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MULTI-AUTHOR REVIEW

Establishment of intestinal homeostasis during the neonatal period

Silvia Stockinger · Mathias W. Hornef · Cécilia Chassin

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Abstract The intestinal mucosa faces the challenge of regulating the balance between immune tolerance towards commensal bacteria, environmental stimuli and food antigens on the one hand, and induction of efficient immune responses against invading pathogens on the other hand. This regulatory task is of critical importance to prevent inappropriate immune activation that may otherwise lead to chronic inflammation, tissue disruption and organ dysfunction. The most striking example for the efficacy of the adaptive nature of the intestinal mucosa is birth. Whereas the body surfaces are protected from environmental and microbial exposure during fetal life, bacterial colonization and contact with potent immunostimulatory substances start immediately after birth. In the present review, we summarize the current knowledge on the mechanisms underlying the transition of the intestinal mucosa during the neonatal period leading to the establishment of a stable, life-long host–microbial homeostasis. The environmental exposure and microbial colonization during the neonatal period, and also the influence of maternal milk on the immune protection of the mucosa and the role of antimicrobial peptides, are described. We further highlight the molecular mechanisms of innate immune tolerance in neonatal intestinal epithelium. Finally, we link the described immunoregulatory mechanisms to the increased susceptibility to inflammatory and infectious diseases during the neonatal period.

S. Stockinger · M. W. Hornef · C. Chassin (✉)
Institute of Medical Microbiology and Hospital Epidemiology, Hannover Medical School, 30625 Hannover, Germany
e-mail: chassin.cecilia@mh-hannover.de

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Introduction

The mammalian mucosal surfaces such as the lung, reproductive tract, urinary tract and intestine are in direct contact with the external environment populated with bacteria, fungi, viruses and parasites. This is particularly evident in the intestine where a dense and highly diverse microbiota exists in a mutually beneficial relationship with the host. Yet, the bacterial colonization of the intestinal mucosa requires a tight epithelial barrier and functional mucosal immune system to ensure maintenance of the epithelial integrity and tissue homeostasis. In addition, the intestinal mucosa is intermittently exposed to potentially harmful pathogenic microorganisms. Thus, the establishment of a mature mucosal immune system able to restrict the microbiota to the intestinal lumen and to discriminate invading pathogens from commensal members of the microbiota is required and represents a unique regulatory challenge for the mucosal immune system.

The mammalian small intestine is composed of three tissue layers consisting of an outer smooth muscle layer, stromal tissue and an inner mucosal layer covered by a single sheet of cuboideal epithelial cells. The epithelial cell layer comprises four different cell types: enterocytes (secreting hydrolases and absorbing nutrients, ions and fluid), goblet cells (producing the mucus layer), enteroendocrine cells (secreting hormones, like serotonine, substance P and secretine), and Paneth cells (secreting antimicrobial peptides like cryptidins or defensins and enzymes like lysozyme). All four lineages derive from pluripotent continuously proliferating intestinal stem cells

that are situated in a protected niche close to the bottom of intestinal crypts. The intestinal epithelium regulates the selective entry of fluids, minerals, vitamins and nutrient substrates, but also forms an active barrier separating the 10–100 trillion microorganisms of the gut microbiota from the largely sterile submucosal tissue [1].

Intestinal epithelial cells express innate immune pattern recognition receptors (PRR), such as Toll-like receptors (TLRs), Nod-like receptors (NLRs) and helicases, and thereby are able to actively respond to exposure of microbe-associated molecular patterns (MAMPs) [2, 3]. The factors that allow the host to discriminate between colonization by commensal microbiota and infection by pathogenic bacteria are still largely undefined [4], but an increasing number of studies start to shed light on the molecular and cellular mechanisms underlying the maintenance of gut homeostasis under conditions of mucosal stress, in most cases using the model of oral dextran sulphate sodium (DSS) treatment. Mucosal host–microbial homeostasis is the result of a complex cross-talk between microbiota, the epithelium and host immune cells [5–8]. Several studies using chimeric animals or mice with cell lineage-specific gene deletions showed that epithelial cells, which were originally considered a simple physical barrier, actively contribute to the regulation of immune responses in the gut [2, 9–11]. Hyporesponsiveness to the microbiota and mucosal homeostasis thus appears to be an interactive, dynamic, regulatory mechanism, rather than be caused by a non-responsive surface layer of cells. In fact, there is evidence that intestinal epithelial-specific dysfunction of innate immune signaling pathways and an impaired interaction between epithelial and submucosal cells might lead to mucosal inflammation or colitis-associated cancer [7, 12].

Several functional and structural aspects of the intestinal mucosa have been identified to contribute to homeostasis in adult mice and may also support the mucosal barrier formation during the postnatal period. Epithelial barrier integrity is enforced by intercellular tight junctions that block paracellular transcytosis and actin-rich microvilli that form a dense brush border to prevent microbial attachment and invasion [5]. Goblet cells produce mucins, heavily glycosylated interlinked protein chains that form a hydrophilic matrix overlaying the epithelial cell layer. The mucus layer physically separates the great majority of luminal microbiota from the apical epithelial surface [13, 14]. The critical importance of an intact mucus layer is illustrated by mice lacking the major mucin protein MUC2 that develop spontaneous colitis [15]. Goblet cells additionally secrete the resistin-like molecule (RELM) β and trefoil factors (TFF) that play a role in intestinal homeostasis, wound healing and the host defense against worm infection [16]. Paneth cells produce antimicrobial peptides (AMPs) to limit bacterial growth and shape the microbiota composition [17, 18]. Resident $CD103^-$ $CX3CR1^+$ phagocytic cells generate dendrite-like protrusions that reach into the intestinal lumen and allow uptake of bacteria and sample luminal antigens [19, 20]. They control T cell activation via secretion of IL-10 and TGF-β but are also able to promote Th1 and Th17 differentiation during inflammation. Also, migratory $CD103^+$ $CCR7^+$ dendritic cells are conditioned by microbial and epithelial-derived factors and promote the differentiation of $CD4^+$ $Foxp3^+$ regulatory T cells (T_{reg}) and the secretion of immunoglobulin A (IgA) by B cells [21]. Under inflammatory conditions, they also promote IL-17-producing helper T (T_H17) cells that in turn induce infiltration by professional immune cells via secretion of IL-17 and IFN-γ [22]. T_{reg} and Tr1-like cells regulate inflammation through IL-10- and TGF-β-dependent mechanisms [21]. $ROR\gamma t^+$ innate lymphoid cells (ILCs), including lymphoid tissue-inducer (LTi) cells and IL-22-producing $NKp46^+$ cells, synthesize IL-22 which induces expression of the antimicrobial protein Reg3γ. Epithelial IL-25 induced by the microbiota is able to control IL-22 production [23]. Also, lymphotoxin (LT) expression by $ROR\gamma t^+$ cells favors epithelial cell repair and and induces epithelial secretion of CXCL1 and CXCL2 and the recruitment of neutrophils and macrophages during infection [24]. Enterocytes inhibit T_H1 differentiation via soluble thymic stromal lymphopoietin (TSLP) [25] and control tumor necrosis factor (TNF)-induced epithelial apoptosis [9]. Finally, continuous signaling in epithelial cells through PRRs like TLR4, TLR5, Nlrp3, Nlrp6 and associated molecules like Myd88, NEMO, IKK1 and 2 as well as Caspase-1 has been shown to protect against colitis [11, 26–28] suggesting an active role of epithelial cells in the maintenance of host–microbial balance.

Whereas the mechanisms that ensure maintenance of immune tolerance and gut homeostasis in the adult host begin to be unraveled, the factors that facilitate the establishment of this surprisingly stable and life-long host–microbial interaction after birth and during the neonatal period are largely unknown. The healthy mammalian fetus develops in a bacteria- and microbial ligand-free environment. Upon rupture of the amniotic membranes and passage through the birth canal, the neonate organism is exposed to maternal bacteria from the vaginal tract, skin and feces and to environmental microbial ligands such as endotoxin [29–31]. During this time, the neonate intestinal epithelium has to adapt to facilitate the robust adaptation from a sterile protected site to a densely colonized surface and to establish a symbiotic interaction with the bacterial microbiota, in order to establish a stable host–microbial homeostasis [32, 33]. Alterations of the microbiota composition have been linked to several diseases such as allergies, vascular diseases, cancer and autoimmunity, as well as inflammatory bowel disease (IBD) and necrotizing

enterocolitis (NEC) [34–37]. In this review, we will focus on the development of the neonatal mucosal immune system, the formation of the intestinal microbiota and the establishment of the host–microbe homeostasis during the neonatal period. Additionally, we will address infant diseases that might result from a dysregulated adaptation process during the postnatal period leading to an impaired immune homeostasis in the gut.

Development of the intestinal mucosa

During the fetal period, tissue morphogenesis and cell differentiation prepare the epithelium for absorption of colostrum and milk. The primitive gut is a pseudostratified layer of endodermal origin surrounded by mesenchymal tissue, appearing early during ontogeny [38]. Later on (around embryonic day E15 in mice), an anterior-to-posterior wave of morphogenic changes occurs and the undifferentiated group of cells converts into a single-layered epithelium with columnar cells and nascent villi [39]. The mesoderm differentiates into smooth muscle and stromal tissue. Cell proliferation, first homogenously present along the intestinal tract, becomes restricted to the intervillus regions, where the crypts begin to develop as soon as epithelial cells penetrate the underlying mesenchyme. Crypts contain a small group of proliferating stem cells, giving rise to the different cell phenotypes that migrate along the crypt–villus axis. The maturity of the epithelial tissue at birth depends on the length of the gestation period. Early crypt development occurs in species with a long gestational period including humans, but not rodents, where crypts develop only after the immediate postnatal period [40]. In some vertebrate species, such as zebrafish, crypts never appear and stem cells remain localized within the intervillus region [41]. Intercellular communication between epithelial cells, which facilitates a coordinated epithelial response upon microbial challenge [42], also represents one of the key mechanisms driving epithelial morphogenic movements, differentiation and migration. The composition of the extracellular matrix detected via cell surface receptors such as E-cadherin and integrins determines intestinal epithelial polarization characterized by the separation of the apical and basolateral plasma membrane through the expression of intercellular tight junction molecules [43, 44]. Hedgehog signaling, through Sonic hedgehog (Shh) and Indian hedgehog (Ihh), plays a role in endodermal and mesodermal patterning, crypt formation and spacing [45]. Conditional deletion of $\beta 1$ integrins in the intestinal epithelium of mice results in a loss of Hedgehog expression and early postnatal lethality [46]. Forkhead box transcription factors, Homeobox genes and Parahox genes, as well as GATA/FOG transcription factors, regulate intestine-specific developmental genes during fetal development [45]. The immature primitive polarized cells lead to the formation of the four different lineages of IECs, enterocytes (90% of epithelial cells), goblet cells (5%), enteroendocrine cells (<1%) and Paneth cells (10–15 per crypt, restricted to the small intestine), that are maintained in the adult gut [47]. Nevertheless, the four cell types do not emerge synchronically, since active enteroendocrine cells are already present around E10 whereas Paneth cells in mice appear only after birth. LGR4, an orphan G-protein coupled receptor, has been shown to be required for Paneth cell differentiation [48]. Epithelial proliferation is known to be low in the intestine of suckling mice and starts to increase approximately at postnatal day P15, correlating with the adaptation of the gut to utilize solid nutrient components and the formation of the crypt–villus architecture of the intestinal epithelium [49–51]. The pool of stem cells, confined to the crypt, allows constant and rapid renewal of the adult gut epithelium. Transit-amplifying cells, the progeny of stem cells, divide approximately five times and then differentiate into specialized epithelial cells [51]. Differentiated cells of the upper crypt and villus epithelium continuously migrate towards the villus tip and are replaced by newly formed cells from the stem cell pool in the crypts, making the small intestinal epithelium an extremely dynamic surface structure. Epithelial cells differentiation and proliferation are controlled by several major signaling pathways. Wnt signaling maintains cellular proliferation in crypts and controls the development of the secretory lineage and the migration along the crypt–villus axis. Bone morphogenetic protein (BMP) signaling negatively regulates cell proliferation. K-RAS regulates cell proliferation and survival. Finally, notch signaling regulates secretory lineage development and crypt proliferation [41, 45]. Recently, an interesting study connected the age-dependent expression of the transcriptional repressor Blimp1 to the developmental adaptation of the murine intestinal epithelium during the postnatal period. Blimp1 expression is high in the embryonic gut and starts to decrease at birth in cells of the intervillus region which subsequently gives rise to developing crypts. Adult enterocytes completely lack expression of Blimp1. Intestinal epithelial-specific deletion of Blimp1 leads to enhanced postnatal lethality with disturbance of small intestinal tissue architecture, vacuolation of intervillous cells and altered differentiation, illustrating the critical importance of the switch in the global transcription profile between fetal and adult intestinal epithelial cells [52].

Also, posttranscriptional regulatory mechanisms such as microRNAs (miRs) have been implicated in the development of the intestinal epithelium. In mice, epithelial-specific ablation of Dicer1, essential cofactor in the maturation of miRs, leads to a disorganization of the epithelium, decrease in the number of goblet cells and an increase in the apoptosis rate [53, 54]. miR-145 has been

shown to play a critical role in promoting the maturation of the zebrafish gut epithelium through the regulation of gata6, essential for intestinal morphogenesis [55]. Also, miR-194 regulates the expression of HNF1α, a Notch signaling activator expressed during organogenesis in the gut, which determines epithelial cell maturation and differentiation in mice [56]. Finally, miR-103 has been shown to control the expression level of proteins involved in the G1/S transition regulatory network during intestinal stem cell proliferation [57]. miRs may also play an important role for epithelial differentiation and barrier homeostasis after the immediate postnatal period [58]. For example, the epithelial di/tripeptide membrane transporter PepT1 was shown to be downregulated by miR-92b. PepT1 is expressed in differentiated IECs at the top of the villi and is involved in the transport of formyl-methionyl-leucyl-phenylalanine (fMLP), muramyl dipeptide (MDP) and L-Ala-D-Glu-meso-DAP (Tri-DAP), and thus contributes to innate immune stimulation via NOD2 [58]. miR-92b via the regulation of PepT1 thereby inhibits the inflammatory response induced by bacterial peptidoglycan fragments [58]. Also, enhanced expression of miR-29a was found in a fraction of patients with irritable bowel syndrome (IBS). The same patients exhibited increased intestinal membrane permeability associated with decreased expression of the glutamine synthetase GLUL (glutamate-ammonia ligase), a target of the miR-29.

In addition to the maturity of the intestinal epithelium, the development of the gut-associated lymphoid tissue (GALT) also correlates with the length of gestational period. Lymphomyeloid precursor cells are present during early development and disseminate to seed progenitors in early structures of Peyer's patches and mesenteric lymph nodes [59]. In mice, the initiation of Peyer's patches genesis starts around E15–E17 [60, 61]. The migration of mature lymphocytes begins at postnatal day P2 and fully organized Peyer's patches with follicular DCs, germinal centers, a B cell and a T cell region are evident at P4 [62]. In contrast, mouse cryptopatches and isolated lymphoid follicles (ILF) are only formed after microbial exposure [63]. In human fetuses, Peyer's patches outlines appear at 11 weeks gestation and functional T cells and B cells are found at 16 and 12 weeks gestation, respectively [64]. At 16 weeks gestation, fully formed Peyer's patches are present and progressively expand. During the neonatal period, the gut immune system is structurally complete, but still undergoes significant expansion and maturation. Also, innate and adaptive immune responses of intestinal immune cells during the neonatal period are different from the adult situation [59, 65]. Neonatal $CD4^+$ T cell are more prone than adult $CD4^+$ T cells to differentiate into T_{Reg} cells upon stimulation [66]. In addition, B cells expand during the postnatal period and develop into plasma cells that produce large amounts of secretory (S)IgA [67]. SIgA prevents inappropriate immune activation by binding to nutritional and microbial antigens. Thus, interactions with microbial ligands and food antigens facilitate the maturation of dendritic cells, T cells and B cells during the postnatal period and drive the development of immune tolerance mechanisms to avoid an inappropriate immune response [64]. As described below, the immature neonate immune system also renders the organism more susceptible towards microbial infection [32, 65, 68].

Maternal influence on postnatal mucosal homeostasis

One unique feature of the neonatal mucosal immune system is the link to maternal immunity through breast feeding. Breast milk stimulates cellular growth and tissue repair, enhances the immunocompetence and provides significant immunoprotection [68, 69]. Early breast milk (called colostrum) contains large amounts of IgA, and also immune cells such as neutrophils, macrophages/colostral corpuscules and lymphocytes, and soluble mediators such as cytokines (interleukins [IL], interferon [IFN]-γ, and TGF-β), hormones and growth factors (insulin, insulin-growth factors [IGF], erythropoietin, colony-stimulating factor [CSF], vascular endothelium factor [VEGF], epidermal growth factor [EGF], nerve growth factor [NGF], hepatocyte growth factor [HGF]), non-specific immune factors (sphingomyelin, oligosaccharides, lactoferrin), and certain miRs [64, 68, 70]. The functional importance of breast milk for the developing intestinal mucosa is highlighted by the finding that breast feeding reduces the risk to acquire inflammatory enteric diseases, such as Crohn's disease, coeliac disease, gastrointestinal infections, NEC and food allergies [64].

Breast milk has been shown to modulate neonatal TLR-mediated microbial recognition. For example, soluble TLR2, found in the maternal milk, may help to restrict innate immune stimulation induced by Gram-positive bacteria in the neonate gut [71]. Milk-derived growth factors contribute to the maturation of the mucosa, reinforce epithelial barrier formation and enhance the ability to selectively transport and absorb nutrients [68]. Macrophages present in colostrum and mature breast milk persist in the lumen of the neonate's gut during the first postnatal week and are able to translocate and reach the systemic circulation [68]. Macrophages are able to secrete cytokines and growth factors that favor epithelial maturation and bind SIgA to enhance the neonate's own immune system [72]. Maternal SIgA also restricts immune activation and microbial attachment by binding to nutritional and microbial antigens. Importantly, the spectrum of the maternal IgA reflects the geographical and temporal environment of both the mother and the child and thus provides highly

specific protection. Maturation of the SIgA-producing plasma cells in the GALT and expression of the polymeric immunoglobulin receptor (pIgR), a molecule that translocates SIgA into the intestinal lumen, occur gradually during the neonatal period and are influenced by environmental conditions [73].

Lactoferrin contained in breast milk limits the pool of free iron and suppressed bacterial growth in addition to its interference with the nuclear transcription factor-κB (NF-κB) [68, 70]. Interestingly, miR-584 has recently been shown to induce expression of the lactoferrin receptor in epithelial cells during the neonatal period [57]. Furthermore, the breast milk constituent lysozyme inhibits bacterial growth by disrupting the peptidoglycan layer of the microbial cell wall [70]. Oligosaccharides have prebiotic effects, but also act as receptor analogs to inhibit attachment of commensal bacteria to the epithelial surface [74]. Maternal cytokines also influence the neonates's immune system. IFN-γ stimulates phagocyte function and TGF-β acts as an immunosuppressor and maintains the integrity of the mucosal barrier. Significant levels of miRs have been detected in breast milk despite the low pH indicating their stability and thereby potential regulatory function of the intestinal mucosa [49, 53]. Particularly, miRs associated with T cell and B cell differentiation and regulation have been observed in breast milk [70].

In addition to their nutritional and innate immune functions, factors present in breast milk also play a role in wound healing and tissue repair. Insulin-like growth factor (IGF) 1 is induced after mucosal injury to promote cell proliferation and is present in the maternal milk. Also, epidermal growth factor (EGF) has been shown to play a role in cell proliferation, maturation and differentiation, and is protective against NEC, a devastating intestinal inflammatory disease predominantly of premature neonates. EGF downregulates pro-inflammatory cytokines such as IL-18, increases anti-inflammatory cytokines such as IL-10, and restores the intestinal barrier [75]. EGF also promotes the generation of the mucus layer by goblet cells which is formed by complex interlinked mucin glycoproteins and shields particularly the colon epithelium from direct exposure to luminal substrates [76].

Immune tolerance of IECs after birth

With rupture of the membranes and passage through the birth canal, the neonate becomes exposed to the maternal microbiota, environmental bacteria and microbial constituents such as lipopolysaccharide (LPS). This first exposure occurs prior to ingestion of breast milk, and thus encounters the naïve fetal intestinal mucosa. Intestinal epithelial cells have been shown to express innate immune receptors, such as TLRs throughout fetal, neonatal, and adult life. Both TLR2 and TLR4 expression were found in human fetal tissue from 18 weeks of gestation [77]. Also, in mice, TLR4 and the accessory protein MD2 are expressed in fetal IECs [78, 79] that are able to respond to LPS [49, 79].

Interestingly, we observed a transient transcriptional postnatal activation of epithelial cells, with a peak of *Cxcl2* chemokine expression between 2 and 4 h after birth followed by rapid normalization [79]. This transient transcriptional epithelial activation was induced by orally ingested LPS since it was absent in vaginally delivered TLR4-deficient mice or mice born by caesarian section and thus without exposure to the maternal mucosal secretions during birth. In accordance, low but detectable amounts of LPS were measured in the neonatal intestinal tissue shortly after birth and oral administration of LPS to cesarean section-born mice readily induced epithelial activation [49, 79]. Immunofluorescence studies confirmed epithelial stimulation demonstrating p65 nuclear translocation and IκB-α phosphorylation in small intestinal epithelial cells after vaginal delivery. These analyses also demonstrated epithelial internalization of orally administered LPS in accordance with the previous finding that TLR4 is localized intracellularly in intestinal epithelial cells and requires ligand internalization [80]. Surprisingly, intracellular epithelial LPS could be detected during the complete postnatal period. Since epithelial activation after vaginal delivery was transient and not accompanied by the recruitment of professional immune cells, the induction of negative regulators of the TLR4 signaling were subsequently studied. However, no increase in the expression of well-established regulator molecules such as Sigirr, ST-2, the spliced form of Myd88, or Tollip was detected in isolated IECs after birth. Yet, an almost complete disappearance of the essential TLR signalling molecule interleukin 1 receptor-associated kinase (IRAK) 1 in epithelial cells isolated from mice shortly after birth was noted. Epithelial IRAK1 downregulation was observed in vaginally delivered mice but neither in caesarian section-delivered animals nor in TLR4-deficient mice, suggesting that it might be a direct consequence of the described postnatal epithelial activation [49, 79]. Also, IRAK1 downregulation might cause epithelial TLR hypo-responsiveness and thus contribute to the observed epithelial innate immune tolerance during the neonatal period. Indeed, significant apoptosis was observed in IRAK1 expressing intestinal epithelial cells from cesarean section-born neonates but not epithelial cells from vaginall delivered mice with reduced IRAK1 expression after oral administration of bacteria.

A similar effect of post-stimulatory IRAK1 downregulation associated with an impaired immune responsiveness had previously been demonstrated in macrophages and

suggested to contribute to the well-known refractory state to secondary TLR4 stimulation named endotoxin tolerance [81]. Subsequent in vitro studies using a well-established murine intestinal epithelial cell line [82] confirmed the downregulation of IRAK1 protein expression following TLR4 activation associated with a lack of responsiveness upon secondary stimulation. In contrast to the situation in macrophages, both proteasomal and lysosomal degradative mechanisms were shown to contribute to IRAK1 downregulation in epithelial cells [49], and the functional relevance was also confirmed in vivo. Administration of proteasome and lysosome inhibitors to vaginally delivered newborns prevented the downregulation of epithelial IRAK1, and also caused epithelial apoptosis following oral administration of bacteria after vaginal delivery. In addition to the proteasomal/lysosomal degradation of IRAK1, translational repression of *Irak1* mRNA by strongly enhanced miR-146a expression was identified in epithelial cells [49, 83, 84]. Epithelial miR-146a expression was induced by the initially observed postnatal epithelial activation and absent in caesarian section-delivered or TLR4-deficient mice. Although initial studies had described miR-146a-mediated *Irak1* mRNA degradation, our results both in vitro and in vivo indicated solely transcriptional repression by miR-146a without any alteration in the level of *Irak1* mRNA [49, 85, 86]. In accordance with a critical importance of enhanced postnatal epithelial miR-146a expression, administration of anti-miR-146a to vaginally delivered newborns restored epithelial IRAK1 expression and epithelial apoptosis upon oral bacterial challenge. Conversely, administration of a miR-146a homologue to caesarian section-delivered mice was sufficient to cause IRAK1 downregulation and protect the epithelium from bacteria-induced damage.

Strikingly, enhanced miR-146a, IRAK1 downregulation and lack of LPS-induced chemokine expression persisted in epithelial cells throughout the postnatal period until weaning, associated with the above-mentioned persistence of intraepithelial LPS. Further analyses revealed that continuous TLR4 stimulation and signal transduction possibly provided by the intraepithelial LPS maintained elevated miR-146a levels and ongoing IRAK1 degradation. In addition, this constant signaling under conditions of high miR-146a and low IRAK1 protein ($IRAK1^{low}$) induced a discrete program of gene transcription, different from the gene pattern induced in naïve, high IRAK1 protein-expressing cells. This gene expression included genes associated with epithelial cell survival, proliferation, cell differentiation, and metabolism [49, 83, 84]. Of note, a similar change in the gene expression pattern after acute (M1 state) versus chronic stimulation (M2) has also been observed in macrophages [87]. In the intestine, the described adaptive process might thus simultaneously protect from inappropriate pro-inflammatory innate immune activation during bacterial colonization of the naive fetal mucosa and drive maturation of the epithelium to establish host–microbial homeostasis.

During the third week after birth, profound changes occur with enhanced stem cell proliferation, crypt formation and the start of the continuous crypt–villus migration and constant renewal of the epithelium. The loss of intraepithelial LPS coincided with reduced miR-146a expression, reappearance of high epithelial IRAK1 levels and inducible chemokine expression, thus providing a fully competent epithelial innate immune system to protect from enteropathogens that might encounter the adult host upon uptake of solid food.

Antimicrobial peptides as a host defense mechanism of the intestinal epithelium

Antimicrobial peptides are ancient gene-encoded natural peptide antibiotics. In mammals, two dominant antimicrobial peptide families are found: defensins and cathelicidins. Defensins are characterized by the presence of three intramolecular disulfide bonds and can be subcategorized into α- and β-defensins based on the interlinkage of the cysteine bonds. Mature defensins consist of approximately 30 amino acids and form a triple-stranded β-sheet structure. In the gastrointestinal tract, expression of α-defensins is confined to Paneth cells which are located at the base of the crypts of Lieberkühn in the small intestine and display a highly secretory phenotype filled with granules [88]. Whereas only two α-defensins, human α-defensins 5 (HD5) and HD6, are expressed in the human small intestinal tissue, more than 20 α-defensins (also named cryptdins) have been sequenced from murine small intestinal tissue. In addition, murine Paneth cells express a related large family of covalently linked homo- or hetero-dimeric antimicrobial peptides, the cryptdin-related sequence (CRS) peptides [89]. The distribution of β-defensins includes the stomach and colon. Although β-defensin mRNA has been detected in small intestinal tissue, its expression on the protein level has not been confirmed. β-defensins are regulated on the transcriptional level and their expression occurs either constitutively or after stimulation by endogenous proinflammatory stimuli or innate immune activation. In contrast, α-defensins are constitutively produced by Paneth cells and posttranscriptionally regulated by proteolytic processing. Proteolytic cleavage in mice is performed by the matrix metalloproteinase 7 (MMP7, also named matrilysin) prior to secretion, whereas human α-defensins are cleaved by the endoprotease trypsin only after secretion within the intestinal lumen. Paneth cells express a selection of PRRs and

α-defensin secretion is induced by endogenous or microbial stimuli. In addition to α-defensins, which account for around 70% of the secreted bactericidal activity [90], Paneth cells also secrete the antimicrobial proteins lysozyme P, secretory phospholipase A2, and the recently discovered C-type lectins, Reg3γ and Reg3β [88, 91].

Defensins display broad spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria with some additional activity against fungi, viruses and protozoa. They are highly cationic and are believed to disrupt the membrane integrity of their bacterial targets by interaction with negatively charged phospholipid groups on the outer membrane. Displacement of lipids by integration between phospholipid groups alters the membrane stability and finally leads to membrane disintegration and potentially pore formation [92]. The biological importance of defensins has been demonstrated by the generation of MMP7-deficient mice, which are more susceptible against oral infection with *Salmonella enterica* ssp. *enterica* sv. Typhimurium (*S.* Typhimurium) [93] and display significant alterations of the enteric microbiota composition [17]. Conversely, transgenic mice expressing the human α-defensin 5 (HD5) display enhanced resistance against *S.* Typhimurium infection [94].

Cathelicidins also represent cationic amphipathic peptides but in contrast to α-defensins form α-helical or β-hairpin structures. They are produced by proteolysis of the C terminus of cathelin-domain-containing protein precursors. In humans and mice, only one cathelicidin precursor, hCAP18 and CRAMP, respectively, is produced, whereas in cattle and pigs this peptide family comprises a large number of members and is highly diverse. Cathelicidins are expressed in the skin, lung and intestinal tract by a variety of cell types including neutrophils, mast cells and epithelial cells.

More recently, the C-type lectin Reg3γ was described as an antimicrobial protein expressed in the intestinal tract both by absorptive enterocytes and Paneth cells. Reg3γ is particularly active against Gram-positive bacteria [95]. Expression of this protein is induced by the presence of the microbiota [95], depends on the IL-1R and TLR adaptor molecule MyD88 and is at least in part mediated by an intrinsic regulatory loop mediated by IL-22 [96]. Activity of Reg3γ is further modulated by proteolystic cleavage of a negatively charged N-terminal inhibitory prosegment from the positively charged core protein by trypsin [97]. Although the processing of Reg3γ resembles the processing of α-defensins, the mechanism of action against bacteria is different. While mature cationic α-defensins bind to negatively charged bacterial phospholipids, Reg3γ specifically interacts with native peptidoglycan at the bacterial surfaces [97, 98]. Reg3γ expression was shown to also contribute to the antimicrobial host defence using the model of oral infection with *Listeria monocytogenes* [96]. In addition to their direct antimicrobial function, antimicrobial peptides have been shown to exert immunomodulatory functions. They bind to LPS and neutralize its proinflammatory activity, exhibit chemoattractive activity, promote wound healing and modulate dendritic cell responses [99].

A number of studies have shown that expression of antimicrobial peptides is also under developmental control. As outlined before, morphogenesis of the mouse small intestine is not completed until the the third week after birth. Crypts form from epithelial cells at the intervillus region after the first week and undergo proliferation by crypt fission between the second and third weeks [100]. The emergence of Paneth cells coincides with crypt morphogenesis and a dramatic increase in epithelial stem cell proliferation and is independent of the presence of the microbiota. The differentiation of Paneth cells includes the sequential expression of α-defensins, phospholipases and lysozyme [101]. The regulation α-defensin expression is generally considered not to occur on the transcriptional level, and total α-defensin mRNA levels were found to be similar in the presence or absence of the intestinal microbiota [102, 103]. Yet, some α-defensins, especially α-defensin 6, might be expressed by epithelial precursor cells before the emergence of Paneth cells [104], although at a much lower level [105]. Also, a significantly higher gene expression of a small group of α-defensins was reported in conventional as compared to germ-free mice [106]. In particular, α-defensin 4 and 5 were found to be significantly reduced in germ-free mice. Differences were also noted in the course of mRNA expression during postnatal development between individual α-defensin isoforms. One group, including α-defensin 1, 3 and 6, show a more gradual increase during the postnatal period whereas another group, including α-defensin 2 and 5, exhibit a rapid increase in gene expression accompanying the onset of Paneth cells. Differences in the experimental approaches (e.g., m-RNA vs. protein level) and the use of oligonucleotide probes that detect several members of this highly conserved large group of peptides simultaneously might account for some observed discrepancies [102, 106, 107]. Although generally coregulated, individual α-defensins might be influenced by endogenous or exogenous factors and play a distinct role in intestinal homeostasis and antimicrobial host defense [106].

In contrast to the delayed appearance of α-defensin expression in Paneth cells of the murine small intestine, high expression of the cathelicidin CRAMP by the intestinal epithelium is found at birth. Strikingly, CRAMP expression is restricted to the first postnatal 2 weeks and gradually disappeares with the appearance of crypts, Paneth cells, and α-defensins [105]. Again, the downregulation of CRAMP during postnatal development is

independent of the enteric microflora. CRAMP-deficient neonates are more susceptible to oral infection with the Gram-positive enteric pathogen *L. monocytogenes*. Whether CRAMP also contributes to the postnatal establishment of the enteric microflora still needs further investigation.

Postnatal development of the intestinal microbiota

The fetus develops in a sterile and environmentally protected environment within the amniotic membranes in the uterus. Microbial exposure and bacterial colonization of mucosal surfaces, however, start immediately at birth. Already with passage through the birth canal and during the immediate postnatal period, maternal and environmental bacteria are transferred to the neonate's body surfaces. The intestinal tract provides a favorable environment for commensal bacteria providing essential nutriments for their metabolism [14, 108]. A number of studies have investigated the postnatal development of the intestinal flora in mice [109–112]. Although neonatal rodents are exposed to greater numbers of environmental microbes than human neonates and differences have been observed on the species level, the principal scenario of the succession of intestinal microbial colonization in rats and mice resembles that in human neonates. Facultative anaerobic or microaerophilic bacteria like *Lactobacilli* and *Streptococci* dominate during the first week after birth, followed a few days later by *Enterococci* and members of the Enterobacteriaceae. These bacteria reduce the local oxygen concentration by their metabolic activity and thereby establish the milieu for the subsequent colonization by strictly anaerobic bacteria like *Bifidobacteria*, *Bacteroides* spp. and *Clostridium* spp. [109–113].

Colonization of the newborn intestinal mucosa is influenced by a variety of factors including the mode of delivery, gestational age, environmental factors such as hygiene and lifestyle and diet (i.e. formula vs. breast milk) [30, 31, 33, 114, 115]. Caesarean section-born infants, for example, undergo delayed colonization with an altered flora compared to vaginally delivered infants [31]. Significant differences in the enteric microbial colonization have also been found between breast-fed infants in which *Bifidobacteria* represent the dominant group whereas formula-fed infants harbor high numbers of *Bacteroides* spp., enterobacteria, *Clostridium* spp. and *Lactobacilli* [30]. The diversity of the infant's intestinal microbiota increases gradually over time with major shifts at weaning or with changes in their diet [33, 116]. In addition to alterations in the environmental exposure, the increased diversification might also be influenced by the decline in maternal SIgA [117]. Most of the intestinal bacteria of adult mice establish within 3–5 weeks after birth [112], and obligate anaerobes of the phylum *Bacteroides* and *Clostridiales* represent the most abundant species after weaning.

The mature microbiota of an adult individual consists of 10^{14} bacteria representing approximately 500 species. Its composition stays relatively stable throughout the whole life. It fulfils a variety of important biological functions. Microbial enzymes help to process ingested nutrients and thereby influence the metabolism and digestive efficiency and regulate host fat storage. The dense population of commensal bacteria at the mucosal surface prevents adhesion and subsequent colonization by pathogenic species, a mechanism termed colonization resistance. Finally, the presence of the microbiota stimulates mucosal angiogenesis and significantly contributes to the maturation of the gut innate and adaptive immune system particularly during the postnatal period [118–121].

Susceptibility of neonates to inflammatory and infectious diseases

The most common inflammatory diseases of the gastrointestinal tract of preterm infants is NEC. Several contributing factors have been identified including breaches in the intestinal mucosal barrier leading to bacterial translocation, transient mucosal ischemia, cytokine induction and enteral feeding. The precise mechanism underlying the pathogenesis, however, is still unclear [122]. Increased adhesion of disease-promoting bacterial species to the immature mucosal surface was identified as possible risk factor for NEC [122]. Also, increased epithelial expression of the lipopolysaccharide (LPS) receptor TLR4 and enhanced TLR4-mediated signaling in response to hypoxia have been associated with NEC in humans and mice [123, 124]. The critical role of TLR4 has been illustrated by the finding that gene-deficient mice are protected against disease in a murine NEC model [124]. More recently, reciprocal expression patterns of TLR4 and TLR9, the receptor for bacterial CpG DNA, have been observed in the developing mouse intestine. TLR9 signalling inhibits TLR4-mediated cell activation in an IRAK-M-dependent manner. In accordance, activation of TLR9 in a murine NEC model ameliorated the tissue damage whereas TLR9-deficient mice exhibited enhanced disease severity [125]. Furthermore, induction of proinflammatory cytokines and reactive oxygen species (ROS), generated as a result of ischemia/reperfusion injury in the gut, have been linked to the development of NEC in premature infants. An important role may be played by the proinflammatory cytokine TNF-α. Whereas TNF-α via the TNF receptor (TNFR)2 induces Muc2 and Muc3 expression by goblet cells in the mature intestinal mucosa, it causes loss of Muc2-containing goblet cells in a TNFR1-dependent manner in immature

pre-weaning mice. Of note, reduced goblet cell numbers were also found in the intestinal mucosa of human infants with NEC [126]. Additionally, TNF-α stimulation induces an increase of intestinal permeability by the degradation of occludin, a component of the tight junction [127].

In both mouse and human neonates, the immune response towards microbial infection is generally reduced as compared to mature adult individuals illustrated by an enhanced susceptible to gastrointestinal infection. A number of enteropathogenic microorganisms including rotavirus, *Shigella*, *Listeria monocytogenes* and *Salmonella enterica* affect neonates and infants more severely.

Rotavirus infection represents one of the leading causes of dehydrating diarrhea among children worldwide with, according to the World Health Organization, approximately half a million deaths per year particularly in areas with insufficient access to medical care [128]. It mainly affects children under the age of 6 years. Similarly, the susceptibility to rotavirus is highest between days 3 and 11 after birth in mice and decreases abruptly at weaning. The age-dependent susceptibility to infection has been associated with postnatal maturation of the intestinal mucosa and can be modulated by administration of glucocorticoids, which induce premature intestinal maturation [129]. The antiviral innate immune response of the neonate intestinal mucosa largely relies on the production of type III interferon and the inhibition of viral spread at the intestinal epithelium [130]. An adaptive T cell-mediated antiviral host response, however, is required to terminate viral replication and eliminate the virus.

The ubiquitous bacterium *Listeria monocytogenes* represents an opportunistic human pathogen which predominantely infects immunocompromised patients, pregnant women, elderly people and neonates [131]. In a murine infection model; increased levels of systemic IL-10 were detected in neonate animals compared to adult mice. Anti-IL-10 treatment decreased the bacterial burden in neonates at early and late time points after infection whereas this treatment was only effective at early stages of infection in adult mice [132]. More recently, lack of cytotoxic T cell activation and poor IFN-γ secretion was shown in neonatal mice after intraperitoneal infection with *L. monocytogenes*. Reduced immune activation was correlated with low expression of the mannose-bind lectin (MBL) and PRRs such as TLRs required to mount an efficient T_H1 response [133]. Due to an amino acid exchange in the mouse gene encoding epithelial E-cadherin, an important epithelial host receptor for enteric *L. monoctogenes* invasion, oral infection of mice requires high doses of infection [134]. Therefore, tudies comparing neonate and adult animals in humanized transgenic mice expressing human E-cadherin still need to be performed [135].

Adult mice are largely resistant to infection with *Shigella flexneri*, the causative agent of human bacillary dysentery characterized by an acute colonic inflammation. In contrast, newborn mice are highly susceptible and develop acute, lethal enteritis [136]. The enhanced susceptibility of neonatal mice during the first week after birth was explained by the lack of intestinal Paneth cells during early postnatal development and thus reduced production of antimicrobial peptides. Accordingly, depletion of Paneth cells in adult animals rendered adult animals susceptible to *Shigella* infection [137]. Also, MMP7-deficient mice unable to proteolytically process mature enteric α-defensins displayed a higher bacterial load and increased inflammation than wild-type animals after oral *Shigella* challenge [138].

Age-dependent differences in susceptibility to infection have also been reported for oral challenge of mice with *S.* Typhimurium [50, 139]. Additionally, mutant bacteria that exhibit an attenuated phenotype in adult mice are still able to infect neonatal and suckling mice systemically [139]. Neonatal mice show an attenuated inflammatory response and a higher systemic bacterial burden. IFN-γ in adult mice is required for an efficient host defence against *Salmonella* and the increased resistance of adult mice was correlated with an age-dependent increase of IFN-γ- and IFN-γ-regulated genes. The source of developmentally regulated IFN-γ most likely is not of epithelial nature. This cytokine, however, targets a number of cell types including epithelial cells and induces a variety of epithelial defence mechanisms against intracellular pathogens [50].

Strikingly, neonatal mice were shown to be more resistant than adult mice to an oral infection with *Yersinia enterocolitica*, an enteric pathogen causing gastroenteritis in humans [140]. These results differ from the situation in humans, in which two-thirds of *Y. enterocolitica* infections occur among infants. After oral infection, *Y. enterocolitica* disseminated to spleen and liver in adult mice whereas the spread to these organs was restricted in neonates. The lower bacterial load in spleen and liver of neonate mice correlated with an enhanced survival. The enhanced resistance of neonates was only observed after oral infection. Since bacterial spread to spleen and liver was largely controlled by neutrophils and the percentage of neutrophils and macrophages was increased in neonatal mesenteric lymph nodes compared to adult tissue. the authors of this study speculated that neonates maybe more resistant due their ability to rapidly mobilize innate phagocytes to the site of infection [140]. Additionally, the strong innate immune response in neonates orally infected with *Y. enterocolitica* promotes a robust protective $CD4^+$ T cell-dependent immune responses [141]. It is unclear, however, whether this rapid mobilization of neutrophils is restricted

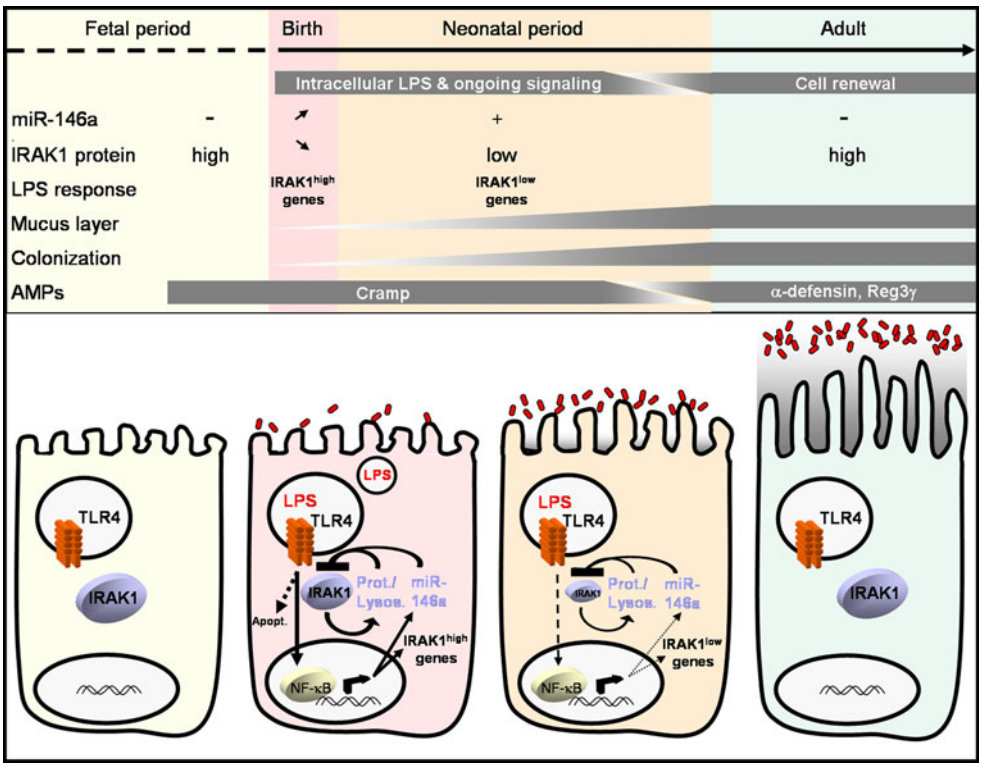

Fig. 1 Summary of changes taking place in the intestine from the fetal period to adulthood

to *Y. enterocolitica* infection and why this mechanism is not protective in other infection models.

Conclusions

Many aspects of the intestinal innate and adaptive immune system as well as the intestinal epithelial barrier undergo significant changes during the postnatal period. This includes the rate of epithelial cell proliferation, cell differentiation and gene expression, the spectrum of synthesized antimicrobial peptides, and maturation of the mucosal immune system, and also environmental factors such as bacterial colonization, nutrient composition, and exposure to immunomodulatory factors in breast milk. Whereas many adaptive changes are induced by exogenous stimuli such as the microbial colonization, developmental regulatory circuits are also involved. Together, the changes characterize a unique adaptive process that governs the transition from a sterile, environmentally protected site in utero to the situation of the adult intestine, densely populated by a highly diverse microbiota and exposed to a large variety of nutritional and environmental substrates (Fig. 1). Further characterization of the mechanisms involved will illustrate the enormous challenge of the mucosal surface to establish the delicate host–microbial interaction and unravel new factors critical to establish, but also to maintain and restore, intestinal mucosal homeostasis. Thus, the analysis of the processes that occur at the intestinal mucosa during the postnatal period might ultimately also lead to a better understanding of inflammatory diseases in the adult host and help to develop strategies to restore a beneficial homeostatic mucosal host–microbial interaction.

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