they are mechanistically related to intestinal immunity development and education. First, we review the susceptibility of neonates to infections and inflammatory diseases, related to their immune system and microbiota changes. Then, we highlight the maternal factors involved in protection and education of the mucosal immune system of the offspring, the role of the microbiota, and the nature of neonatal immune system until weaning. We also present how the development of some immune responses is intertwined in temporal and spatial windows of opportunity. Finally, we discuss pending questions regarding the neonate particular immune status and the activation of the intestinal immune system at weaning.

## Introduction

The adult gut mucosa is well equipped to tolerate the enteric microbiota while protecting against enteropathogenic infections or food-borne toxins. Barrier-strengthening defense mechanisms promote homeostasis by keeping microorganisms at bay. In the small and large intestine, the secretion of antimicrobial peptides (AMPs) by Paneth cells and enterocytes creates a chemical barrier [1–3]. In addition, goblet cells

generate a mucus layer that forms a physical barrier to withhold luminal bacteria, especially in the colon, where commensal bacteria exhibit the highest density [4,5]. Below this mucus layer, a thick glycocalyx enables access of soluble molecules but not virus or bacterium-sized microparticles to the intestinal epithelial cells (IECs) [6,7]. Moreover, tight junctions seal the epithelial cell–cell contacts and regulate the

## Abbreviations

AMP, antimicrobial peptides; cDC, conventional dendritic cell; CRAMP, cathelicidin-related antimicrobial peptide; CTL, cytotoxic T cells; EGF, epidermal growth factor; EPEC, enteropathogenic *Escherichia coli*; FAE, follicle-associated epithelium; GAP, goblet cell-associated antigen passage; GC, germinal center; HMO, human milk oligosaccharides; IEC, intestinal epithelial cell; ILC3, type 3 innate lymphoid cells; ILF, isolated lymphoid follicle; LOS, late-onset sepsis; MLN, mesenteric lymph node; NEC, necrotizing enterocolitis; pIgR, polymeric Ig receptor; PP, Peyer's patch; SARS-CoV2, severe acute respiratory syndrome coronavirus type 2; SCFA, short-chain fatty acid; sIgA, secretory immunoglobulin A; Tfh, follicular helper T cell; TLR, Toll-like receptor; Treg, regulatory T cells.

paracellular passage of solutes [8]. Finally, the intestinal immune system comprises many mechanisms of surveillance, defense, and tolerance, which provide a tailored response to encountered antigens [9,10]. In the small intestine, the immune system is compartmentalized between organized inductive sites, that is, the isolated lymphoid follicles (ILFs) and Peyer's patches (PPs), and diffuse effector sites, that is, the lamina propria (LP) and the epithelium. In absence of infection, the intestinal immune system promotes tolerance to soluble antigens transported by LP migratory conventional dendritic cells (cDCs) through afferent lymphatics to the draining mesenteric lymph nodes (MLNs) [11–13]. By contrast, PPs do not have afferent lymphatics but are in direct contact with luminal antigens [14]. PPs consist of clustered B-cell follicles forming domes covered by the follicle-associated epithelium (FAE) containing M cells that are specialized in the transcytosis of luminal particulate antigens [15–17]. These antigens are then taken up by subepithelial macrophages and specialized monocyte-derived DCs termed LysoDCs [18]. Upon stimulation, subepithelial LysoDCs migrate to the T-cell zones of PPs to induce an immune response [19]. In addition, subepithelial phagocytes interact with and activate naïve B cells [20]. Due to continuous access to luminal antigens, PPs serve as the main inductive sites for B-cell priming and maturation, leading to their homing to the LP where they differentiate into immunoglobulin A (IgA)-secreting plasma cells [21,22]. Secreted IgA, transported by villus epithelial cells into the lumen via the polymeric Ig receptor (pIgR), plays a pivotal role in homeostasis and during infection, notably by excluding pathogens and neutralizing toxins, but also by regulating the microbiota composition [23–26].

In neonates, the situation largely differs. At birth, the neonatal gastrointestinal tract is rapidly colonized by trillions of bacteria, which initiate the development of the mucosal immune system [27], while breastfeeding provides neonates with factors regulating the microbiota composition and the immune response but also protecting against infections [28]. At weaning, cessation of breastfeeding and the resulting change in diet now containing nutrients with a complex composition induce a diversification of the microbiota and trigger the maturation of the mucosal immune system, notably leading to the production of endogenous IgA. During the last decade, several early-life key age-related events that are essential for the development of optimal intestinal immunity in adults have been described, introducing and supporting the concept of time windows of opportunity [27,29,30]. These events are not only dependent on time but also on the sites where

they occur. Thus, sampling of luminal antigens is tightly regulated both temporally and spatially in early life as is the proximity between the microbiota and the mucosa and the type of luminal antigen encountered, with a clear distinction between the small intestine and the colon [31]. In this review, we first discuss the susceptibility of neonates to infections and inflammatory diseases as an initial evidence that intestinal immunity in neonates is not absent or immature but actually strongly different from that of adults. Then, we briefly summarize the multistep process of microbiota assembly as a key element of intestinal immune education, before focusing on maternal factors that are the primary actors to be involved in neonatal protection and mucosal immune system education. We also describe neonatal defense mechanisms and immune responses of neonates. Finally, we discuss how microbiota is involved in several spatial and temporal key steps of immune education and imprinting. Altogether, this review highlights the microbiota, breast milk factors, and the host's response to environmental changes as three major factors supporting the multistep evolution of early-life homeostasis.

## **Susceptibility of the neonatal host to inflammation and infection**

Clinical studies describe the neonatal immune status as hyperinflammatory, that is, prone to an inappropriate inflammatory reaction. At the same time, the neonatal host is generally more susceptible to infection although exceptions have been described. The molecular mechanisms that determine these age-dependent differences are only beginning to be unraveled but they likely include differences in the organization and gene expression of stromal cells, particularities in the differentiation and adaptation of innate and adaptive immune cells as well as the reduced diversity and density of the neonatal microbiota.

One example for the hyperinflammatory status of the neonatal immune system is necrotizing enterocolitis (NEC), a severe inflammation of the colonic tissue affecting mainly preterm infants [32]. The inflammatory response is thought to be driven by the early bacterial colonization of the intestinal tract and caused by the immature mucosal tissue in preterm infants, unable to cope with postnatal bacterial colonization. Certain pathobionts have been associated with NEC. Their expansion in the neonatal intestine is facilitated by the lack of a dense and diverse microbiota that limits pathobiont growth by competition. Consistently, antibiotic treatment that reduces microbiota diversity is associated with an enhanced risk of developing NEC

(Fig. 1) [33,34]. Indeed, studies comparing the fecal microbiota between preterm infants indicate a lower bacterial diversity and certain alterations of the microbiota composition in infants developing NEC [32,35]. Hence, the use of probiotics (single or combination of beneficial live bacteria), especially the administration of strains of *Lactobacillus* spp. and *Bifidobacterium* spp., has been linked to the prevention of NEC in preterm neonates [36–38]. Probiotics enhance the abundance of beneficial bacteria, reduce pathobiont numbers, and lower the compositional variation. In addition, they exert an anti-inflammatory effect, decreasing the expression of innate immune receptors and increasing expression of the anti-inflammatory cytokine IL-10 in the small intestine [39].

Another example is neonatal sepsis. In the first days after birth, neonatal sepsis, either as early-onset sepsis developing within 72 h after delivery or as late-onset sepsis (LOS) developing between 72 h and 28 days after delivery, is associated with a severe systemic inflammation and represents a leading cause of neonatal death [40]. The commensal bacterium of the maternal vaginal tract *Streptococcus agalactiae* (also called group B streptococci, GBS) and *Escherichia coli* K1 represent typical pathogens linked to neonatal sepsis. In developing countries, systemic neonatal infections with enteric *Salmonella* are also increasingly recognized [41]. Similar to NEC, a low bacterial diversity of the early postnatal microbiota has been associated with a higher risk of developing neonatal sepsis (Fig. 1) [42]. A low microbiota complexity facilitates the persistence and expansion of sepsis-causing pathobionts in the gut. In a mouse model of LOS driven by systemic dissemination of the opportunistic pathogen *Klebsiella pneumoniae*, Singer *et al.* [43] demonstrated that neonatal microbiota composition influences sepsis outcome. Oral vancomycin treatment promoted high abundance of naturally vancomycin-resistant members of the *Lactobacillus* genus and protected against LOS, whereas gentamicin treatment decreased the relative abundance of *Lactobacillus* and aggravated disease severity.

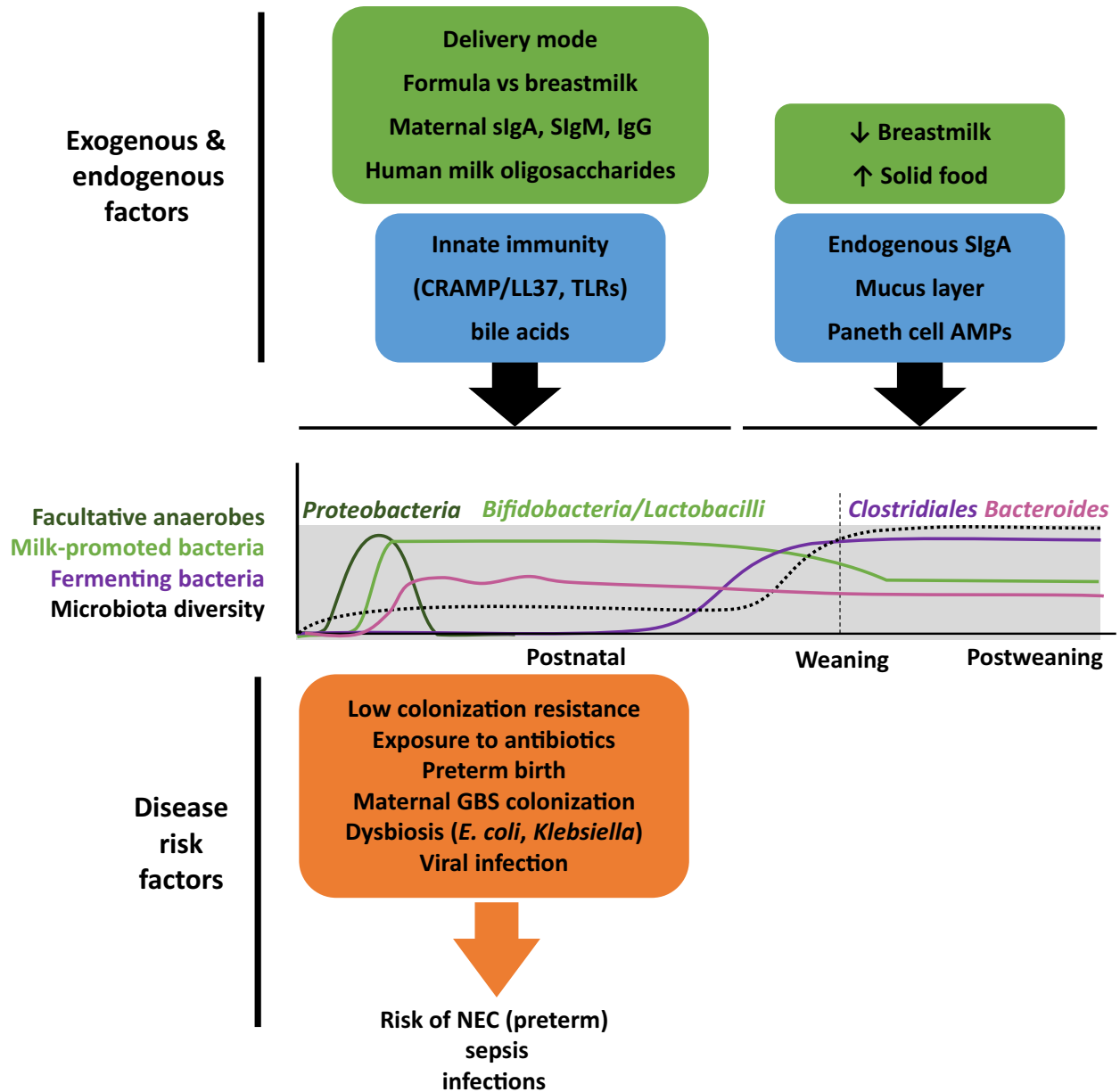
On the other hand, neonates exhibit an enhanced susceptibility to infections, in particular caused by pathogens that target the mucosae of the respiratory and gastrointestinal tract (Fig. 1) [44]. Infections of the respiratory tract, for example by respiratory syncytial virus, or of the gastrointestinal tract by rotavirus, cryptosporidium, enteropathogenic *E. coli* (EPEC), or *Shigella* spp., represent the most prominent causes of infant morbidity and mortality worldwide [44].

The intestinal parasite *Cryptosporidium parvum* persistently infects young mammals, leading to watery

diarrhea [45]. In mice, symptomatic infection is limited to the neonatal period. It increases intestinal permeability, due on one hand to *C. parvum* disrupting enterocyte tight junctions, and on the other hand to the recruitment of monocytes that secrete the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ . *Cryptosporidium parvum* also reprograms LP CX<sub>3</sub>CR1<sup>high</sup> macrophages from expressing high levels of the anti-inflammatory cytokine IL-10 to the pro-inflammatory TNF- $\alpha$ . Infected enterocytes upregulate several chemokines, especially CXCL9 and CXCL10, attracting LP CD103<sup>+</sup> cDCs, which control parasitic infection through secretion of IL-12 p40 and IFN- $\gamma$  [46].

Viral infection with rotavirus still represents one of the leading causes of diarrhea-associated mortality in children under five [47]. In the mouse model of rotavirus infection, neonates exhibit a high viral load and diarrhea, whereas adult mice remain asymptomatic [48]. This age-dependent infection susceptibility is related to the limited expression of Toll-like receptor (TLR) 3 by IECs between birth and postnatal day 10 (P10). TLR3 recognizes double-stranded RNA molecules and its increased expression after P10 dampens the susceptibility to rotavirus infection through induction of a protective type III interferon-mediated antiviral host response [49]. Of note, endoscopic biopsies from healthy human infants below the age of 6 years also revealed lower TLR3 expression levels.

Oral exposure of adult mice to EPEC is not associated with signs of infection. However, neonates infected with EPEC before P10 exhibit the typical attachment and effacement (A/E) lesions with EPEC tightly attached to the apical plasma membrane of IECs [50]. The close adherence to IECs stimulates immunoregulatory protein serum amyloid A3 expression and enhances Reg3 $\gamma$  secretion by goblet cells. Neonatal infection resolves spontaneously during the weaning period when the microbiota composition changes. This phenomenon, known as colonization resistance, is conferred by the highly competitive dense and diverse microbiota of the adult host. Also, some individual species such as bacteria belonging to the *Clostridium* clusters IV and XIVa may play a particularly strong protective role against enteropathogens [51]. Although germ-free or antibiotic-treated adult mice are readily colonized by EPEC, they fail to develop A/E lesions, whereas EPEC microcolonies are present in infected germ-free neonates, indicating that the microbiota is not involved in lesion formation at that age. Thus, lack of colonization resistance facilitates neonatal infection but age-dependent host factors independent of the microbiota play a critical role in EPEC pathogenesis [50]. In the adult murine small



**Fig. 1.** Multistep assembly of the postnatal enteric microbiota and influencing factors. Maternal fecal bacteria represent the primary source of the neonate's early bacterial colonization. The composition of the initial microbiota depends on the mode of delivery, diet (breast milk vs. formula), and environmental exposure (upper left green box). Aerobic conditions initially favor facultative anaerobic species, such as *Escherichia coli*, that subsequently lower oxygen levels and facilitate colonization by microaerophilic and anaerobic bacteria (middle panel). Breast milk constituents such as lactose, sIgA, and HMOs direct the composition of the early human microbiota characterized by Lactobacilli and *Bifidobacteria* (upper left green box). Mucosal host factors influence the postnatal microbiota in an age-dependent manner (Blue boxes). Although the murine neonatal epithelium is characterized by the expression of the cathelicidin CRAMP, TLR5-mediated Reg3 $\gamma$  expression, TLR4 tolerance, and low mucin production, the adult intestine secretes defensins and TLR5 expression is restricted to Paneth cells. Oral acquisition of pathogens such as GBS at birth can lead to systemic infection (sepsis), whereas an inappropriate mucosal inflammation to dysbiotic colonization in the preterm host can cause NEC. At weaning, the uptake of solid food with complex carbohydrates and fibers and the reduction of breast milk-derived factors induce a major change in the composition of the microbiota, favoring the expansion of fermenting bacteria such as members of the Clostridiales phylum (upper right green box). Endogenously generated sIgA, Paneth cell-derived defensins, and the mucus layer stabilize the microbiota composition in the adult host (upper right blue box).

intestine, *Salmonella* targets M cells of terminal ileum PPs to facilitate infection and systemic dissemination [52]. In contrast, they readily invade IECs in neonatal mice, proliferate intracellularly, and disseminate to liver and spleen [53]. *Salmonella* translocates a number of virulence factors via its pathogenicity island (SPI) 1-encoded type 3 secretion system to modulate its intraepithelial compartment and form so-called *Salmonella*-containing vacuoles. Interestingly, enterocyte invasion and intraepithelial proliferation resemble *Salmonella* infection as it occurs in humans. It therefore provides a relevant *in vivo* model to study bacterial effector molecules involved in the *Salmonella*-IEC interaction [54].

Although neonates are highly susceptible to many infectious diseases, there are notable exceptions to the rule. Oral infection with the Gram-negative bacterium *Yersinia enterocolitica* triggers a strong immune response that increases the survival rates in 1-week-old mice as compared to adults [55]. Indeed, neonates exhibit an early recruitment of macrophages and neutrophils as well as an enhanced expression of pro-inflammatory genes in the MLNs upon *Yersinia* infection [56,57]. Besides the strong activation of innate immunity, infected neonates efficiently develop *Yersinia*-specific memory B cells along with IgG1 and IgG2a antibodies [56]. This enhanced protection requires IFN- $\gamma$  secretion in two sequential waves: The early protection depends on its secretion by CD8<sup>+</sup> T cells [58], whereas the late protection is ensured by CD4<sup>+</sup> T cells [56]. Another example where children seem to respond to a pathogen in a more appropriate manner and are able to balance pathogen clearance vs. immune-mediated pathology in a more beneficial way than adults is the infection with the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV2). Children are susceptible to the infection [59] and contain comparable amounts of the virus in the nasopharynx [60]. However, the morbidity and mortality are strongly reduced in the pediatric population even though the development of the multisystem inflammatory syndrome has been documented as a rare complication in children previously infected with SARS-CoV2 [59,61]. Interestingly, children display lower levels and a narrower repertoire of neutralizing antibodies after infection suggesting a more efficient pathogen control at the innate level [62]. Natural cross-reactive antibodies have been suggested to contribute to the successful disease control in children [63]. Further studies need to elucidate the age-dependent differences in the immune response over the course of a SARS-CoV2 infection.

In conclusion, neonates generally present an increased susceptibility to infectious diseases relying on an easier access to the subepithelial tissue and a

reduced or altered antimicrobial host defense. However, they are not devoid of defense mechanisms and can mount specific immune responses following infection with bacterial, viral, or parasitic pathogens. In fact, their ability to mount a strong inflammatory response makes them, under certain conditions, susceptible to inappropriate inflammation and functional organ impairment, as exemplified by NEC and neonatal sepsis. The need to maintain immune homeostasis particularly at mucosal surfaces draws attention to the process of postnatal colonization and establishment of the enteric microbiota.

### Multistep assembly of the microbiota

Although experimental evidence strongly supports the sterile womb hypothesis, the presence of a placental microbiome is still debated [64–66]. During delivery, bacteria are transferred from the mother to the child. Vaginally delivered neonates receive a microbiota closely related to the maternal fecal microbiota [67]. In contrast, the primary microbiota of newborns delivered by caesarian section is closer to skin microbiota (e.g., *Staphylococcus*, *Corynebacterium*) [68]. The initial microbiota exhibits a low bacterial diversity, little difference between the different parts of the gastrointestinal tract and its composition is influenced by environmental and geographical cues [68,69].

The empty niche of the neonatal gut favors colonization by fast-growing, aerotolerant bacteria such as members of the Enterobacteriaceae family that includes *E. coli* or the *Enterococcus* genus (Fig. 1) [69–72]. This initial colonization decreases oxygen availability within the gut lumen and makes it suitable for anaerobic genera, for instance *Clostridium* and *Bifidobacterium*. Thus, initially dominated by members of the phyla Proteobacteria and Actinobacteria, the healthy microbiota diversifies as bacterial species from the Firmicutes and Bacteroidetes phyla expand, turning the tide in dominant phyla. Besides the mode of delivery and its circumstances, the neonatal diet (i.e., breast milk vs. formula) shapes the microbiota composition [73]. Breast milk carries microorganisms from the mother, thus directly transferring bacteria to newborns [74]. In addition, breast milk in humans is highly enriched in human milk oligosaccharides (HMO) that put a selection pressure favoring bacterial species able to catabolize these complex sugars, especially *Bifidobacteria* (Fig. 1) [75]. Moreover, immunoglobulins present in breast milk shape the neonatal gut microbiota and influence intestinal homeostasis until adulthood [76]. Breastfeeding also has an influence on the virome that is established in two sequential steps [77].

First, in parallel with bacterial colonization, viral sequences are detected in newborn stool and their abundance further increases with age. These viruses are mainly prophages that integrate in bacterial genomes, for instance, belonging to *Bifidobacterium* and *Lactobacillus* genera. Secondly, at 4 months of age viruses that infect human cells are increasingly found in fecal samples. However, their abundance is lower in breastfed as compared to formula-fed children.

Weaning represents an important step into microbiota diversification and selection. Weaning is characterized by a change in diet, from breast milk to solid food, restricting breast milk-derived immunomodulatory factors and changing nutrient availability ultimately reshaping microbial communities (Fig. 1) [31,78]. The solid diet favors bacterial species able to metabolize nondigestible fibers and produce short-chain fatty acids (SCFAs), whereas the ‘milk-oriented microbiota’ decreases [72]. In humans, from weaning (at approximately 6 months) until 2–3 years of age, the bacterial diversity increases, and the temporal variability of the microbiota composition gradually decreases until resembling the bacterial composition and richness of adults. In mice, cessation of breastfeeding and weaning occurs at 21 days. This time point marks a major shift in the microbiota composition and initiates the formation of an organ-specific microbiota in small and large intestine. Bacterial richness continues to rise thereafter until approximately 6 weeks of age [69,79–82].

Prolonged maintenance of an enteric microbiota with low microbial diversity may have long-term consequences. The western lifestyle, characterized by high fat low-fiber diet, antibiotic use, few contacts with animals, fewer infections, and birth by caesarian section, has been associated with changes in the enteric microbiota composition leading to a lower bacterial diversity as compared to the one found in inhabitants of rural areas or indigenous tribes [83–86]. The ‘hygiene hypothesis’ suggests a link between western lifestyle factors and an impaired education of the tolerance-inducing arm of the immune system, leading to an increased prevalence of allergies as well as inflammatory and auto-immune diseases [87,88]. The ‘disappearing microbiota’ hypothesis further adds insights on the consequences of the loss of bacterial species [89]. For instance, the prevalence of *Helicobacter pylori*, the main bacterial species found in the stomach, has been greatly declining over the last century. *Helicobacter pylori* has both detrimental and beneficial effects. On the one hand, it can cause gastritis and duodenal ulcers and represents a contributing factor to stomach cancer. Notably, the age of the host at the

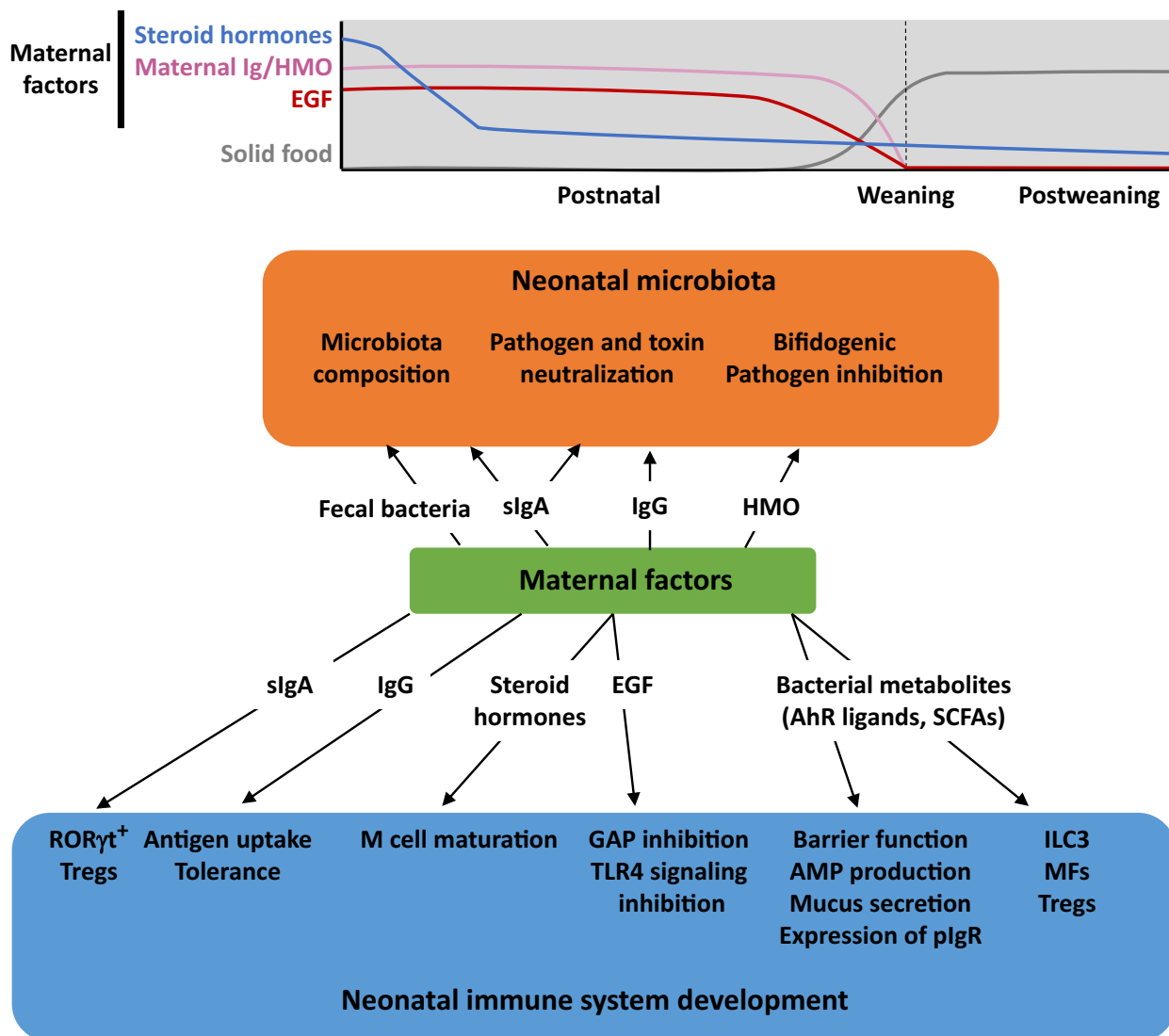
time of initial infection may account for the susceptibility to *H. pylori*-associated disease manifestations [90]. On the other hand, *H. pylori* is linked to increased levels of regulatory T cells (Tregs) promoting immune tolerance [91,92]. Thus, the disappearance of *H. pylori* may affect the host’s health both in a positive and negative way. In addition, individual bacterial species also affect each other by competing for ecological niches. Thus, the disappearance of a commensal bacterium may provide a niche for an enteric pathogen.

### **With a little help from your mother: maternal factors involved in neonatal protection and mucosal immune system education**

#### **The maternal microbiota**

In addition to providing the newborn with beneficial commensal bacteria upon delivery, the influence of the maternal microbiota on the fetus significantly impacts both intestinal innate immunity and energy metabolism in the offspring [93,94]. Using a model of transient colonization of germ-free dams with an auxotroph *E. coli* strain, Gomez de Agüero *et al.* [93] showed that transient bacterial colonization of the dam increases intestinal type 3 innate lymphoid cells (ILC3) and macrophages in the gut of the offspring. It also stimulates expression of genes related to mucosal immunity, such as members of the antibacterial Reg3 protein family, lysozyme, pIgR, and mucus secretion-inducing genes. Altogether, these factors reinforce mucosal integrity and reduce translocation of bacteria to the neonatal MLNs. Mechanisms of protection by the maternal microbiota rely on the transfer of microbial-derived metabolites, such as aryl hydrocarbon receptor ligands, from the mother to the offspring (Fig. 2). This transfer seems partly dependent on maternal antibody-mediated retention and transmission of microbial-derived products during pregnancy but also lactation [93]. Among these metabolites, SCFAs stimulate the free fatty acid G protein-coupled receptors 41 and 43 in the embryo and neonate, promoting the development of thymic and peripheral Tregs [95] and conferring protection against metabolic disorder, obesity, and asthma in the adult offspring [94,96].

Hence, the maternal microbiota during pregnancy and lactation produces factors that reach the offspring and shape intestinal homeostasis by promoting enterocyte maturation, epithelial barrier formation, intestinal innate immunity, and metabolism (Fig. 2).



**Fig. 2.** Maternal factors influencing neonatal microbiota composition and immune maturation. Even prior to birth, bacterial metabolites from the maternal intestine reach the fetus and influence the maturation of ILC3 and macrophages (MFs). Maternal factors such as steroid hormones, breast milk-derived immunoglobulins (Ig), HMO, and EGF are provided during breastfeeding (upper panel) and influence the neonatal microbiota composition (orange box) and mucosal immune maturation (blue box) after birth. Breast milk secretory IgA (sIgA) protects neonates via immune exclusion and toxin neutralization and influences the development of ROR $\gamma$ t<sup>+</sup> Tregs. IgG promotes epithelial FcRn-mediated antigen uptake and immune tolerance. Breast milk HMO are bifidogenic promoting a healthy ‘milk-oriented microbiota’. High EGF concentrations in breast milk inhibit acetylcholine-mediated activation of the GAPs in the small intestine and colon. Decrease of EGF allows temporal GAP-mediated antigen sampling in the colon between postnatal day 10 and 21, which facilitates tolerance induction. Decreasing concentrations of the maternal steroid hormones estradiol and progesterone in the neonates over time may favor PP M cell maturation after 1 week of life.

### Maternal secreted immunoglobulins

The passive immunity, transmitted from the mother to the newborn through breastfeeding, greatly influences neonatal health. In the lactating mother, intestinal plasma cells migrate in a CCR10/CCL28-dependent manner to the mammary glands where they secrete

immunoglobulins into breast milk [97]. Those maternal antibodies include mostly secretory IgA (sIgA) followed by IgM and IgG, and reflect specificity to the maternal microbiota. As vaginally delivered newborns have a primary microbiota similar to the maternal fecal microbiota [67], transfer of these specific antibodies gives a clear advantage to control bacteria in the



neonatal gut [28]. Thus, passive immunity provides protection to neonates by shaping the microbiota and keeping it at bay from the sterile tissue, thus preventing invasion of pathobionts and inflammation (Fig. 2). Weanling mice nursed by dams deficient for Ig secretion (pIgR<sup>-/-</sup>), compared to those nursed by wild-type dams, have a different microbiota composition and deregulated colonic epithelial cell gene expression leading to an impaired barrier function, with increased translocation of enteric bacteria, such as *Ochrobactrum anthropi*, but also worsened inflammation in dextran sodium sulfate-induced colitis [76]. However, it is not clear whether IgA has a direct effect on colonic epithelial cells or an indirect effect through alteration of the microbiota. Passive immunity also prevents early activation of the adaptive immune system. An early germinal center (GC) activation and IgA production in PPs at P13 was observed in pups nursed by females deficient in adaptive immunity [98]. Moreover, pups nursed by dams deficient in antibody production have increased levels of activated T cells and IgA<sup>+</sup> plasma cells [99]. This premature development is likely induced by an increased bacterial translocation across the neonatal intestinal epithelium due to a deficit in bacterial coating by maternal antibodies, leading to an early activation of the mucosal immune system. Upon challenge with *Enterobacter cloacae*, a fast-growing facultative anaerobe of the adult microbiota, these prematurely produced neonatal IgA appear as effective as maternal antibodies in preventing bacterial translocation to the MLNs [99]. The induction of endogenous IgA in neonates in the absence of breast milk Ig is likely a downstream effect of a premature activation of follicular helper T cell (Tfh). Indeed, Koch *et al.* [100] demonstrated that transferred maternal IgG2b and IgG3 limit activation of Tfh cells as well as GC expansion in pup PPs and MLNs. Early activation of the adaptive immune system also occurs during infectious challenge. Thus, neonates develop reovirus-specific antibody-secreting cells in PPs and MLNs upon challenge at P10 [101]. However, this early virus-specific IgA response is abrogated when pups receive passive immunity from an immunized foster dam, probably due to an efficient neutralization of the virus by maternal antibodies. Passive transfer of maternal pathogen-specific antibodies does indeed provide protection in neonates against rotavirus [102], poliovirus [103], or HIV [104] through their neutralizing activity. In humans, high titers of maternal-specific IgA also correlate with a better protection of neonates against diarrheagenic enteropathogens such as *E. coli*, *Vibrio cholerae*, *Shigella*, and *Campylobacter* [105]. However, immunoglobulins from unimmunized mothers

can also protect against enterotoxigenic *E. coli* infection through IgG coating, suggesting a contribution of polyreactive and nonspecific immunoglobulins [106]. Overall, these studies show that the transfer of maternal secreted immunoglobulins, either polyreactive (IgG) or antigen-specific (IgG and IgA), plays an important role in neonatal protection by exerting their immune exclusion activity, allowing symbiosis with the microbiota, and preventing infection (Fig. 2). Nevertheless, it is important to keep in mind that immunized mothers confer a transient immunity to neonates that vanishes after the breastfeeding period, thus supporting the need for the establishment of endogenous active immunity by the young host (e.g., vaccination) [107].

Lastly, passive immunity is also involved in shaping the neonatal adaptive immunity and thus might aid to set a trajectory for health and disease in later life. This is facilitated through the bidirectional transport of maternal IgG between intestinal lumen and the mucosal tissue via epithelial expression of the neonatal Fc receptor (FcRn) [108]. In humans, IgG transfer from mother to offspring is largely limited to the fetal period via placental transfer. In rodents, however, this transfer continues during the neonatal period in which IEC expresses extremely high levels of FcRn, enabling the translocation of milk IgG from the gut lumen to the mucosal tissue [108]. Yoshida *et al.* [109] showed that weanling mice are less susceptible to *Citrobacter rodentium* infection when FcRn is expressed in IEC. FcRn allows the delivery of IgG-bound bacterial antigen to LP CD11c<sup>+</sup> cells, leading to specific T-cell activation in the MLNs. Thus, IgG transport from the intestinal lumen to the mucosal tissue supports the transfer of antigens and their presentation, leading to an appropriate immune response (Fig. 2). In addition to initiating adaptive immune responses against pathogens, transfer of breast milk IgG immune complexes can induce tolerance to food allergens in the offspring. A thorough study by Ohsaki *et al.* demonstrated that breast milk ovalbumin immune complexes are delivered to LP CD11c<sup>+</sup> cells through the transport of IgG by FcRn [110]. These phagocytes then present the antigen and activate specific Tregs protecting the offspring from allergy-mediated immunopathology. Interestingly, this also involves expression of FcRn in CD11c<sup>+</sup> cells.

Passive immunity can also shape the B-cell receptor repertoire, likely by interfering with presented epitopes and limiting long Tfh-GC B-cell interactions, thus reducing plasma cell generation [111]. Recently, Ramanan *et al.* [112] demonstrated an unprecedented mechanism of immune profile transmission across multiple generations regulated by levels of breast milk IgA

rather than through genetic background or microbiota composition. Taking advantage of immune phenotypic traits specific to C57BL/6 or BALB/c mouse strains, that is, high levels of colonic ROR $\gamma$ t<sup>+</sup> Tregs and high levels of IgA, respectively, they studied the transmission of these traits across generations by cross-fostering pups with dams of the opposite genetic background. They showed that variations in breast milk IgA production condition colonic ROR $\gamma$ t<sup>+</sup> Treg proportions later in life. High levels of IgA-coated bacteria affect antigen detection and limit expansion of ROR $\gamma$ t<sup>+</sup> Tregs. On the contrary, low levels of IgA lead to higher frequencies of ROR $\gamma$ t<sup>+</sup> Tregs, which in turn suppress B-cell activation and IgA induction. It is noteworthy that this mechanism is active during the first week of life, long before endogenous plasma cells and ROR $\gamma$ t<sup>+</sup> Tregs appear in the offspring. This timeframe suggests that maternal IgA influences the intestinal microenvironment, either by acting on the microbiota, the nonhematopoietic, or hematopoietic compartment, leading to an early immune imprinting.

In summary, in addition to protection against pathogens, passive immunity impacts neonatal immune cell development and the establishment of immune homeostasis with consequences far beyond the postnatal phase (Fig. 2).

### Maternal leukocytes

Besides immunoglobulins, murine and human breast milk contains immune cells, of which approximately 80% are myeloid cells and 20% are lymphocytes, mainly T cells [113]. The findings on the fate and function of these cells are controversial. Maternally derived lymphocytes have been reported in mouse offspring tissue although other studies were unable to confirm this finding [113,114]. Nevertheless, the possibility exists that maternal immune cells are able to survive the adverse environment of the upper gastrointestinal tract and penetrate the epithelium. This would lead to microchimerism and provide neonatal tissues with mature and active leukocytes [113,115]. For instance, using GFP expressing dams nursing WT neonates, it was shown that from P6, milk CD8<sup>+</sup> cytotoxic T cells (CTL) expressing the gut homing proteins  $\alpha$ 4 $\beta$ 7 and CCR9 engraft in neonatal PPs, and to a lesser extent in the MLNs and the villus LP [116]. Their number was reported to decline after weaning. These maternal CTL may be active and functional, as they express granzyme B, TNF- $\alpha$ , IFN- $\gamma$ , and IL-18.

Although it is not possible to track maternal cell transfer in the tissues of human neonates, immune cells have been characterized in human breast milk. For

instance, a recent study identified MAIT cells,  $\gamma\delta$  T cells, ILC1, and ILC3 in breast milk [117]. Interestingly, breast milk also contains pathogen-specific lymphocytes. Thus, breast milk of HIV-infected mothers can contain HIV-specific CD8<sup>+</sup> T cells, which exhibit a memory T-cell phenotype and express the gut homing receptor CCR9 [118]. One could hypothesize that these activated CTL take part in preventing HIV transmission in newborns and that other types of pathogen-specific lymphocytes could be transmitted from infected mothers. As described above, plasma cells migrate during lactation to the mammary glands where they mainly secrete IgA. Human breast milk also contains  $\alpha$ 4 $\beta$ 7<sup>+</sup> plasma cells and plasmablasts [119]. However, only one study performed in mice reported maternal IgG-secreting plasma cells in the spleen and bone marrow of B-cell-deficient ( $\mu^{-/-}$ ) pups [120].

In summary, the breast milk-mediated transfer of intact maternal immune cells may help to maintain gut barrier function, promote the establishment of symbiosis, and protect against pathogens. The exact contribution of maternal cells, however, remains to be established.

### Maternal soluble factors

Highly abundant in human breast milk, HMOs influence the neonatal host-microbial interaction in several ways [121,122]. Indigestible for the infant host, they serve as substrate for bacteria such as *Bifidobacterium* and *Bacteroides* [123]. HMOs prevent bacterial adhesion and act as a nonimmune exclusion factor (Fig. 2). HMOs can also be recognized by different families of immune cell receptors, such as C-type lectins and sialic-acid-binding immunoglobulin-like lectins (Siglecs). Thus, HMOs can bind the C-type lectin receptor DC-SIGN on human monocyte-derived dendritic cells [124,125]. It has been proposed that such binding could promote tolerogenic properties and/or block pathogen interactions with these dendritic cells [124,126]. However, the exact role of these interactions is still unclear.

A large number of milk-derived peptides and proteins harbor antimicrobial and immunomodulatory activity. For instance, the iron-binding glycoprotein lactoferrin efficiently decreases the growth of bacteria associated with LOS [127]. The murine cathelicidin-related antimicrobial peptide (CRAMP) or the human homologue LL-37 inhibits growth of *Staphylococcus aureus*, *E. coli*, and *Streptococcus pyogenes* (group A streptococci) [128]. Similar to  $\alpha$ -defensins,  $\beta$ -defensins,  $\beta$ -casein, and lysozyme, these antimicrobial molecules have therapeutic potential to prevent

neonatal infection [129,130]. In addition, human breast milk contains soluble CD14 (sCD14) that binds lipopolysaccharide (LPS) from Gram-negative bacteria [131]. Milk-derived sCD14 promotes LPS recognition by TLR4 leading to the secretion of pro-inflammatory cytokines [132]. Finally, human breast milk (especially the breast milk produced during early lactation called colostrum) contains additional unidentified proteins that enhance innate immune recognition by neonatal cells [133].

Along with antimicrobial molecules and sCD14, many cytokines, such as IL-10, TGF- $\beta$ 1, TGF- $\beta$ 2, IL-6, TNF, and IFN- $\gamma$ , are transferred through breastfeeding. They promote oral tolerance as well as IEC proliferation and survival, thus supporting mucosal barrier function [134].

Finally, breast milk-derived epidermal growth factor (EGF) plays a critical role in maintaining epithelial barrier integrity and orchestrating the establishment of neonatal mucosal immunity (Figs 2 and 3) [135]. Interestingly, milk-borne EGF is at its highest concentration during early lactation and decreases over the lactation period (Fig. 2). During the early postnatal phase, EGF inhibits goblet cell-associated antigen passage (GAP) formation, prevents bacterial translocation, inhibits TLR4 signaling, and is protective in experimental models of NEC and LOS [31,136,137]. The decline of EGF facilitates GAP-mediated translocation of antigens and immunostimulatory molecules prior to weaning, which is critical for the initiation of intestinal immune system maturation [31,78,135,138].

In summary, many milk-derived compounds promote pathogen detection and killing, intestinal immune system maturation and oral tolerance development, maintenance of gut barrier function, and modulation of the microbiota composition in neonates (Fig. 2).

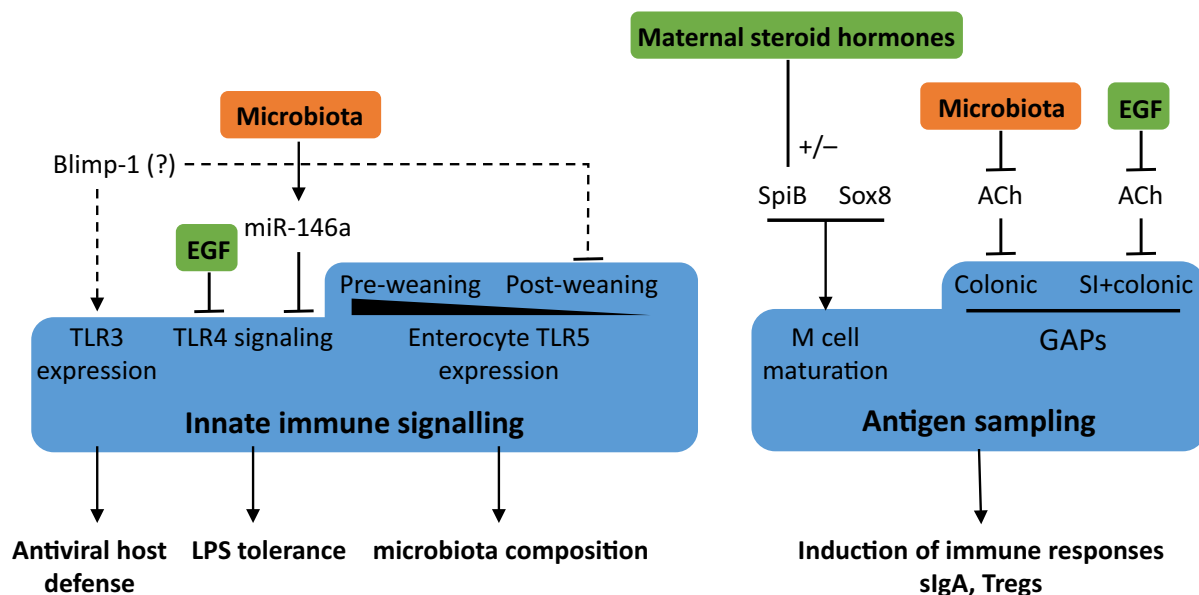
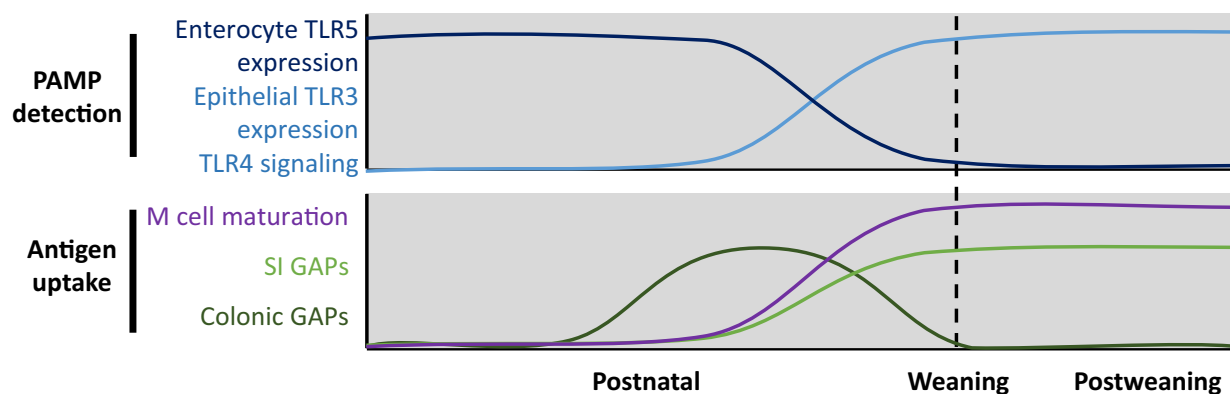
## Early-life defense mechanisms and immune responses

### Postnatal intestinal tissue development

Mice have a short gestation period of only 21 days and exhibit substantial postnatal tissue maturation. Although small intestinal villi develop prenatally at E15 [139], intestinal crypts develop only from P12 to P15 [140]. Neonatal IECs exhibit a low proliferation rate, and the continuous renewal of the epithelium is not initiated until 10–12 days after birth. Crypt-villus structures elongate from birth in the duodenum and from P4 in the ileum until P16 [141]. Mature Paneth cells appear upon crypt formation around 2 weeks after birth [142]. Colonization by the microbiota

significantly influences IEC maturation as illustrated in germ-free mice; within 2 days of conventionalization, colonic enterocyte proliferation intensifies and crypts elongate [143]. During the suckling period, enterocytes are equipped to metabolize milk nutrients [144]. Their neonatal phenotype is mainly regulated by expression of Blimp-1, a transcriptional repressor that prevents adult-like enterocyte differentiation until weaning. Consistently, transgenic mice with epithelial cell deletion of Blimp-1 (Blimp-1<sup>ΔIEC</sup>) are born with continuous epithelial cell turnover, the presence of small intestinal crypts and increased numbers of Paneth cells. IECs from Blimp-1<sup>ΔIEC</sup> neonates also exhibit a transcriptional profile similar to the adult epithelium, that is, low expression of enzymes involved in milk constituent degradation and elevated expression of enzymes that allow degradation of complex carbohydrates such as starch [144,145]. This adult-like epithelium makes the neonate ill-adapted to neonatal life and results in reduced weight gain and increased mortality [144]. The secretion of mucins by goblet cells is also markedly reduced in neonates, leading to a less prominent mucus layer. Consequently, colonizing bacteria get in closer contact with the mucosal tissue surface. This spatial proximity may contribute to the enhanced susceptibility of neonate mice to *E. coli* K1 infection [146]. Zhang *et al.* [53] showed that mucin gene expression and mucus layer thickness in mice increase at P15. Similarly, the small intestinal mucus layer of neonate rats increases until it covers the villi during the first 10 days postpartum. Thus, age-dependent differences between the adult and neonatal epithelium such as reduced IEC proliferation and exfoliation, lack of mature Paneth cells and Paneth cell-derived AMPs and a reduced mucus layer may contribute to the enhanced susceptibility of the neonate host to infection.

Mouse PPs and ILFs, which are gut immune inductive sites containing B-cell follicle(s), develop at different time points pre- and postnatally, respectively. Prenatal formation of PP anlagen is initiated at embryonic day 12.5 (E12.5) as VCAM-1<sup>+</sup> stromal cells begin to recruit cKit<sup>+</sup>LT $\beta$ <sup>+</sup>CD11c<sup>+</sup> cells to the intestinal tissue via the receptor tyrosine (RET) kinase -RET ligand axis [147]. Next, recruitment of lymphoid tissue inducer (LTi) cells that are essential for the formation of secondary lymphoid organs occurs through several signals. In fetal gut tissue, neuronal stimulation triggers expression of retinoic acid, which induces the expression of the chemokine ligand CXCL13 on stromal cells, attracting ROR $\gamma$ <sup>+</sup> LTi cells forming clusters at E15.5 [148,149]. Additionally, IL-7 expression in the tissue leads to the upregulation of lymphotoxin (LT)



**Fig. 3.** Innate immune recognition and antigen sampling in neonates. Pathogen-associated molecular pattern (PAMP) detection and antigen uptake are under strict temporal regulation (upper panels). Epithelial TLR3 expression is increased at weaning, likely through downregulation of the transcriptional regulator Blimp-1, and contributes to the antiviral host response (lower left blue box). Microbiota-derived endotoxin induces epithelial expression of the microRNA miR-146a that reprograms TLR4 signaling and promotes LPS tolerance (lower left blue box). TLR4 signaling is also regulated by maternal EGF. TLR5 is expressed by the postnatal intestinal epithelium and via Reg3 $\gamma$  influences the colonization efficacy of flagellated bacteria (lower left blue box). After weaning, TLR5 expression is restricted to crypt Paneth cells. Concentrations of maternal steroid hormones in neonates after 1 week of life may favor Spi-B expression and the postnatal maturation of M cells, responsible for luminal sampling of particulate antigen in PPs (lower right blue box). Antigen uptake and tolerance induction by GAP are inhibited by high concentrations of EGF during early lactation. The decline of breast milk EGF transiently activates GAP in the colon before it is inhibited again at weaning by the increasing bacterial load (lower right blue box). In contrast, GAP and antigen sampling in the small intestine (SI) is only facilitated at weaning and remains functional thereafter.

$\alpha 1\beta 2$  in ROR $\gamma$ t<sup>+</sup> LTi cells, necessary for PP formation [150,151]. At E17.5, the interaction between LT $\alpha 1\beta 2$ <sup>+</sup> LTi cells and LT $\beta$ R<sup>+</sup> mesenchymal cells leads to the development of a microenvironment enriched in VCAM-1<sup>+</sup>ICAM-1<sup>+</sup> mesenchymal cells expressing the chemokines CXCL13, CCL19, and CCL21, which promote the recruitment of lymphocytes to the PP anlagen [152–155]. Four days after birth, lymphocytes seed

the anlagen and a mature adult-like lymphoid structure of the PPs is detected at P7, with a compartmentalized distribution of T cells in the interfollicular region (IFR), B cells and follicular dendritic cells in follicles, and high endothelial venules in the parafollicular areas [156]. In contrast to lymph nodes, the main route of antigen transport to PPs is carried out by M cells. At birth, the FAE typically lacks mature M cell

markers that gradually appear during postnatal development (Fig. 3) [53]. Recently, Hays *et al.* [157] showed that maternal steroid hormones that reach the newborn could be involved in the regulation of neonatal M cell maturation. Indeed, maternal estradiol and progesterone concentrations decrease after birth to reach levels at P7 that favor M cell maturation (Figs 2 and 3). Spi-B represents the master regulator of M cell maturation [158]. *Spib*-deficient mice lack expression of typical mature M cell markers, such as GP2, a surface receptor for type-I-piliated bacteria, and CCL9, a chemokine involved in the recruitment of CCR1-expressing immune cells, whereas early markers, such as *Marks11* and *Anxa5*, are still expressed. In neonates, expression of *Spib* is only detected from P8, followed by GP2 and CCL9 expression at P15 [53]. Finally, glycoconjugates with  $\alpha$ -linked fucose residues recognized by the lectin UEA1 start to be expressed at P21 [53]. Another regulator of M cell differentiation, Sox8, could also play a role in the delayed maturation of M cells in neonates [159]. Transcytosis of luminal particulate material is ineffective in immature M cells, which limits antigen transport to the subepithelial dome until the PP immune system is ready to handle pathogens. Consistently, invasiveness of *S. agalactiae* (GBS), responsible of many LOS cases, increases in adults after treatment with estradiol and progesterone concentrations that mimic neonate hormonal levels reached at P7 [157]. Such hormonal concentrations were shown to promote M cell maturation and transcytosis of the bacteria.

Cryptopatches, the small clusters of lymphoid cells giving rise to ILFs, are detected at the base of intestinal villi between crypts 2 weeks after birth [160]. Their postnatal development requires ROR $\gamma$ <sup>+</sup>IL-7R $\alpha$ <sup>+</sup> LTi cells and IL-7 signaling. Diet-derived aryl hydrocarbon receptor ligands are needed for their maintenance, and diet-derived vitamin A and its metabolite, retinoic acid, have also been shown to influence ROR $\gamma$ <sup>+</sup> ILC3 cell numbers and the further development of cryptopatches [161,162]. Thus, murine gut-inductive sites undergo important structural changes postnatally and their architecture is completed only within 2 weeks postpartum.

Unlike rodents, the long gestational period of humans allows the gut mucosa to develop during fetal life. The human gut mucosal tissue development is completed by 19 weeks of gestation with its final architecture already in place at birth [140]. Thus, the human newborn intestine contains PPs [163], but their size and number gradually increase until puberty [164]. ILFs, with similar structure and immune cell composition as in mice, are present in human small intestine

and colon [165]. However, whether human ILF development is similar to mice remains to be determined. Human cryptopatches have been reported in only one study using CCR6 to identify putative clusters of LTi cells [166]. Nevertheless, humanized mice reconstituted with human hematopoietic stem cells that express IL-2R $\gamma$  are able to develop cryptopatches, whereas the same mice deficient for IL-2R $\gamma$  are not, suggesting that cryptopatches can also develop in humans [167].

### Antimicrobial peptide production and innate immune stimulation in neonates

While still developing, the neonatal mucosal immune system faces two challenges: firstly, to avoid any inappropriate inflammatory response that could compromise the gut barrier integrity and, secondly, to protect the organism from infection by enteropathogens. The lack of mature Paneth cells and AMP secretion (e.g.,  $\alpha$ -defensins and lysozyme) in the small intestine before P14 makes murine neonates more vulnerable to infection [168]. Instead, neonatal IECs express the CRAMP during the first 2 weeks of life. CRAMP expression does not depend on the presence of the microbiota, which suggests a strictly developmental regulation. CRAMP exhibits antimicrobial activity against pathogenic bacteria (e.g., *Salmonella* Typhimurium, *E. coli* K1) but also against some commensal bacteria (e.g., *Lactobacillus reuteri* or *Lactobacillus murinus*). *Cramp*<sup>-/-</sup> neonates are more susceptible to *Listeria monocytogenes* infection, thus illustrating the role of CRAMP to prevent infection *in vivo* [168].

Two weeks after birth, ROR $\gamma$ <sup>+</sup> ILCs populate the small intestinal tissue in higher numbers and with a higher expression of IL-22 than in adults [169]. ROR $\gamma$ <sup>+</sup> ILC3-derived IL-22 promotes Reg3 $\gamma$  expression and epithelial barrier function. Although the function of ILC3s in neonates remains to be established, they most likely contribute to the mucosal host defense during the late neonatal period.

Microbial detection through epithelial pattern recognition receptors must be tightly regulated to avoid inappropriate innate immune stimulation and inflammation-driven disruption of mucosal barrier integrity (Fig. 3). This particularly accounts for the neonate intestine characterized by reduced mucus production, AMP secretion, and epithelial cell regeneration. During the first 3 weeks of murine life, epithelial TLR4 signaling is reprogrammed by enhanced production of the small hairpin mRNA miR-146a, which inhibits transcription of the TLR signaling molecule interleukin 1 receptor-associated kinase 1 (IRAK1) [170]. This mechanism facilitates LPS tolerance by

preventing the secretion of pro-inflammatory mediators and mucosal damage until weaning [171]. Interestingly, miR-146a induction and neonatal epithelial TLR4 tolerization depend on microbial signals (Fig. 3), since TLR4 deficient mice fail to develop epithelial innate immune tolerance [170]. This may explain why milk proteins have been shown to enhance TLR4 recognition ensuring miR-146a upregulation and TLR4 tolerization [133]. On the other hand, caesarian section-born mice maintain epithelial IRAK1 protein expression promoting epithelial stimulation and cell death [170]. Interestingly, NEC in human pre-term infants has been linked to an inappropriate TLR4 stimulation [37,39,172].

TLR5, the receptor for bacterial flagellin, is highly expressed by neonatal IECs prior to weaning (Fig. 3) [173]. TLR5 expression during the neonatal period limits colonization by flagellated bacteria and has a long-term impact on the microbiota composition [173]. After weaning, its expression is restricted to Paneth cells [174]. Conversely, intestinal epithelial TLR3 expression is found only after weaning and confers protection against viral infection (Fig. 3) [48]. This age-dependent expression does not depend on microbial signals but may be linked to IEC maturation through downregulation of the transcriptional regulator Blimp-1 [144].

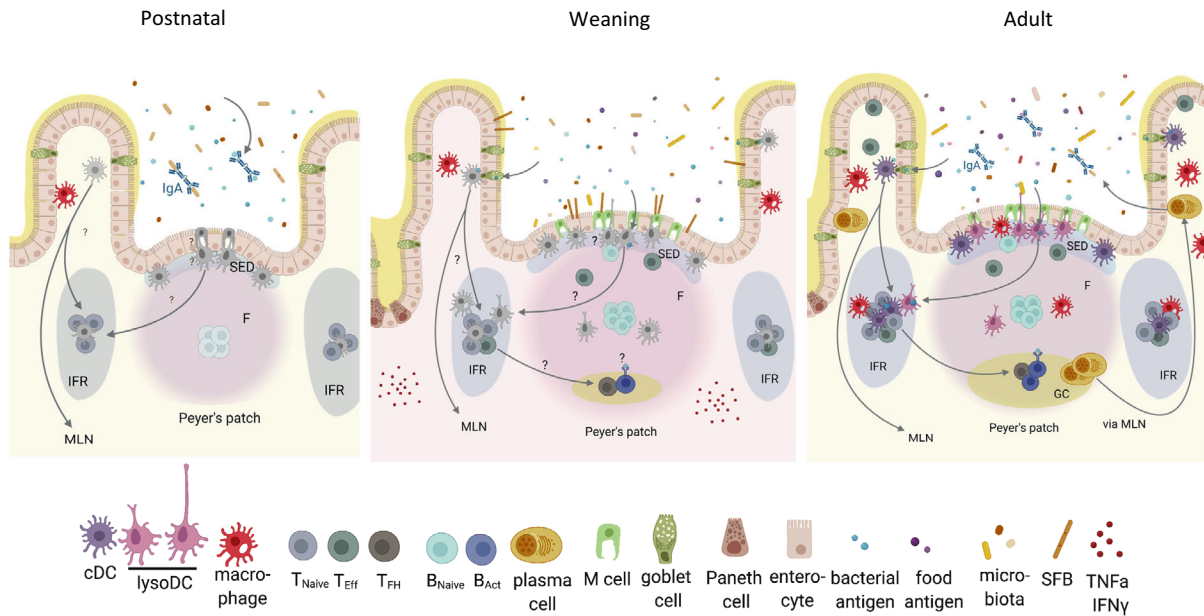
In summary, the intestinal epithelium gets in close contact with microorganisms during the neonatal period. A shift in the AMP spectrum and changes in the expression and function of innate immune receptors may help to establish postnatal host-microbial homeostasis.

### Neonatal immune responses

At steady state, the adult small intestinal tissue is populated with numerous conventional and nonconventional lymphocytes and most lymphocyte populations reflect a continuous cross talk with the microbiota as germ-free mice have greatly reduced numbers of these cell populations. The murine gut is devoid of adaptive immune cells at birth and is seeded during the first week of life. Interestingly, although the neonate is rapidly colonized after birth by commensal bacteria, T and B cells during the postnatal period display a naïve phenotype and are only found in the gut-associated lymphoid tissues but not the LP (Fig. 4) [114]. Activated lymphocytes are only detected after weaning, both in gut-associated lymphoid tissues and at effector sites but they reach adult levels long after [114,175]. Thus, there is a relatively long period, during which the adaptive immune system of the gut under

homeostatic conditions remains oblivious to microbial, food, and environmental stimulation (Fig. 4).

Induction of tolerance to orally ingested molecules requires antigen delivery to cDCs, which is tightly regulated during the neonatal period. GAP-mediated transport of luminal antigens represents a substantial sampling mechanism taking place at the small intestinal and colonic epithelium [176,177]. In early life, GAPs are inhibited by breast milk-derived EGF, limiting antigen exposure and preventing bacterial translocation in colon and small intestine (Fig. 3) [31,137]. In the preweaning period, the decline of EGF in breast milk limits EGFR activation and allows acetylcholine signaling that leads to GAP formation in the colon, thereby promoting transport of luminal antigens exclusively to the colonic LP. Antigens delivered by GAPs are preferentially taken up by CX<sub>3</sub>CR1<sup>+</sup>CD103<sup>+</sup> cDCs [177], which are known to promote Treg expansion and tolerance [178,179]. As such, preweaning induction of colonic GAP triggers the activation of T cells and preferential development of Tregs specific for bacterial antigens in the colon-draining MLNs followed by their migration in the colon LP where they establish tolerance to the microbiota [31]. Around weaning, the detection of the increased bacterial load by colonic goblet cells via a Myd88-dependant pathway inhibits colonic GAPs whereas small intestinal GAPs start to form, are maintained throughout life, and by contrast play a key role in the tolerance to food antigens [180,181] (Fig. 3). Therefore, GAP formation is tightly regulated both temporally and spatially in early life to support induction and maintenance of tolerance in adults and to avoid immune responses to gut microbiota in the colon and to food antigens in the small intestine. In adult mice, the induction of Tregs and tolerance rely on the ability of CD103<sup>+</sup> cDCs to metabolize vitamin A into retinoic acid through their RALDH activity [178,179]. In contrast, in 1-week-old mice, MLN CD103<sup>+</sup> cDCs exhibit weak RALDH activity as compared to 3-week-old mice and are consequently less efficient in activating antigen-specific T cells, rendering neonates more prone to developing allergy [182]. Nevertheless, upon diet supplementation with vitamin A, neonatal MLN CD103<sup>+</sup> cDCs mature and secrete retinoic acid. In this model, neonatal tolerance is induced through expansion of IFN- $\gamma$  producing T helper cells in contrast to the induction of FoxP3<sup>+</sup> Tregs in the adult [182]. Recently, it was demonstrated that during the first 2 weeks of life, neonates have myeloid-derived suppressor cells that are induced by breast milk-derived lactoferrin [183]. These cells can inhibit T-cell expansion in co-culture via secretion of prostaglandin PGE<sub>2</sub>, nitric oxide, S100A8, and



**Fig. 4.** Age-dependent immune maturation of the PPs and villus LP. During postnatal development (left panel), commensal bacteria and bacterial antigen as well as breast milk IgA are found in the intestinal lumen. GAPs and M cells that allow antigen uptake in the adult have not developed/matured, yet. The mononuclear phagocyte (MNP) compartment is ill-defined. The follicle (F) and IFR are present in PPs but B and T lymphocytes within are not primed and do not migrate to the LP under homeostatic conditions. At weaning (mid-panel), solid food and food antigens are found in the luminal content whereas maternal IgA disappears and is not yet replaced by self-production. Microbiota displays significant dynamics and undergoes diversification due to the complex and diverse nutrients of the solid food. The status of the MNP compartment is still ill-defined. GAPs and M cells are present and allow for the uptake of luminal antigens that are then presented to T cells and a homeostatic/tolerogenic bias is imprinted. The pro-inflammatory cytokines of the weaning reaction  $IFN\gamma$  and  $TNF\alpha$  are transiently present and shape the immune responses, ultimately leading to the adult-state homeostasis. T follicular helper cells primed at weaning aid the activation of B cells that recognize luminal antigen. In the adult (right panel), the organization of the MNP compartment is well characterized with regional specificities in PP. Thus, cDCs are enriched in the IFR where naïve T cells reside, whereas LysoDC and macrophages are enriched in the subepithelial dome (SED) where constant uptake of luminal antigen occurs. Presentation of these antigens leads to the presence of antigen-experienced T cells in the LP and PPs. In GC, B cells undergo homeostatic activation and differentiate into IgA-producing plasma cells that migrate through the MLNs to the LP where they secrete IgA into the intestinal lumen and control microbial composition, localization, and density. Created with BioRender.com.

S100A9. *In vivo*, myeloid-derived suppressor cells improve the outcome of NEC in preterm neonates by promoting Tregs over Th17 cells, leading to decreased intestinal permeability and inflammation, and increased survival.

Neonatal T cells have long been considered immature but this concept is an oversimplification and poorly reflects the plethora of data on neonatal T-cell responses collected to this point (thoroughly reviewed by [184]). Regarding their phenotype and location, murine T cells found in the neonatal intestine are more similar to those found in sterile secondary lymphoid organs than in their adult intestinal counterparts. In contrast to the situation in mice, activated T cells have been found in human fetal intestine devoid of living microorganisms, but their origin and function require further investigations [185,186]. Neonatal T cells are

able to respond to stimulation, but they do so in a different fashion as compared to adult T cells. This reflects their adaptation to the neonatal environment and their origin, since neonatal T cells by definition represent recent thymic emigrants [187]. Historically, one of the neonatal features that has been first described is their Th2 bias during secondary responses and thus a limited Th1 response in the first week of life [188]. This bias is mediated by neonatal type 1 cDCs (cDC1) that are phenotypically and functionally distinct from their adult counterparts [189]. Neonatal cDC1 produce IL-10 to limit cytotoxic T-cell activation but can prime *bona fide* protective Th1 responses after stimulation via TLR3 [190]. Despite their Th2 bias, a type 1 response can still be elicited in newborns under specific settings. For example, DNA vaccines promote Th1 cell expansion in neonatal mice, whereas

vaccination with a live virus leads to Th2 responses [191].

### **Microbiota imprinting: the neonatal windows of opportunity**

In recent years, several studies have highlighted that the long-term development of a mature and homeostatic intestinal immune system entails a crosstalk with the microbiota in specific time windows of opportunity starting even prior to birth [27,29,30].

During pregnancy, maternal microbiota-derived metabolites shape the neonate's innate immune system and this influence extends to the time of breastfeeding [93]. Dietary fibers strongly influence the nature of these maternal microbiota-derived metabolites, notably SCFAs. Maternal SCFAs promote the development of Tregs [95] and confer protection against metabolic dysregulation and obesity in the adult offspring [94]. They also protect the offspring against house dust mite-induced asthma, regardless of the age at the time of sensitization [96].

At birth, the exposure to the microbiota is crucial to induce the secretion of TNF- $\alpha$  by spleen myeloid cells that in turn is necessary to induce the differentiation of neonatal IL-10-producing pre-cDC1 into IL-12-p40-producing cDC1 [192]. Importantly, this maturation of pre-cDC1 allows neonates to generate a protective cytotoxic T-cell response against *L. monocytogenes*.

The crucial role of host-microbiota interactions during early life was demonstrated by colonization of germ-free mice at different time points after birth. Although many phenotypical traits were reversed by conventionalization of adult germ-free animals [193], other immune parameters were found to require the presence of the microbiota during a postnatal time window. For example, only microbial exposure during early life but not in adults is able to inhibit the expression of CXCL16 in lung and colonic mucosa, allowing the subsequent control of invariant natural killer T (iNKT) cell numbers, which is protective against immune-mediated diseases (e.g., colitis) in later life [194]. The intestinal expansion of iNKT cells during the neonatal period is controlled by sphingolipids derived from *Bacteroides fragilis*, a member of the commensal microbial community [195]. Similarly, early microbial exposure is required to control the levels of IgE [196] and the influx of Tregs to the neonate's skin [197]. Another study showed that exposure to bacterial species able to synthesize riboflavin, such as the pathobiont *Prevotella mirabilis*, during the second week of life promotes the expansion of MAIT cells, especially in the skin where they favor tissue repair [198].

Finally, GAP-mediated antigen sampling in the colon must happen during the second week of life to ensure appropriate immune responses in adulthood [31]. Inhibition of colonic GAP formation in the preweaning phase or their maintenance after weaning lead to impaired Treg development and worsen inflammation in the DSS-induced colitis model.

### **Weaning-induced immunity**

#### **The weaning reaction, the last window of opportunity**

At weaning, the passive protection provided by breast milk-derived proteins is replaced by a stepwise maturation and establishment of the endogenous adaptive immune system. At the same time, the introduction of solid food in the diet reshapes the microbiota composition and promotes its diversification, reprogramming bacterial metabolic pathways [145]. Thus, major changes occur in the gut at weaning leading to the final education of the intestinal immune system during what has been called a weaning reaction [78]. The opening of this last window of opportunity at 3 weeks of age in mice is regulated by the decreasing concentration of breast milk EGF and is characterized by a strong and transient upregulation of IFN- $\gamma$  and TNF- $\alpha$  expression [78]. Similarly, during the first week after weaning, piglet intestinal tissues undergo an inflammatory response, notably with the upregulation of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  [199]. Although TNF- $\alpha$  expression induced by microbial colonization at birth is restricted to the spleen and promotes pre-cDC1 maturation [192], TNF- $\alpha$  expression induced by the weaning reaction occurs in the gut [78,199]. In adult mice, the gut TNF- $\alpha$  expression that follows TLR7 stimulation is known to induce a massive and rapid migration of cDCs from the villus LP to the MLN and from the PP dome-associated villus LP to the IFRs enriched in naïve T cells [200,201]. Whether such migration occurs during the weaning reaction-induced TNF- $\alpha$  expression in infant animals remains to be established. Anyhow, in weanling mice microbial antigens, along with SCFAs and retinoic acid promote the expansion of colonic ROR $\gamma$ t<sup>+</sup> Tregs that enhance protection against DSS-induced colitis in adults [78]. Mice that missed the weaning reaction are ill-equipped to adequately respond to pathological inflammations such as colitis, allergy, or cancer in adulthood. The critical role of innate immune signals during this transition has been highlighted in humans by the work of Vatanen *et al.* [202]. They showed that the production of highly stimulatory hexa-acylated LPS by members of the infant



microbiota was associated with protection from allergic disease.

### Mucosal immune cell changes induced during weaning

A major change driven by weaning is the expansion and activation of mucosal B cells, inducing the maturation of PPs, ILFs, and cryptopatches and marking the start of endogenous IgA production, crucial for maintaining intestinal barrier, controlling the microbiota, and fighting against pathogens. In the small intestine, peptidoglycan from Gram-negative bacteria is recognized by epithelial nucleotide-binding oligomerization domain-containing protein 1 (NOD1) [203], which leads to the expression of  $\beta$ -defensin 3 and CCL20. In turn, CCL20 recruits CCR6-expressing B cells into cryptopatches. B-cell expansion induces the maturation of cryptopatches into ILF through TLR2- and TLR4-mediated microbial recognition. Consecutively, ILF B cells modulate the commensal microbial community through IgA induction.

Germinal center formation and the subsequent generation of antigen-specific IgA characterize PP maturation (Fig. 4). Lack of receptor activator of nuclear factor kappa-B ligand (RANKL) expression in IEC blocks M cell differentiation at a very early stage [204]. In the absence of M cells, the uptake of particulate antigens is strongly inhibited and a delay in GC formation and plasma cell generation after weaning leads to decreased levels of IgA in the intestinal lumen and reduction of IgA-coated bacteria that persist in adulthood. The transcription factor Sox8 is expressed downstream of RANKL signaling and involved in the late stage of M cell maturation (Fig. 3) [159]. Impaired antigen uptake in Sox8-deficient mice leads to delayed GC reaction and IgA induction at weaning that do not persist into adulthood. Other mechanisms for soluble and particulate antigen uptake that contribute to the establishment of intestinal homeostasis in absence of mature M cells remain to be identified and the stage of the immune development during which those mechanisms are activated needs to be defined. Moreover, the relative contribution of the different PP phagocyte subsets to the weaning-induced immune reaction has to be determined. In adults, subepithelial LysoDCs and macrophages are the main phagocyte subsets involved in particulate antigen uptake (Fig. 4) [19,205,206]; whether it is also the case in neonates and weanling mice and whether they can be activated the same way than in adults is currently unknown.

In mice and humans, B-cell clones that are generated in early life persist into adulthood, even those

specific of bacterial species encountered at weaning but lost afterward due to the diversification of the microbiota [207]. Activation of B cells in the PPs may be influenced significantly by dietary antigens, as rats that are colonized with a commensal microbiota but are kept on a protein-depleted diet have smaller PPs mainly containing immature B cells, a phenotype that can be rescued when complementing the diet with casein [208].

Many changes in the intestinal immune system occur at weaning with the induction of microbially induced ROR $\gamma$ t<sup>+</sup> Tregs and IgA being the best-characterized examples. CX<sub>3</sub>CR1<sup>+</sup> macrophages make up a great proportion of the gut immune cell compartment and react very rapidly to changes in homeostasis. In the adult, intestinal macrophages are renewed relatively quickly and differentiate from incoming monocytes that were generated in the bone marrow [209]. The neonatal colonic tissue, however, contains macrophages that mainly stem from embryonic hematopoiesis [209]. From weaning onwards, there is an influx of CCR2-dependent monocytes, which with time replace embryonic-derived macrophages and constantly replenish the intestinal macrophage pool. The seeding of the colon with an adult macrophage compartment requires microbial exposure as this process is significantly dampened in germ-free mice. Interestingly, fetal-derived macrophages residing far from the intestinal lumen and its content do not completely disappear in the adult tissue. A small subset of T-cell immunoglobulin and mucin domain containing 4 (TIM-4)-expressing macrophages are indeed long-lived and derived from fetal hematopoiesis, whereas the TIM-4-negative macrophages derived from CCR2-dependent monocytes, quite rare at birth, increase in frequency at 4 weeks of age, and further expand in adults [210]. Another study showed that whereas macrophages close to the gut lumen are rapidly renewed, a large part of muscularis and submucosal macrophages, distant from the lumen, are long-lived, self-maintaining cells barely replaced by circulating monocytes [211]. These macrophages are associated with and support neurons and gut vasculature and their loss typically results in vascular leakage and reduced transit.

Similar to their adult counterparts, neonatal colonic macrophages display an anti-inflammatory profile that is critical for gut homeostasis. IL-10 receptor signaling is essential to induce these anti-inflammatory properties in macrophages and its loss in mouse and man results in spontaneous colitis and severe early-onset inflammatory bowel disease, respectively [212,213]. Loss of autocrine IL-10 signaling in intestinal macrophages actually reshapes them into IL-23-producing

cells, which leads to IL-22-driven colitis [214]. Interestingly, unlike the colon where IL-10 production by macrophages as well as Treg expansion and maintenance depend on microbial exposure, macrophage production of IL-10 and the generation of Tregs in the small intestine require dietary amino acids [215,216], indicating a regionalized regulation of intestinal immunity based on intestinal lumen content sampling, possibly by GAPs [31,180].

## Conclusions and perspectives

The results discussed in this review illustrate that the neonatal mucosal tissue and immune system undergo gradual maturation processes within defined time windows and depend on developmental programs and/or exogenous signals such as microbial exposure. This assigns the neonatal period a critical and nonredundant role in the individual's life to set the stage for future host-microbial interactions and to establish immune homeostasis. This relates to cell homing and early suppression of immune activation, the acquisition of tolerance to innocuous antigens during a time-window controlled sampling activity, and, finally, the generation of large amounts of effector T lymphocytes and plasma cells. The discovery of the succession of necessary and nonredundant phases during ontogeny has given rise to the concept of layered immunity [217], which combined with the concept of windows of opportunity, may help to identify important endogenous and exogenous stimuli that act on the host during the different phases and ultimately allow to better understand the situation of the adult host [30]. It may also help to define the cause of immune- and inflammation-mediated diseases of the intestinal tract and identify possible strategies to reinstall immune homeostasis and preserve health.

However, important questions remain. For example, what are the functional mechanisms that explain the prolonged period of immune naïveté of the healthy murine neonate? The bacterial colonization of the mucosal surfaces starts at birth and quickly reaches high bacterial numbers. However, the adaptive immune system in the small intestine is maintained in a naïve, silent state until after weaning (Fig. 4). In addition, what are the mechanisms that suppress early immune priming? First, the microbial antigen diversity and antigen concentration in the intestinal lumen of neonates may—despite high bacterial density—be too low and fail to reach the threshold required to allow cognate antigen recognition to occur. A combined approach using metagenomics, metaproteomics, and antigen prediction might help to answer that question.

Second, the mechanisms that allow antigen uptake, processing, and presentation may not be fully functional at birth. The main routes of antigen sampling may indeed be less functional since GAPs are inhibited in neonates and mature M cells only appear in PPs shortly before weaning [31,53]. Moreover, subepithelial phagocytes may be unable or less efficient to prime B and T cells before weaning. Third, breast milk factors strongly involved in the regulation of the neonatal intestinal immune response [31,78,137] may restrain PP activation before weaning. Lastly, neonatal T-cell intrinsic features may preclude effective activation under homeostatic conditions. Consistently, neonatal T lymphocytes by definition are recent thymic emigrants that have been shown to exhibit significant differences after activation [187,218].

Other open questions relate to the emergence of immune activation at weaning. In particular, the role of specific commensal bacteria involved in this process such as the so-called segmented filamentous bacteria (SFB) that intimately interact with the apical plasma membrane of the intestinal epithelium in the ileum and the FAE overlaying the PPs (Fig. 4) [219,220]. Notably, SFBs show the highest abundance at 3 weeks after birth [221,222] and exhibit strong immunomodulatory properties characterized by a strong IgA and Th17 inducing activity in PPs or ILFs [223–225]. In PPs they also stimulate the expression of IL-21, aiding the induction of commensal-specific IgA and creating a regulatory loop controlling SFB abundance [226]. Another candidate might be *Alcaligenes* spp. that reside within subepithelial phagocytes in PPs and induce IgA, Th17, and ILC3 responses [227,228]. Besides specific viable microorganisms, bacterial metabolites have also been identified as potent inducers of immune responses [229]. They are also involved in PP activation and might initiate immune priming at weaning. Finally, although the landscape of PP mononuclear phagocytes has been well characterized in the adult [18] their presence, location, and function during the postnatal development and weaning remains to be established (Fig. 4).

A better understanding of the neonatal and infant mucosal immunity has important medical implications. Children exhibit the highest risk of enteric infections, and diarrheic diseases significantly contribute to childhood mortality worldwide. A mechanistic understanding of the influence of maternal and dietary factors as well as microbial metabolites and immunostimulatory molecules is instrumental to improve oral vaccines and develop therapeutic strategies to prevent early-life infections. Given the life-long priming during the neonatal window of opportunity, this time period

might also represent the prime period for interventions that aim to prevent inflammatory and immune-mediated diseases in later life.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

CW, NT, MWH, and HL wrote the manuscript. CW and NT designed the figures.

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