The Journal of Immunology

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J Immunol 2017; 198:557-563; ;
doi: 10.4049/jimmunol.1601253
http://www.jimmunol.org/content/198/2/557

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Brief Reviews

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The Neonatal Window of Opportunity: Setting the Stage for Life-Long Host-Microbial Interaction and Immune Homeostasis

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The existence of a neonatal window was first highlighted by epidemiological studies that revealed the particular importance of this early time in life for the susceptibility to immune-mediated diseases in humans. Recently, the first animal studies emerged that present examples of early-life exposure–triggered persisting immune events, allowing a detailed analysis of the factors that define this particular time period. The enteric microbiota and the innate and adaptive immune system represent prime candidates that impact on the pathogenesis of immune-mediated diseases and are known to reach a lasting homeostatic equilibrium following a dynamic priming period after birth. In this review, we outline the postnatal establishment of the microbiota and maturation of the innate and adaptive immune system and discuss examples of early-life exposure–triggered immune-mediated diseases that start to shed light on the critical importance of the early postnatal period for life-long immune homeostasis. *The Journal of Immunology*, **2017, 198: 557–563.**

Immune-mediated diseases, such as allergies and inflammatory bowel disease (IBD), are highly prevalent in western countries and are associated with significant morbidity. Despite decades of intensive research on the associated functional and structural alterations, the disease etiology and, thus, the decisive molecular mechanisms underlying disease initiation have not been resolved. For example, the search for unknown pathogenic microorganisms revealed some interesting candidates but no uniform causative agent. Also, genome-wide association studies identified a large number of susceptibility loci, but many individuals that carry these mutations never experience clinical symptoms. Therefore, the identified factors might enhance disease susceptibility and/or promote the progression and severity of clinical symptoms rather than play a decisive role during the initiation of the disease. However, only the identification of the first step in disease pathogenesis will allow us to develop effective strategies for future disease prevention.

Approximately three decades ago, researchers noticed dramatic changes in the interaction of the human host with pathogenic microorganisms in industrialized countries. The number of infections decreased very significantly during the second half of the twentieth century as a consequence of improved medical healthcare, including effective vaccine strategies and antibiotics, as well as better hygiene and living standards (1, 2). The concomitant increase in immune-mediated diseases, such as Crohn's disease and asthma, as well as diabetes and multiple sclerosis, led to the hygiene hypothesis that proposed a causative relationship between the decrease in infectious diseases and the increasing burden of immune-mediated and allergic diseases. Consistent with this idea, a steady increase in IBD and asthma was subsequently observed in other geographical areas with previous very low incidence following the implementation of effective healthcare systems and infection-control measures (3, 4). Even more strikingly, nematode infection, a highly endemic type of infection in geographic areas with low IBD incidence, resulted in a significant clinical improvement in IBD patients (5). Later, epidemiological studies extended this view to include exposure to environmental microbial constituents, as well as commensal bacteria (6, 7). This was first noted when farm children were compared with their urban counterparts (8). Raw milk consumption and exposure to the livestock-produced feces within the stable environment with a high microbial load and potent immunomodulatory activity were identified as critical factors (9). High endotoxin concentrations in animal feces and the farm environment were identified as functional triggers of regulatory mechanisms and immune homeostasis, yet other less-well detectable microbial stimuli or even viable bacteria might contribute to this effect (10, 11). Indeed, the reduction in the prevalence of certain infectious diseases was paralleled by major alterations in the enteric microbiota composition (12, 13). Loss of individual bacterial members of a healthy microbiota and a general reduction in bacterial diversity were noted and may contribute to enhanced disease susceptibility (13). Consistently, individual members of the microbiota were assigned a specific preventive or disease-promoting function in immune modulation. For

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Received for publication July 19, 2016. Accepted for publication October 17, 2016.

This work was supported by the Deutsche Forschungsgemeinschaft (Ho2236/8-1, Priority Program 1656, and Priority Program 1580 [to M.W.H.] and TO 1052/1-1 [to N.T.]).

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Abbreviations used in this article: DC, dendritic cell; IBD, inflammatory bowel disease; iNKT, invariant NKT; pTreg, peripheral-induced Treg; Treg, regulatory T cell; tTreg, thymus-derived regulatory T cell.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1601253

example, the segmented filamentous bacterium *Faecalibacterium prausnitzii* and the mucin-degrading bacterium *Akkermansia muciniphilia* were shown to promote mucosal immune cell maturation, immune homeostasis, or a beneficial host metabolism in mice and men (14–16). Yet, fecal transplantation, despite impressive clinical benefits in certain patient cohorts, such as patients with recurrent *Clostridium difficile* infection (17), has failed to provide a uniform and lasting clinical response.

Soon, epidemiological studies identified the particular influence of age. For example, farm exposure during fetal and early postnatal life exerted the strongest protective effect (8, 18). Also, antibiotic use during pregnancy and early childhood was reported to represent a risk factor for allergic disease development (19). These findings introduced yet another model in which exogenous factors present during early development could also directly or indirectly influence immune homeostasis and disease susceptibility during later life. Indeed, the immune system at birth differs significantly from that of adult individuals, and the postnatal period contributes significantly to immune development and maturation. Also, microbial colonization starts at birth and eventually generates a life-long, relatively stable ecosystem. This early priming was later described as the neonatal window to highlight the exclusive and nonredundant function of the early postnatal and infant period for life-long immune homeostasis (Fig. 1). A number of well-controlled animal studies has begun to unravel the mechanisms that underlie the observations from epidemiological studies (Table I) (20–23). In this article, we discuss human and mouse studies that reveal the critical and nonredundant role of the neonatal window for immune homeostasis and its implication for future research.

Early microbial exposure and generation of the mature microbiota

It is generally accepted that the healthy fetus is devoid of colonizing viable microorganisms. However, reports on the PCR- or culture-based detection of bacteria in presumably sterile mouse and human fetal or placental tissue samples stimulated discussion on the possible existence of a fetal or placental microbiome (reviewed in Ref. 24). The common idea is that only with rupture of membranes do viable commensal bacteria start to colonize the newborn's body surfaces. It is thought that the fetus first encounters bacteria derived from the maternal vaginal microbiota during passage through the birth canal. Thus, the vaginal microbiota represents the first inoculum to colonize the newborn. Of notable difference, cesarean section–delivered human neonates first encounter bacteria of the skin of the mother or health care worker; thus, in this case, the skin microbiota represents the primary source (25). High interindividual variation, but low diversity and density, characterize this early colonization phase that is dominated by bacteria specialized in milk fermentation, such as *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*, in humans (26, 27). Given the nutrient-rich environment provided by breast milk in the neonatal gut lumen, microbial density reaches plateau levels only a few days later (28). In both mouse and man, the microbiota at this stage is highly sensitive to exogenous perturbations (e.g., formula feeding or antibiotic administration) that delay the development of a mature diverse microbial community (29). Microbiota alterations, in turn, render the bacterial ecosystem less resilient to further perturbations (30). Subsequently, population dynamics of the commensal bacterial communities are shaped by newly introduced bacterial species, the local conditions in the neonatal intestine, and niche and nutrient availability (31). With the cessation of breastfeeding and the introduction of solid food, the microbiota composition changes dramatically, and the diversity rises, mirroring the increasing complexity of nutrients. At this stage, predominance of Clostridiales and Bacteroidetes that is typical of the adult colonic microbiota in mice is noted in most individuals (32). The diversity of the adult microbiota is reached in humans after ~1–3 y and correlates with an increase in the overall compositional stability (27, 33). This compositional stability progressively generates what is called colonization resistance. Newly introduced bacteria barely gain access to niches in the intestinal lumen and nutrient supply and fail to become a permanent member of this ecosystem (34–36). This strongly enhances the resistance to many enteric pathogens in adult individuals. Thus, the lower compositional stability and colonization resistance in neonates and young infants may explain the particular susceptibility of the neonate to certain foodborne infectious agents (35, 37). It may also allow longer persistence of probiotic bacteria and provide an explanation for the stronger effects observed after administration of probiotics in the human pediatric population (38). Although environmental factors, such as mode of delivery, nutrition, or antibiotic treatment, significantly alter the human neonatal microbiota composition, perturbations generally resolve within 2 y (29). The first known perturbing factor in life, cesarean section, was associated with an increased risk for immune-mediated diseases, such as asthma, allergy, and celiac disease, in childhood (39–42). Also, the use of antibiotics was associated with atopic diseases, such as asthma and atopic dermatitis, in human infants (43, 44). It is under debate whether microbiota alterations, such as those detected in cesarean section–delivered children, exert functional consequences. Only the generation of a disease state after transfer of the altered microbiota is able to prove this direct functional link.

Establishment of the mucosal innate immune defense

The mucosal innate immune system undergoes major alterations after birth that might help to define the neonatal window. These alterations occur as part of a developmental program or in response to environmental stimuli. They are particularly dominant in mice that exhibit a very short gestational period and are born with an immature intestinal epithelium. For example, the newborn small intestinal epithelium exhibits enhanced permeability to soluble Ags and is devoid of small intestinal crypts that only develop at weaning and harbor intestinal stem cells and antimicrobial peptide-producing Paneth cells (45, 46). The lack of Paneth cell–derived antimicrobial peptides may be compensated for by the cathelin-related antimicrobial peptide that is produced by murine enterocytes in the neonatal small intestine (47). Also, expression of mucins, building blocks of the intestinal mucus layer, is low in the neonate and rises at weaning (35). Finally, epithelial innate immune recognition varies in an age-dependent manner. Epithelial expression levels of the pattern recognition receptors Tlr3, Tlr4, and Tlr9 are expressed in an age-dependent fashion in mice (48, 49). Also, postnatal

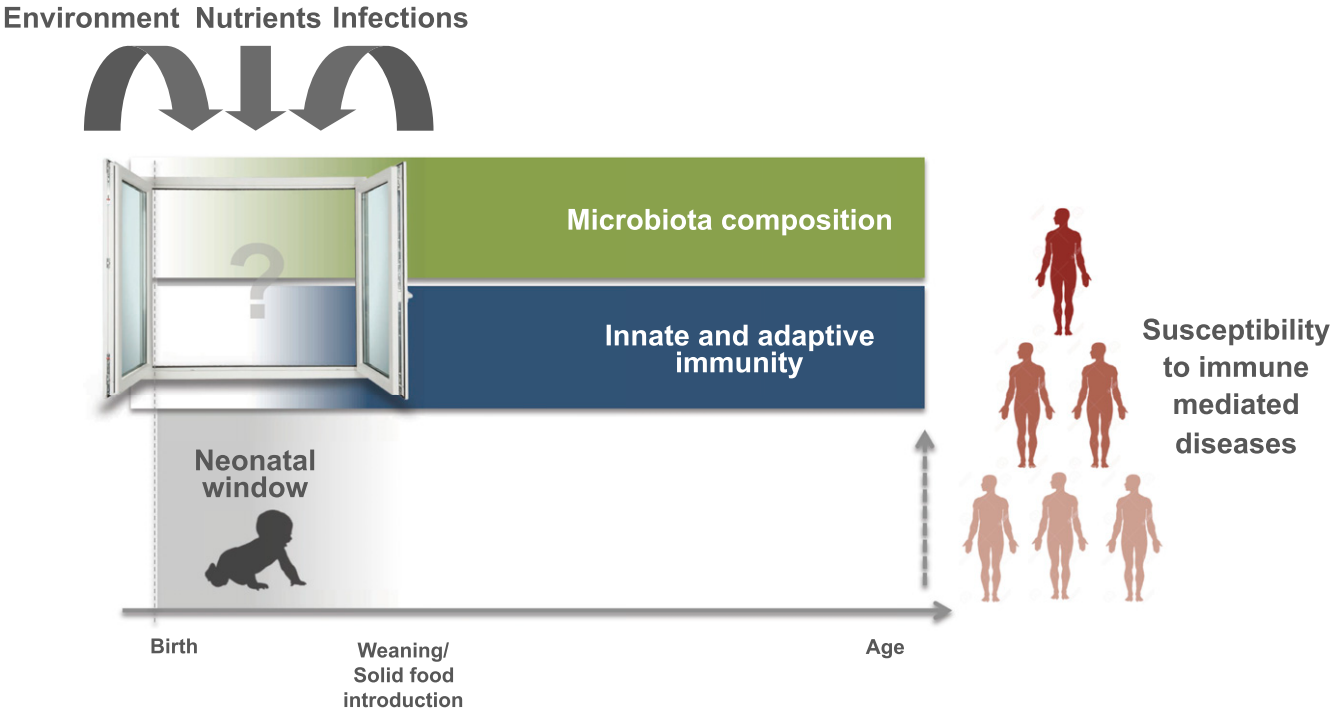

FIGURE 1. The neonatal window of opportunity. Environmental factors (e.g., rural versus urban), nutrients (e.g., breast milk versus formula versus solid food), and infections (e.g., antibiotic treatment) impact the dynamic development of the postnatal microbiome and the maturation of the innate and adaptive immune system during the neonatal phase. The mechanisms of these interactions remain mostly unresolved. The functional consequences skew the delicate balance toward an increased susceptibility to immune-mediated diseases, such as asthma, IBD, and allergy, far beyond the neonatal phase.

exposure to bacterial endotoxin induces a transient state of innate immune tolerance of the intestinal epithelium that helps to prevent an exaggerated Tlr-mediated immune stimulation during early colonization in mice (50, 51). In contrast, the structure of the human intestinal epithelial layer is fully developed at birth. Functional analyses in the healthy intestinal epithelium are limited by ethical concerns. Nevertheless, lower expression of TLR3 and enhanced expression of the NF-κB RelA inhibitor IκB-α were demonstrated in the human neonatal epithelium (49, 52). Also, postnatal innate immune tolerance by induction of the regulatory secretory leukocyte protease inhibitor was observed in the human upper gastrointestinal tract epithelium (53). Interestingly, an altered concentration of immunostimulatory endotoxin within the gut lumen as a consequence of an altered microbiota composition was reported recently (13). Thus, an impaired balance between microbial stimulation and tolerance to innate immune stimuli might promote the prevalence of autoimmune diseases in humans (11, 13). A recently reported novel approach analyzing enterocyte transcription in fecal material of term and preterm human neonates by RNA-sequencing identified differential expression, primarily in genes involved in lipid metabolism and immunity (54). This approach also might help to examine age-dependent changes in cell differentiation and correlate mucosal immune signaling with bacterial colonization in the future.

Maturation of the adaptive mucosal immune system during the postnatal period

Similarly, the adaptive immune system in the neonate host differs significantly from the adult situation in mice and men. This was long believed to represent a state of immaturity in neonates that explains the increased susceptibility to many infectious diseases (55). However, we have come to understand that the neonate is well able to mount adult-like immune responses under certain conditions, such as a reduced pathogen inoculum or increased adjuvant dose (56–58). Also, specific features of the neonate mucosal immune system have been unraveled that suggest a specialized (rather than immature) nature of the neonate immune system.

In the murine host, naive TCRαβ and B lymphocytes are released approximately at birth from the primary lymphoid organs and home to the periphery, including mucosal body sites (59). In contrast to the adult host, homing of lymphocytes to the intestine is independent of microbial cues (60). At the mucosa, lymphocytes encounter myriads of mostly innocuous Ags derived from the environment, the rapidly evolving microbiota, and diet. Several mechanisms were identified that might influence the interaction of the neonatal immune system with the postnatal environment. In the murine intestine, the general cell composition and immune tissue architecture are markedly different from the adult state (60). TCRαβ$^+$ and B cells are localized exclusively to the Peyer's patches and exhibit a naive phenotype until weaning under steady-state conditions (60). This is in stark contrast to the situation in the adult tissue, wherein microbiota and food Ags engage the adaptive immune system in a continuous interplay, resulting in the presence of Ag-experienced lymphocytes in Peyer's patches, lamina propria, and the intraepithelial space at steady-state (60–63). In humans, the thymus produces functional lymphocytes at the beginning of the second trimester, and lymphocytes are found in all compartments of the intestine at birth (64, 65). However, lymphocyte maturation is also delayed in human infants and, thus, may

Table I. Experimental studies that demonstrate the influence of microbial exposure during the fetal/neonatal period on the susceptibility to immune-mediated diseases

Age compared	Exposure modifications	Mechanism in the neonate	Mechanism in the adult	Disease susceptibility	Refs.
Birth vs. adult	Abx/GF/*B. fragilis* monocolonization vs. SPF	?	Lack of *Cxcl16* suppression (lung and gut); *B. fragilis* derived sphingolipids; expansion of iNKT cells	AHR, colitis	(20, 21)
Birth vs. adult	GF/simple(r) Schaedler flora vs. more complex Schaedler flora	?	T cell-dependent class switch of B cells in Peyer's patches; hyper IgE	Allergy	(22)
Birth vs. adult	GF vs. monocolonized	?	Lack of oral tolerance	Allergy	(88, 89)
1w, 2w, and 3w	SPF only	Vitamin A deficiency; impaired DC function; lack of IFNγ	Lack of oral tolerance	Allergy	(46)
3d, 15d, 60d	GF vs. SPF		PD1-PD1L interaction between pTreg and DCs	AHR	(74)
7d vs. adult	SPF+Bacteroides vs. SPF	Expansion and activation of Treg	Neonatal Treg-mediated tolerance to skin commensals		(75)
2w	Abx vs. SPF	?	Suppression of lymphocyte mediated IL-22 production; barrier defect	Food allergy	(85)
Birth vs. adult	Abx vs. SPF	?	Enhanced IL-22 production by innate and adaptive lymphocytes	Psoriasis	(23)
Postnatal vs. adult	SPF only	Generation of tTreg in the neonatal thymus	Enhanced suppressory capacity of neonatal PD1+ Tregs	Certain types of autoimmunity	(68, 94)
Prenatal	Reversible gestational colonization vs. GF	Transfer of bacterial constituents across the placenta; transfer of bacterial constituents from dam to neonate; trancriptional changes in intestinal tissue, increased ILC3 and myeloid cells	Increased ILC3 and myeloid cells numbers	Increased barrier permeability	(82)

Abx, antibiotic; AHR, airway hyperresponsiveness; GF, germ-free; ILC3, type 3 innate lymphoid cell; PD1, programmed cell death protein 1; SPF, specific pathogen–free.

underlie similar mechanisms (66, 67). Ethical concerns limit the access to intestinal tissue samples from healthy human neonates and have restricted in-depth functional analyses.

Homing of lymphocytes to the periphery is accompanied by a concomitant release of thymus-derived regulatory T cells (tTregs) at the fetal and neonatal stage in mice and men, respectively (68, 69). Human fetal lymph nodes contain Tregs at higher densities compared with their adult counterparts and are essential to maintain self-tolerance (69). In the murine host, tTregs are required to mediate life-long immunity to self-antigens and contribute to the general state of tolerance toward the enteric microbiota (68, 70). They suppress the homeostatic activation of naive T cells within the Peyer's patches during the early postnatal period that is characterized by the establishment of the microbiota in the murine host (60). With weaning approximately 3 wk after birth, a second population of Tregs, peripheral-induced Tregs (pTregs), is observed that provides an additional regulatory control of the host-microbial interplay in the intestine (71). Food Ags represent the major inducer for pTregs in the small intestine, whereas microbiota-derived Ags prime pTregs in the colon (72). Whether pTregs are already present among the gut Treg population in the human neonate is unclear (73). Also, in the murine lung, Helios$^+$ tTregs dominate during the early phase of the host-environmental interaction that is prone to the development of an airway hyperresponsiveness. These cells expand greatly during the first week of life dependent on microbial exposure. Only after the second week of life do programmed death ligand-1–dependent pTregs develop that efficiently control the susceptibility of the airways to environmental stimuli and inappropriate responses (74). Also, in the skin, significant expansion and activation of (presumably) tTregs are observed around the second week of life, promoting tolerance to commensal bacteria that colonize the dermal tissue (75). Interestingly, the increase in Tregs in the murine skin coincides with the morphogenesis of hair follicles, which provide a reservoir of commensal bacteria (76).

An additional mechanism of tolerance induction acts during fetal and postnatal development in mice and men: the tolerization against noninherited alloantigens and other Ags present in the maternal organism. During fetal development, this takes place by transfer of maternal cells via the placenta (77, 78). Interestingly, maternal-derived Ags can also be transferred postnatally by breastfeeding and lead to tolerance in transplantation, as well as allergy models (79, 80).

IgA-producing plasma cells are only detected after weaning in mice (60–63). However, maternal Igs transmitted transplacentally and by breast milk facilitate high concentrations in serum and at mucosal body sites. Of note, the mother also represents the most important source of the microbiota by transferring the newborn's first inoculum at birth and providing a constant supply of viable microorganisms in breast milk (25, 81). Although the specificity of the mucosal Abs is ill defined, it may allow the simultaneous transmission of commensal bacteria with their corresponding Igs. This could restrict bacterial penetration of the neonatal mucosa and may explain why maternal Igs contribute significantly to control T and B cell activation in the neonatal intestine (60, 61). Maternal microbiota-induced Igs might thereby help to

cope with the presence of proinflammatory microbial constituents and prevent inflammatory responses (82). Particularly the transfer of microbiota-specific IgG2b and IgG3 together with IgA may contribute to the inhibition of mucosal T cell responses during the postnatal period in mice (83).

The impact of early-life exposure on later-life health and disease susceptibility

The important role of microbiota-derived factors, particularly in immune development and maturation, is best illustrated in animals raised in germ-free environments (84). Germ-free mice exhibit a number of significant differences in their immune system, including the cellular composition and maturation status at mucosal sites. Interestingly, the phenotype of germ-free mice is only partially rescued by bacterial colonization after weaning, demonstrating the critical role of early-life exposure for life-long immune homeostasis (Table I). Among these distinct features is the excessive homing of invariant NK T (iNKT) cells to the lung and colon (but not small intestinal) tissue in germ-free animals. iNKT cells expand in gut and lung tissue of germ-free mice in response to Cxcl16. The presence of the microbiota prior to weaning (but not thereafter) suppresses Cxcl16 expression by epigenetic regulation, explaining the high iNKT cell count in germ-free animals (20). An alternative Cxcl16-independent mechanism to suppress the expansion of iNKT cells was identified after monocolonization of neonates with the commensal *Bacteroides fragilis*. *B. fragilis* glycosphingolipids that represent cognate Ags of iNKT cells are also able to suppress the expansion of iNKT cells in the gut (21). Importantly, enhanced iNKT cell numbers confer increased susceptibility to mucosal inflammation in lung and colon tissue, thus linking neonatal microbial exposure with the susceptibility to inflammatory diseases in later life (20, 21). Another example is the elevated systemic IgE levels found in germ-free animals. In the absence of microbiota, IgE class switch takes place in a T cell– and IL-4–dependent manner within Peyer's patches. This phenotype can be reversed, and an atopic (anaphylactic) reaction can be prevented only if the mice are colonized with a relatively diverse microbiota at birth (22).

Administration of antibiotics transiently reduces the microbial load and alters the bacterial composition. Although still poorly understood, the developing microbiota in human neonates and infants may be particularly sensitive to antibiotic-induced disturbance, with potential lasting consequences (22, 29). Antibiotic exposure during pregnancy or the first year of life in humans slightly, but significantly, enhanced the risk for childhood asthma and atopic dermatitis (43). Similarly, antibiotic administration to mice prior to weaning (at 2 wk of age) led to significant microbiota alterations and enhanced the sensitization to food allergens (85). Administration of spore-forming clostridial species was sufficient to reverse the phenotype and protect from sensitization. In addition to enhanced production of IgA and Tregs, clostridial species stimulated innate lymphoid cell– and adaptive lymphocyte-derived IL-22. In turn, IL-22 tightened the epithelial barrier and reduced the penetration of sensitizing luminal Ags to inductive lymphoid organs, possibly by the induction of antibacterial C-type lectins (85). Although the critical role of IL-22 in epithelial regeneration and antibacterial barrier function is well described, its effect on Ag translocation has not been investigated (86, 87). Similarly, antibiotic administration during the postnatal period rendered mice more susceptible in a model of imiquimod-induced psoriasis (23); however, IL-22 plays an adverse role in this model. A disturbed microbiota with enhanced IL-22 and IL-23 levels was identified to contribute to the more severe disease outcome. This may be explained by a distorted induction of immune tolerance to skin commensal bacteria, presumably by thymus-derived Tregs, by antibiotic treatment during the postnatal period (75). The effect would be expected to be age specific. Indeed, antibiotic treatment of adult animals reduced the expression levels of IL-17 and disease severity after imiquimod administration (23). Whether similar age-specific mechanisms are active at other body surfaces remains to be investigated.

In addition to microbial factors, dietary factors contribute significantly to the mucosal immune response in the neonate. Neonatal mice are more susceptible to allergic sensitization, and their ability to establish oral tolerance increases during the first 3 wk of life (46, 74). The ability to induce oral tolerance (to food and microbiota-derived Ag) critically depends on the presence of microbial stimuli during the postnatal phase (88, 89). A recent study also identified endogenously low levels of vitamin A in the breast milk as a causative mechanism for impaired oral tolerance acquisition in neonates. Low dietary retinol was associated with enhanced para- and transepithelial permeability, as well as reduced expression of RALDH by $CD103^+$ dendritic cells (DCs) and decreased IFN-γ induction in $CD4^+$ T cells (46). Vitamin A supplementation, in turn, enhanced epithelial barrier function, RALDH-expressing $CD103^+$ DCs, and T cell proliferation and facilitated oral tolerance induction shortly after birth. Several studies are underway to examine the effect of early vitamin A supplementation in human children; a report from Ghana did not provide evidence for improved survival (90).

Most studies compared germ-free mice colonized at birth versus those colonized as adults. However, to understand the processes that take place within the neonatal window, it is critical to define its precise time frame. In fact, even fetal exposure appears to exert a major influence on immune maturation (82). Using a reversible colonization model, it was recently shown that even in utero microbial constituents, such as aryl hydrocarbon ligands, can be detected and induce major transcriptional changes in the fetal gut and enhance the cellularity of the innate immune system (82). The underlying functional mechanisms through which microbial cues are conveyed to the neonatal immune system remain largely undefined. Reversible colonization at different time points during the postnatal phase could provide useful insights into the actual critical age that defines the neonatal window.

Conclusions

Environmental stimuli and microbial exposure during the early postnatal period exert a major influence on life-long immune homeostasis and disease susceptibility (Fig. 1). The first microbial encounter appears to set the stage for the subsequent long-term host-microbial relationship. Over the course of evolution, this relationship has been strongly interwoven with infectious diseases that represented the most important cause of death. Only with the late nineteenth century did improved sanitary conditions, such as drinking water supply, wastewater treatment, housing conditions, and, later, effective vaccine

strategies and antibiotic drugs, provide successful preventive measures against infectious diseases. As a consequence, life expectancy increased dramatically, and infectious diseases now rank far below cardiovascular diseases and cancer in developed countries. With the increasing incidence of immune-mediated diseases, we may now experience the downside of our success in preventing the infectious disease burden (13). Therefore, we need to address the question of how to compensate for the lack of microbial exposure during early development without the risk for potential life-threatening infections. Clearly, we do not want to sacrifice our achievements in infection control and prevention (13). Nevertheless, we may have overshot the mark with our attempts to reduce the risk of pathogen exposure. Therefore, we should seek a more moderate attitude that facilitates exposure of our children to benign nonpathogenic microorganisms (91). The first attempts, such as exposure to maternal vaginal fluid after caesarean section or the administration of probiotic bacteria to preterm neonates, are being made (25, 92, 93). An improved understanding of the underlying molecular mechanisms and the identification of strategies to reestablish a healthy host-microbial relationship need further attention. Although recent studies provided first insights into mechanisms that lead to disease in the adult after inappropriate exposure during the postnatal phase, we still lack understanding of what actually happens during the neonatal window itself. We need to address how a distorted microbiota composition may persist and how microbial cues are transferred to the developing immune system in the neonate to confer disease resistance or susceptibility (94).

Disclosures

The authors have no financial conflicts of interest.

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