

Protecting the Newborn and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny

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Infections in the first year of life are common and often severe. The newborn host demonstrates both quantitative and qualitative differences to the adult in nearly all aspects of immunity, which at least partially explain the increased susceptibility to infection. Here we discuss how differences in susceptibility to infection result not out of a state of immaturity, but rather reflect adaptation to the particular demands placed on the immune system in early life. We review the mechanisms underlying host defense in the very young, and discuss how specific developmental demands increase the risk of particular infectious diseases. In this context, we discuss how this plasticity, i.e. the capacity to adapt to demands encountered in early life, also provides the potential to leverage protection of the young against infection and disease through a number of interventions.

Introduction

Infectious diseases are a predominant cause of childhood death (Bhutta and Black, 2013; Hostetter, 2012; Liu et al., 2012). Neonatal infection in particular remains a common tragedy, with ~7 million cases and ~700,000 deaths per year, currently accounting for 40% of mortality in those under 5 years of age (Bhutta and Black, 2013; Blencowe et al., 2013; Darmstadt et al., 2014; Ghazal et al., 2013; Hostetter, 2012; Lawn et al., 2014, 2014; Liu et al., 2012; Murray et al., 2012; Seale et al., 2014). Although neonatal morbidity and mortality due to infection also represents a significant hurdle in resource-rich countries, newborns in resource-poor areas are most severely affected (Agarwal, 2016; Bhutta and Black, 2013; Chan and Lake, 2012; Sepúlveda and Murray, 2014). Given the magnitude of this problem, even modestly effective interventions would save millions of lives and billions of dollars. Broadly enhancing protection from infection and disease through immune modulation offers a feasible approach. However, optimal design and implementation of immunomodulatory interventions requires a deeper understanding of the developmental changes occurring in the neonatal immune system at the cellular and molecular levels.

Human immunity develops from a single-cell state in the early stages of embryonic life during which cell autonomous immunity (CAI) provides protection, to one of biochemical communication amongst collections of cells at which point nutritional immunity plays an increasing role. After several weeks of gestation, specialized cells within the developing fetus provide barrier and innate immune protection. Only after these stages does T- and B-cell-based adaptive immunity in the fetus become

effective, as these require highly specialized tissues (Turvey and Broide, 2010). All of these components of immunity remain active throughout postnatal life, with substantial interaction and cross-regulation.

Furthermore, each has been shaped throughout evolution to ensure our species' survival (Figure 1). In addition to these genetically "hard-wired" programs, the immune system also remains responsive to the rapidly changing demands of each individual's specific environment. This requires straddling of sometimes opposing demands such as preservation of semiallogeneic existence in utero while protecting against a multitude of potentially infectious microbes. The ability of the immune system in early life to handle both, genetically encoded and environmental-driven programming emphasizes its enormous dynamic capacity. As a result, well-placed immunomodulatory interventions can leverage this powerful early life plasticity and direct the trajectory of immune ontogeny to enhance resistance to infectious disease while maintaining immune homeostasis.

Herein we review how the immune system contributes to distinct aspects of host defense in early life, and how specific developmental demands increase the risk of particular infectious diseases. We place these insights into context of interventions that have the capacity to broadly enhance immune-mediated protection from a wide range of infectious diseases in the newborn period and early infancy.

Cell Autonomous Immunity: The Most Ancient Form of Self Defense

Cell autonomous immunity (CAI) guards cells against intracellular infection via a system of compartmentalization, responsive to the

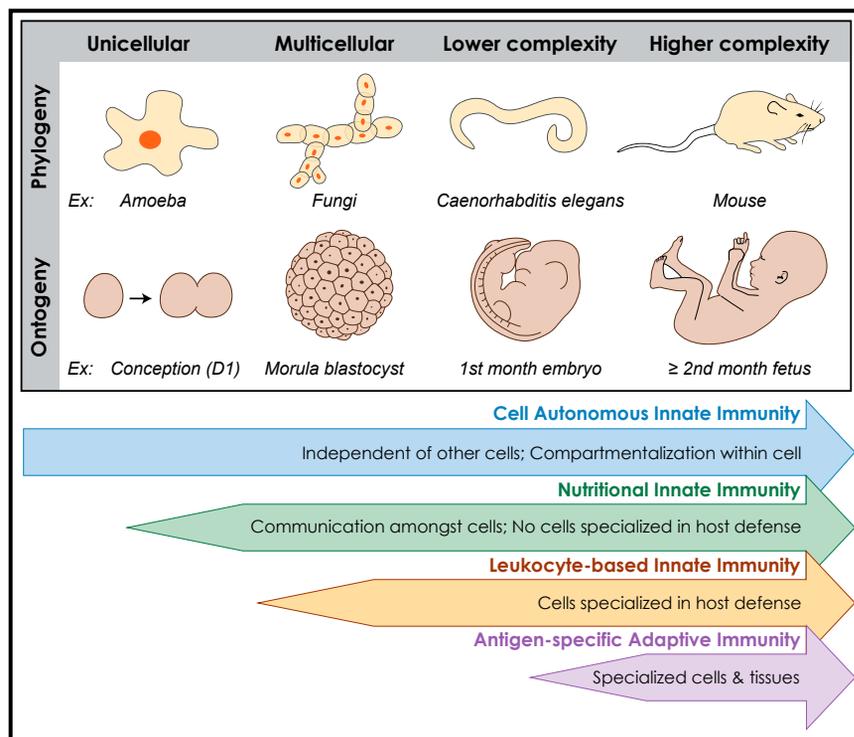


Figure 1. Key Elements of Immunity Share a Common Phylogeny and Ontogeny

Parallels are drawn between the phylogeny and ontogeny of immunity. Host protection from infection has developed in evolution (phylogeny) and develops in a given human across an individual's lifespan (ontogeny) from (1) a unicellular state wherein cell-autonomous immunity (CAI) is key to survival as noted in single cell organism such as an amoeba and early after conception; (2) multicellular organisms such as fungi that expresses biochemical communication via nutritional immunity (NI) amongst collections of cells as noted in ontogeny during the morula blastocyst stage; (3) multicellular animals of lower complexity such as *C. elegans* that express leukocyte-based innate immunity as is noted in the first month embryo; and (4) animals of higher complexity such as mice that express classic adaptive immunity with specialized cells (e.g., T and B cells) and tissues (e.g., lymph nodes) as is noted in the human fetus of 2 months or greater gestational age. All of these aspects of immunity contribute to perinatal host defense.

(CREB)-binding protein (CBP) and their target DNA sequence (Kollmann et al., 2012). The relevance of this pathway for host defense has been confirmed for humans, as genetic mutations in the

threat of pathogens able to cross cell membranes (Randow et al., 2013). Host cells express sensory machinery at these boundaries such as pattern-recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) and receptors that respond to danger-associated molecules (DAMPs) (Oh and Lee, 2014). For example, viral nucleic acids in the cytosol induce a type 1 IFN-dependent CAI response via RIG-I-like receptors (RLRs) that activate autophagy (Richetta and Faure, 2013; Wu and Chen, 2014). Newborns demonstrate age-dependent differences in CAI such as autophagy, likely because the mechanisms involved in regulation of CAI change during normal development responsive to physiological needs beyond those related to host defense against infection (Di Bartolomeo et al., 2010). Not surprisingly then, disruption of the normal physiological development via activation of CAI following, for example, viral infection, can have devastating consequences especially during early life. For example, Zika virus infection activates autophagy in infected human fetal neural stem cells via Akt/mammalian target of rapamycin (mTOR), which in turn inhibits normal neurogenesis during development (Liang et al., 2016). On the other hand, herpes simplex virus (HSV) encephalitis is particularly severe in young children in part because of lower neonatal HSV-induced type 1 IFN production relative to the adult, which results in impaired autophagy and decreased viral control resulting in permanent central nervous system damage (Gantt and Muller, 2013; Wilcox et al., 2015). The molecular mechanisms of lower IFN production in infected cells of the nervous system of newborns vs. adult have not been delineated. But newborn plasmacytoid DCs (pDCs), key to anti-viral defenses, demonstrate impaired production of type 1 IFN as compared to adults due to reduced interaction of IFN-regulatory factor 3 (IRF3) with cAMP-responsive-element-binding protein

PRR-IRF3-IFN signaling cascade predispose to severe HSV encephalitis in childhood (Abel et al., 2010; Casanova et al., 2011). Of note, environmental influences such as nutrient availability alter CAI, indicating that targeted interventions could optimize CAI in early life (Cuervo and Macian, 2012).

Nutritional Immunity Provides Broadly Effective Protection

Nutritional immunity provides host protection via regulation of metabolic pathways and availability of essential nutrients (Beisel, 1992). An example of relevance for immune ontogeny is iron (Fe), an element essential for the survival of all living organisms (Hood and Skaar, 2012). The human body is a rich reservoir of Fe and to prevent infection, it restricts access of microbes to Fe. The master-switch for free Fe levels is hepcidin, a peptide produced in the liver, where its expression is increased in response to inflammation or pathogen recognition. Specifically, interleukin (IL)-6 and other cytokines activate the signal transducer and activator of transcription 3 (Stat3) pathway thereby upregulating hepcidin expression and reducing free Fe (Recalcati et al., 2012; Ruchala and Nemeth, 2014). This hepcidin pathway is likely an important component of newborn host defence, as human neonatal plasma Fe levels directly correlate with susceptibility to sepsis (Bullen et al., 2000; Johnson and Wessling-Reisnick, 2012; Nairz et al., 2014; Oppenheimer, 2012; Wander et al., 2009). Specifically, a dramatic physiological drop in serum Fe within hours of birth reduces the risk for neonatal sepsis (Bullen et al., 2000; Hay et al., 2007; Recalcati et al., 2012; Sturgeon, 1954; Szabó et al., 2001). Conversely, supplemental Fe given to Fe-replete infants increases the risk for sepsis and death (Oppenheimer, 2012; Oppenheimer, 2001; Sazawal et al., 2006). Neonatal and even prenatal (i.e. maternal) nutritional immunity is

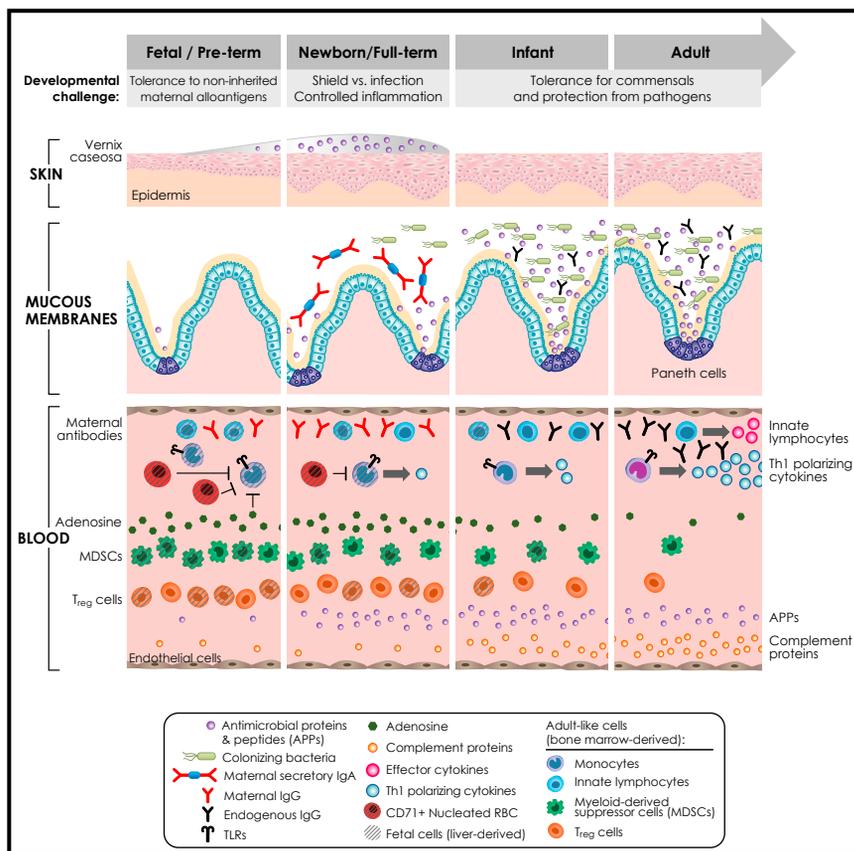


Figure 2. Ontogeny of Fetal, Neonatal, and Infant Host Defense

Host-protective barrier functions include physical, chemical, and functional components of the epithelial of skin and mucous membrane of the fetus, neonate (birth to 28 days of age), and infant (1 month – 1 year of age). These have to be understood in the context of age-specific developmental challenges as outlined near the top of the figure.

(A) Skin: While physical and chemical barriers are reduced early in life, especially in the preterm, the *vernix caseosa* and skin epithelia of full-term newborns robustly expresses APPs.

(B) Mucous membranes: In parallel with and induced by an increasingly complex microbiota, the newborn intestinal mucosal epithelium rapidly changes structurally with increase in crypts, and crypt-based Paneth cells, as well as functionally with increasing APP expression (Maynard et al., 2012).

(C) Blood: The composition of neonatal blood is distinct, with relatively low concentrations of complement components and APPs (Dorschner et al., 2003; Hackam et al., 2013; Hong and Lewis, 2015; Hornef and Fulde, 2014; Marchini et al., 2002; Tourneur and Chassin, 2013; Underwood et al., 2005; Visscher and Narendran, 2014; Yoshio et al., 2003) and high concentrations of the immunosuppressive purine metabolite adenosine. Plasma also contains maternal antibodies transferred beginning mid-gestation, and supplemented by postnatal factors derived from breastmilk (Hanson and Korotkova). Innate immunity is detectable from the end of the first month of gestation, with changes driven largely by the increasing exposure to environmental microbes (De Kleer et al., 2014; Dowling and Levy, 2014; Kollmann et al., 2012; Pettengill et al., 2014;

Vermijlen and Prinz, 2014). Neonatal APCs such as blood monocytes express PRRs (e.g., TLRs) with distinct functional responses including limited Th1-polarizing cytokine production to most stimuli. Age-dependent differences in activity of interferon response factor (IRF) transcription factors as well as epigenetic changes contribute to this cytokine ontogeny (Buffen et al., 2014; Danis et al., 2008; Kleinnijhuis et al., 2015). Adaptive immunity develops from 4 weeks of gestation onwards, with changes driven by an evolving chimerism reflecting fetal (liver-derived, shaded cells) Treg-rich lymphocytes and more adult-like (bone marrow-derived, not shaded cells) lymphocytes (Krow-Lucal and McCune, 2014) with distinct epigenetically encoded functional programs (Hanson and Korotkova, 2002; White et al., 2006).

thus a highly effective means of host protection that is tightly regulated yet could readily be manipulated, providing a distinct avenue to enhance early life protection from infection (Rochette et al., 2015).

Physical Barrier Functions Are Enhanced by Antimicrobial Effector Molecules

Protective barrier functions include physical and chemical components of placenta, skin, and mucous membranes. The outermost layer of the skin acts as a physical barrier; however, its toughest layer, the *stratum corneum*, only fully develops during the first two weeks of life (Figure 2, Skin) (Marchant et al., 2013). As some measure of counterbalance, the skin of full-term newborns displays high production of antimicrobial proteins and peptides (APPs). APPs are in fact expressed in an age-dependent pattern by nearly all human cells that have been exposed to microbes (Wiesner and Vilcinskas, 2010). Examples of APPs include defensins such as human β -defensins, bactericidal/permeability-increasing protein (BPI), whey acidic protein motif-containing proteins, secretory leukocyte protease inhibitor, elafin (antiproteinase 3; skin derived antileuko-proteinase), lactoferrin, and lysozyme (King et al., 2007; Wiesner and Vilcinskas, 2010). The skin in particular produces β -defensins

and cathelicidins (Dorschner et al., 2003). Of note, a waxy coating (*vernix caseosa*) produced by fetal sebaceous glands in utero covers the full-term newborn as a microbicidal shield for the first few days of life. The vernix contains multiple APPs such as lysozyme, β -defensins, ubiquitin, and psoriasin, as well as antimicrobial free fatty acids (Tollin et al., 2005). As the *vernix caseosa* is mainly formed during the last trimester of gestation, the preterm newborn is left relatively more exposed and vulnerable (Marchant et al., 2013).

Similar to the skin, the host defense of the intestinal mucosa via expression of APPs is also developmentally regulated and equally dependent on colonization with beneficial microbes (Figure 2, mucous membranes) (Hornef and Fulde, 2014). The small intestinal epithelium of neonatal mice expresses the cathelicidin cathelin-related antimicrobial peptide (CRAMP) that exerts antibacterial activity against commensal and pathogenic bacteria. Production of Paneth cell-derived APPs like cryptidins and cryptin-related sequence (CRS)-peptides on the other hand begins only after birth, due to the delayed appearance of small intestinal Paneth cells during the postnatal period. Intestinal epithelial CRAMP expression wanes after the postnatal period, reflecting a switch in the APP repertoire and production site from epithelial CRAMP expression to Paneth cell-secreted

cryptidin and CRS peptides after weaning. The mucosa of the respiratory tract also produces a variety of APPs, such as BPI, lysozyme, lactoferrin, and defensins (Diamond et al., 2000; Travis et al., 1999). As in the intestine, APPs in the respiratory tract are mainly produced in response to PRR stimulation following microbial encounters, a response that increases with gestational age and correlates with decreased susceptibility to infection, for example with *Bordetella pertussis* (Elahi et al., 2006; Starner et al., 2005).

Overall, the development of barrier function appears strongly influenced by postnatal microbial exposure. In this context, deliberate exposure to specific non-pathogenic microbes could provide a means of accelerating barrier-mediated host protection. As preterm birth negatively impacts several barrier defense functions (Marchant et al., 2013), trials of prophylactic or therapeutic application of APPs administered to preterm newborns have been undertaken and provide promising results (Battersby et al., 2016). For example, oral administration of bovine lactoferrin, an 80 kDa cationic multi-functional protein with iron-binding, immunomodulatory, and direct membrane-perturbing microbicidal activity, to preterm, very low birthweight newborns (<1,500 g) was associated with reduced incidence of late-onset sepsis, necrotizing enterocolitis, and fungal infection (Legrand, 2016; Manzoni et al., 2014; Manzoni et al., 2009; Manzoni et al., 2012).

Innate Immunity Integrates Environmental Signals and Provides Immediate Effector Function

Coordination of host immune responses plays an increasingly prominent role in host protection of multicellular organisms as well as in the progressively more complex tissues of the human fetus (Figure 1). Via only recently appreciated immunometabolic pathways, innate immunity coordinates CAI and nutritional immunity (O'Neill et al., 2016). For example, innate immune activation following treatment of human monocytes with the Dectin-1 agonist β -glucan induces a shift to cellular aerobic glycolysis via an Akt/HIF1 α -mediated activation of mTOR pathway resulting in increased tumor necrosis factor (TNF) responses to PRR agonists such as LPS or heat killed bacteria (Cheng et al., 2014). This immunometabolic pathway directs the memory-like function of innate immunity, i.e. epigenetic changes that lead to long-lasting alteration of innate immune memory, also known as "trained immunity" (Cheng et al., 2014; Levy and Netea, 2014; Netea et al., 2015; O'Neill et al., 2016).

The effector activities of innate immunity are both rapid (preventing microbial proliferation/spread) and broad (enabling protection against multiple diverse pathogens at the same time) (Buchmann, 2014), and expressed through soluble (e.g. complement and APPs) as well as cellular components (Figure 2) (Pettengill et al., 2014). With respect to soluble factors, levels of most individual complement proteins are lower in neonates compared to adults, resulting in lower complement activity in early life. But while most APPs (e.g. Lactoferrin, BPI, and cathelicidin anti-microbial peptide 18, also called LL-37) have lower constitutive plasma concentrations in preterm and low birth weight newborns, term infant plasma contains substantially higher levels (Singh et al., 2013; Strunk et al., 2009). The importance of APP production for early life innate immunity becomes evident in cases of selected APP deficiency, because lower

serum levels of cathelicidin, for example, are associated with increased severity of acute bacterial respiratory infection in early childhood (Battersby et al., 2016; Mansbach et al., 2012).

Specialized innate immune cells include the myeloid lineages, namely granulocytes (e.g. neutrophils), monocytes, macrophages, and dendritic cells (DCs), as well as innate lymphocytes. While neutrophils are present in human fetal liver parenchyma by as early as 5 weeks gestation (De Kleer et al., 2014), neutrophils in early life demonstrate quantitatively and qualitatively different responses under stress conditions as compared to adult neutrophils, including reduced chemotaxis, respiratory burst and formation of extracellular traps, scaffolds for APPs that serve to capture and kill extracellular bacteria (Carr, 2000). The ability of neonatal neutrophils to ingest opsonized Gram-positive bacteria however appears to be similar to adults, while phagocytosis of Gram-negative bacteria is impaired, suggesting a microbe-specific impact of neutrophil ontogeny (Carr, 2000). While some of the limitations in neonatal neutrophil function might reflect higher expression levels of inhibitory receptors (De Kleer et al., 2014), a unifying explanation of the mechanisms that lead to differences between newborn and adult neutrophils has not been provided.

Monocytes appear in the fetal circulation as soon as self-renewing hematopoietic stem cells (HSPC) have seeded the fetal liver (De Kleer et al., 2014). During early development, monocyte progenitors also colonize various organs and differentiate into tissue-resident macrophages that self-maintain throughout life (De Kleer et al., 2014). Following PRR stimulation, human newborn monocytes/macrophages and DCs produce a cytokine profile that differs substantially from those of their adult counterparts (Figure 2, Blood) (De Kleer et al., 2014; Kollmann et al., 2012). Specifically, upon stimulation of individual TLRs in vitro, neonatal APCs typically produce less proinflammatory (interleukin-1 β [IL-1 β], TNF- α) and T helper 1 (Th1) promoting cytokines (IL-12p70, type 1 IFN), but equal or greater amounts of Th17 promoting cytokines (IL-23, IL-6) compared with adult cells. Robust neonatal production of IL-6 (1) induces a physiological hepatic acute phase response at birth, including induction of mannose binding lectin (MBL), C-reactive protein (CRP), and LPS-binding protein (LBP) that rise in the first week of life, possibly broadly enhancing resistance to infection, and (2) contributes to healing of tissues injured during birth (Jones, 2005; Levy, 2007). Newborn monocytes and conventional DC (cDC) also produce more IL-10 compared to adults, likely reflecting the importance of anti-inflammatory responses in early life. Mechanisms that lead to this early life pattern of innate cytokine response include (1) high mononuclear cell levels of intracellular cyclic adenosine monophosphate (cAMP), a secondary messenger that suppresses Th1 but enhances Th2 and anti-inflammatory cytokine production (Levy et al., 2006) and (2) altered DNA binding capacity of transcription factors such as IRF3 to the promoter regions of cytokine genes secondary to age-specific chromatin remodelling (Lissner et al., 2015). Of note, simultaneous stimulation of TLR and C-type lectin receptors (CLR) reveals marked synergy in immune activation as compared to single PRR stimulation, a synergy that strongly varies with age (Lemoine et al., 2015; van Haren et al., 2016a). Specifically, co-activation of newborn DCs via the CLR agonist Dectin or macrophage-inducible C-type lectin (Mincle), and TLR7/8 potently drives caspase-1 and NF- κ B activation and Th1-supporting

cytokine production (including IL-12p70, overcoming the age-specific epigenetic hurdle in early life for IRF3 function) that results in autologous T cell polarization toward a Th-1 phenotype. Importantly, this synergistic effect of TLR plus CLR stimulation is greatest in newborns, with synergy decreasing as age increases.

Innate lymphocytes develop early during human gestation (Figure 2, Blood). Fetal $\gamma\delta$ T cells are dominated by cells expressing a canonical V γ 9V δ 2 TCR and are programmed to express type 1 effector molecules (Dimova et al., 2015; Vermijlen and Prinz, 2014). Non-V γ 9V δ 2 effector T cells are known to be induced in utero by congenital CMV infection (Vermijlen et al.). Innate lymphocytes expressing rearranged TCR, including iNKT cells and mucosal-associated invariant T (MAIT) cells, as well as non-TCR-expressing innate lymphoid cells (ILCs), also develop early during fetal life and are programmed to express effector functions (Chan et al., 2013; Hong DK; Nakazawa et al., 1997). Although some of these effector functions appear lower in early life, comprehensive analysis across the ages is lacking.

Collectively, these observations indicate that many cellular and molecular components of innate immune responses develop early during fetal life, and on the molecular level are regulated distinctly from their adult counterparts. However, much remains to be learned regarding the molecular basis for these regulatory differences.

Adaptive Immunity—Diverse yet Targeted Effectors

Development of adaptive immunity requires close cooperation between multiple elements of the innate immune system, as well as nutritional and innate immunity via metabolic pathways that shape the function of effector and memory lymphocytes (Buck et al., 2015; Iwasaki and Medzhitov, 2015).

Mature fetal $\alpha\beta$ T lymphocytes can be detected from ~14 weeks of gestation onward, i.e. several months later than a number of innate lymphocyte subsets (Hong and Lewis, 2015). The repertoire of fetal T cell receptors diversifies during the second and third trimesters of gestation (Rechavi et al., 2015). In the fetus and the newborn, the majority of $\alpha\beta$ T lymphocytes are recent thymic emigrants (RTEs) (Haines et al., 2009). In comparison to mature naïve T lymphocytes, RTEs have a distinct functional program involving epigenetic modifications at key cytokine loci (Fink, 2013). Newborns often display limited Th1-type responses to some vaccines and pathogens, correlating with a lower capacity of CD4 T cells to produce IFN- γ and of APCs to produce Th1-polarizing cytokines (Debock and Flamand, 2014; White et al., 2002). However, this reduced Th1 capacity is not absolute, because newborns and young infants develop adult-type Th1 responses to BCG or whole cell pertussis vaccines, for example, and fetuses can develop Th1 responses to CMV infection (Huygens et al., 2015; Marchant et al., 1999; Mascart et al., 2003). This indicates that the quality and magnitude of signals present at the time of naïve CD4 T cell priming determine the development of immune responses in early life. Specifically, the profile of cytokines produced by newborn APCs, including IL-6 and IL-23 suggests a robust ability to mount Th17 and follicular helper T (TFH) cells at similar or even higher levels than adults (Debock and Flamand, 2014). Of note, fetal $\alpha\beta$ T lymphocytes might already acquire a specific phenotype of memory cells in utero, programmed to effectively produce Th1-, Th2-, or Th17-cytokines (Zhang et al.,

2014). Furthermore, multiple pathogens, including CMV, HIV, and *T. cruzi* can induce effector CD8 T lymphocytes in the fetus, clearly indicating that cell-mediated immune responses are not intrinsically deficient in early human life (Hermann et al., 2002; Muenchhoff et al., 2014). However, the anti-microbial properties of fetal and newborn effector T lymphocytes might be limited by a more rapid onset of functional exhaustion (Huygens et al., 2015).

Mature B lymphocytes can be detected in the fetal liver from 8 weeks of gestation (Hong and Lewis, 2015). Somatic hypermutation of peripheral and marginal zone (MZ) B cells develops and isotype switching begins in utero already and leads to fetal diversification of the B cell receptor repertoire (Hong and Lewis, 2015; Rechavi et al., 2015). The capacity of the newborn to develop antibody responses depends on the nature of the immune stimulus as reflected in the immunogenicity of standard childhood vaccines. For example, hepatitis B vaccine (HBV) immunization induces at least equivalent antibody responses in newborns and adults; in contrast, antibody response to oral polio, measles, and rubella vaccines increase with age at immunization (Ota et al., 2004; Siegrist and Aspinall, 2009). Importantly, irrespective of their primary response, neonatal immunization can induce potent memory B cell responses that promote immunogenicity of subsequent vaccine booster doses (Halsey and Galazka, 1985). The mechanisms underlying the early life maturation of effector B lymphocyte responses are currently unclear but could involve the upregulation of complement receptors, of the ecto-enzyme CD73, of T cell co-stimulatory molecules expressed by neonatal B lymphocytes, or the gradual age-dependent enhancement of interactions between infant B and TFH cells, all of which are likely influenced by maternal antibodies (Debock and Flamand, 2014; Pettengill and Levy, 2016; Siegrist and Aspinall, 2009).

Microbial Colonization Provides Key Signals for Immune Development and Protection

Within hours after birth, the neonate is colonized by bacteria (Figure 2) (Arrieta et al., 2014). While the composition of this microbiota rapidly evolves during the first 2–3 years of life, microbial communities are largely unique to each individual, as both host genetic and environmental factors influence the composition of the intestinal microbiota via crosstalk between microbes and their hosts (Arrieta et al., 2014; Chu and Mazmanian, 2013; Dorrestein et al., 2014; Landwehr-Kenzel and Henneke, 2014). Bacterial colonization is in fact essential for optimal host immune development, illustrated by the finding that germ-free mice are at increased risk for infectious as well as autoimmune diseases (Arrieta et al., 2014; Chu and Mazmanian, 2013; Khosravi et al., 2014; Renz et al., 2012). Germ-free mice also display reduced hematopoiesis of macrophages from both the bone marrow and yolk sac early in life, leading to impaired clearance of systemic *Listeria monocytogenes* infections (Khosravi et al., 2014). And in mice, LPS derived from Gram-negative bacteria induces microRNA-146a, that downregulates IL-1 receptor associated kinase 1 (IRAK1), thereby changing TLR4 signaling towards a state of tolerance following bacterial colonization (Lotz et al., 2006). The human commensal *Bacteroides fragilis* produces polysaccharide A (PSA) that induces TLR2-mediated development of regulatory T (Treg) responses (Chu and Mazmanian, 2013), an activity also found in other microbes such as the

probiotic *Bifidobacterium breve*. Beyond their impact on immune tolerance, commensal microbiota also prepare the host to rapidly mount immune responses upon pathogen encounter, as germ-free or antibiotic-treated mice demonstrate impaired clearance of systemic bacterial infection. As mentioned above, interactions of the infant gut with the microbiota are likely important for maturation of mucosal APP expression (Figure 2B), whose dysregulation has been associated with necrotizing enterocolitis (Maynard et al., 2012; Salzman et al., 2007). This physiological process is readily altered by administration of antibiotics in early life. For example, treatment of mice with broad-spectrum antibiotics to deplete the microbiota reduces circulating peptidoglycan and leads to impaired neutrophil-mediated killing of *Streptococcus pneumoniae* and *Staphylococcus aureus*. (Chu and Mazmanian, 2013) This likely relates to the ability of commensal microbes to promote maintenance of neutrophils and other myeloid cells (Arrieta et al., 2014; Renz et al., 2012). Furthermore, antibiotic treatment reduces (1) expression of IFN-responsive genes in peripheral blood monocytes and DCs of young mice due to epigenetic changes including reduced H₃K₄me₃ deposits at specific promoter regions (De Kleer et al., 2014); (2) adaptive immune responses to intranasal infection with influenza virus, reducing virus-specific antibody titers and frequency of virus-specific CD4 and CD8 T cells (Chu and Mazmanian, 2013); and (3) flagellin production by commensal flora important for driving TLR5-mediated lymph node plasma cell differentiation to enhance antibody responses to inactivated influenza and polio vaccines (Oh et al., 2014). These data also highlight the still largely underappreciated role for microbiota in influencing immunity to immunization (Huda et al., 2014).

Importantly, there appears to be an age-restricted “window of opportunity,” as colonization of germ-free or antibiotic-treated mice early in life with commensal microbes promotes normal immune function while colonization of adult germ-free or antibiotic-treated mice fails to do so (Chu and Mazmanian, 2013; Khosravi et al., 2014). For example, invariant NKT (iNKT) cells appear to be activated and home to the gut and promote inflammation in germ-free mice; colonization with a complex microbiota in early life restores homeostasis, but not when gut bacteria are introduced later in adulthood (Olszak et al., 2012). A critical early life period of beneficial microbial-host immune interaction has also been identified in studying the impact of feeding- or birth-mode (Ardeshir et al., 2014; Cox et al., 2014). For example, the decreased diversity of the dominant *Bacteroidetes* phylum in infants born by Caesarean section is associated with lower plasma concentrations of CXCL10 and CXCL11, two IFN-dependent chemokines important for leukocyte migration to sites of infection (De Kleer et al., 2014). These effects likely relate to the impact of the maternal microbiome on newborn immune function; interestingly, this impact is dependent on maternal antibody transfer, suggesting complex cross-regulatory interactions between host and microbiota even across generations (Gomez de Agüero et al., 2016). Finally, although controversial, a fetal microbiome has been described, suggesting this host-microbe interaction might already begin prior to birth (Aagaard et al., 2014). Overall, these observations suggest a critical perinatal (and possibly even prenatal) window wherein the interaction of the host with the microbiome drives optimal immune develop-

ment and exposure to specific microbes, including probiotics, might enhance immune mediated homeostasis (Arrieta et al., 2015; Dominguez-Bello and Blaser, 2015).

Regulation of Immunity in Early Life

The transition around birth from a largely shielded environment in utero to postnatal life as an “animal in a microbial world” (McFall-Ngai et al., 2013) represents the most dramatic life event for our mammalian immune system. Specifically, the semi-allogeneic state of the mother/fetus requires suppression of rejection, in part accomplished via a “default” tolerogenic immune response involving adaptive Treg cells as well as pronounced PRR-mediated IL-10 production by neonatal APCs (Kollmann et al., 2012). The normal birth process on the other hand is the result of targeted inflammation necessary to separate the maternal-fetal layers, requiring immune suppressive counter-regulation to prevent systemic inflammation (Gomez-Lopez et al., 2014). In addition, hypoxia suffered during labor can cause tissue damage that in turn enhances inflammation, causing further damage (Sharma et al., 2012). Such potential for perinatal inflammatory immunopathology is somewhat counterbalanced by a strong immune bias towards resolution of inflammation and healing, i.e. the well-described Th2-dominated response in early life (Iwasaki and Medzhitov, 2015). Furthermore, the postnatal period is characterized by rapidly changing environmental and microbial exposures that relentlessly stimulate immune development, requiring immune regulation to preserve homeostasis (MacGillivray and Kollmann, 2014).

Managing such rapidly shifting, diverse functional demands is achieved in part through compartmentalization where immune responses appear strictly confined to specific tissue niches (Iwasaki and Medzhitov, 2015; Kollmann et al., 2012; Thome et al., 2016). Such “spatial” regulation is complemented through regulatory mechanisms that are “compartmentalized in time,” for example through layered immune maturation, whereby fetal immune cells, functionally distinct from their adult counterparts, arise from discrete HSPC at different stages of development (Krow-Lucal and McCune, 2014). Such layered immunity also appears to provide for the abundance of active immune suppressive cells and functions in fetal and early postnatal life, including Treg cells, myeloid-derived suppressor cells (MDSCs), and erythroid (nucleated RBC) suppressor cells (Elahi, 2014; Gantt et al., 2014; Gervassi et al., 2014; Pandiyan et al., 2011; Power Coombs et al., 2013). Natural Tregs develop in the thymus in parallel with naïve T cells during fetal life (Hong and Lewis, 2015), and such Treg cells continue to comprise a larger proportion of CD4 T cells in peripheral tissues in young children as compared to adults, supporting their role in the maintenance of immune homeostasis in early life (Thome et al., 2016). Furthermore, fetal naïve CD4 T cells preferentially differentiate to Treg cells in peripheral tissues in response to non-inherited maternal HLA antigens and could thereby contribute to fetal tolerance to maternal cells (Boer et al., 2015; Mold et al., 2008; Mold et al., 2010). These Tregs impact immunity even beyond birth—even across generations—enhancing reproductive benefits (Kinder et al., 2015).

MDSCs are a heterogeneous population of granulocytic or monocytic cells that suppress innate as well as adaptive immune function (Gantt et al., 2014; Gervassi et al., 2014). MDSCs express suppressive factors such as arginase-1, reactive oxygen species,

and inducible nitric oxide synthase, which inhibit T cell proliferation and cytotoxicity, induce the expansion of Treg, and block NK cell activation. Granulocytic MDSCs are present in large numbers in pregnant women and in cord blood, yet wane rapidly during infancy. Cord blood MDSCs suppress CD4 and CD8 T cell and NK cell responses, suggesting a significant role in immune homeostasis in early human life. Similarly, physiologically abundant CD71⁺ erythroid cells in neonatal mice and human newborns exhibit immunosuppressive properties (Elahi, 2014). Specifically, neonatal CD71⁺ cells express the immunosuppressive enzyme arginase-2 and ablation of CD71⁺ cells in neonatal mice increases resistance to the perinatal pathogens *L. monocytogenes* and *E. coli*, yet not to polymicrobial sepsis (Elahi et al., 2013; Wynn et al., 2015). Lastly, compared to adults, neonatal cord blood plasma has higher amounts of adenosine-generating enzymes (soluble CD73 and alkaline phosphatase) and lower levels of adenosine deaminase (ADA), the enzyme that metabolizes and inactivates adenosine, resulting in high plasma concentrations of adenosine, an endogenous purine metabolite that inhibits TLR-mediated Th1-polarizing cytokine induction (Pettengill et al., 2013; Power Coombs et al., 2013), and also reduces neutrophil activation (Haskó and Cronstein, 2013).

Behind the complexity of regulating immune ontogeny stands a surprisingly simple basic principle: balancing cost to the species vs. the individual of an immune response directed at the microbial world while maintaining homeostasis (Iwasaki and Medzhitov, 2015). Specifically, all immune effector responses can be viewed on a spectrum defined by the costs, including potential immunopathology, associated with their maximal deployment. Evolution has presumably selected for immune responses that minimize cost while at the same time providing sufficient protection for survival of the human species. In this light, the development of multiple highly effective immune regulatory strategies in parallel with immune effector functions in early life indicates that the human newborn immune system is not “immature” (meaning “not ripe,” “not perfect”), but perfectly shaped to satisfy the complex demands of early life. Given the particular demands in early life are focused on maintaining tolerance and balancing the perinatal exposure to a range of pro-inflammatory stimuli, it appears that the threshold required to reach in order to initiate immune effector responses is set higher in early as compared to adult life. However, once this higher set-point is reached, an immune response of substantial magnitude is unleashed (Ghazal et al., 2013). And this can be associated with higher immune pathology (Iwasaki and Medzhitov, 2015), explaining in part the profound morbidity observed during infections incurred early vs. later life. It follows that increased protection from certain infectious diseases in early life could benefit from a decrease rather than increase, i.e. a more balanced immune response resulting in decreased immunopathology. The potential benefit of such attenuated immune responses in early life is evident for viral infections such as hepatitis B virus (HBV) or HIV, where the immune regulatory mechanisms dominant during early life prevent the immune-mediated harm readily observed in infected adults (Bertoletti and Hong, 2014; Huygens et al., 2015; Muenchhoff et al., 2016).

Age-Dependent Susceptibility to Infection in Early Life

The intensity and rapid kinetics of the opposing immunologic demands of host protection in early life versus immunoregulation

to avoid tissue damage suggests that alterations in a given individual along these physiological trajectories can have dire consequences. In this context, the clinically observed increased risk for severe infection in early life is best viewed as an imbalance of the phylogenetically selected beneficial survival programs vs. specific environmental demands exerted on the individual during ontogeny. This concept appears useful in correlating the particular aspects of immune regulation during ontogeny with the most pertinent infections during the same age period. For example, infection of fetal mice with HBV activates the prevailing tolerogenic responses in hepatic macrophages that in turn suppress antiviral cytolytic effector T cell responses; while decreasing acute immune pathology. This age-specific host-pathogen interaction appears causally related to the observed increased risk of vertical HBV transmission and the ensuing chronic infection in the offspring (Tian et al., 2016). Furthermore, the decreased CAI and barrier function of the skin and mucous membranes of pre-term infants predicts the known risk for invasion with skin- and mucosa-colonizing microbes (Marchant et al., 2015). Furthermore, the relative deficiencies in the complement system and APPs along with reduced phagocyte migration indicate a potential increased vulnerability of the newborn infant, especially those born preterm, to systemic spread and infection with extracellular microbes. Indeed, newborns display heightened susceptibility to pyogenic infection with Gram-positive extracellular bacteria such as *Staphylococcus* spp. (Power Coombs et al., 2013) and *Streptococcus agalactiae* (Group B Streptococcus) (Landwehr-Kenzel and Henneke, 2014), Gram-negative infections such as *E. coli*, and certain fungal infections (Hsieh et al., 2012; Rao and Ali, 2005; Vergnano et al., 2005). The distinct aspects of early life innate and adaptive immune ontogeny summarized above would further predict an increase of infection in early life with pathogens controlled by Th1 type immune responses. This is also the case, as an increased risk for severe infection with intracellular pathogens requiring Th1 protective responses for effective host defense, including bacteria (e.g., *L. monocytogenes*, *Salmonella* spp.), mycobacteria, and viruses is observed in newborns and young infants (Chirico et al., 1985; Garcia-Vidal et al., 2013; Sherrid and Kollmann, 2013; Speer et al., 1988; Vanden Driessche et al., 2013; Weiner and Kaufmann, 2014). Indeed, viral infections including respiratory syncytial virus and influenza virus are often more severe and/or prolonged in early life as compared to adult life (Bertoletti and Hong, 2014; Clark and Lynch, 2011; Gantt and Muller, 2013; Huygens et al., 2015; Muenchhoff et al., 2014). However, given the current limited insights into cause-effect relationships, linking susceptibility of particular infections in early life to specific immune parameters during immune ontogeny remains an area that needs more detailed studies.

Opportunities for Intervention

As discussed above, the early life immune system is not in a fixed state of “immaturity,” but rather rapidly adapts to environmental cues. It is thus possible, and indeed likely, that protection from infection can be enhanced by providing broadly active immune modulatory stimuli during early life. We already mentioned some specific examples in the respective sections on immune ontogeny above; we here focus on a range of interventions that can provide broad protection against a wide range of pathogens (Figure 3).

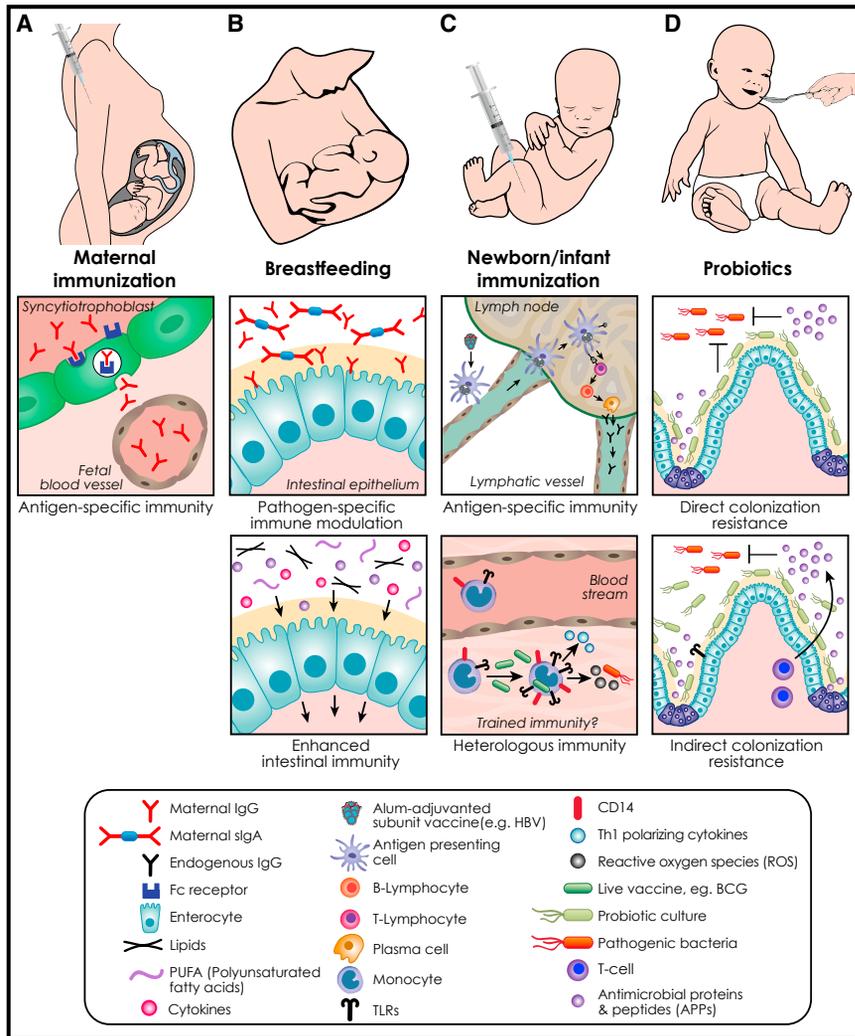


Figure 3. Interventions that Broadly Enhance Host Defense against Infectious Disease in Early Life

There are key windows of opportunity during prenatal life and early postnatal life to enhance host resistance to specific infections via homologous—i.e., pathogen and thus classic antigen-specific responses (top panels)—as well as broadly protective heterologous (“non-specific”) responses (bottom panels).

(A) Maternal immunization leverages passive transfer of maternal IgG antibodies across the placenta that can protect the fetus and newborn. The specificity of the maternal IgG reflects past maternal exposures thereby targeting specific pathogens.

(B) Top panel shows that breastfeeding provides secretory IgA, with specificities reflecting maternal microbiota, transferred across the gut along with maternal IgG bound to antigen; whereas the bottom panel shows that breastmilk also contains soluble factors, including cytokines, lipids, and fatty acids, that broadly enhance mucosal resistance to infection.

(C) Early life immunization of the newborn or young infant reduces risk for infection with (C-top) specifically-targeted pathogens (Clemens et al., 2010; Levine, 2011); (C-bottom) live attenuated vaccines such as Bacille Calmette Guérin (BCG) provide broader heterologous (“non-specific”) protection, possibly via “trained immunity” mediated by epigenetic reprogramming of monocytes (Aaby et al., 2014).

(D) Probiotics reduce infection (AlFaleh and Anabrees, 2014; Oncel et al., 2014; Roy et al., 2014; Samanta et al., 2009). Mechanisms underlying probiotic effects remain under study and may include, for example, (D-top panel) enhancement of colonization resistance (Buffie and Pamer, 2013; Sassone-Corsi and Raffatelli, 2015), wherein bacteriocin production by probiotic bacteria targets specific pathogens without affecting commensal flora, and (D-bottom) mucosal PRR signalling-mediated enhancement of immune development, including intestinal epithelial cell expression of antimicrobial protein and peptide (APPs) as well as innate lymphoid cells and mucosal Th17 and Treg development.

The traditional pathogen-centric approach has led to the successful development of childhood vaccines that prevent ~2.5 million deaths each year worldwide (Bärnighausen et al., 2014; Clemens et al., 2010; Levine, 2011; UNICEF, 2014). However, vaccine-mediated prevention of infections occurring at birth or soon after birth is limited by reduced or slower immune responses to a number of vaccines administered in early life. In the future, it might be possible to enhance responses to early life vaccines by inclusion of novel adjuvants that demonstrate age-specific immune-enhancing activity (Oh et al., 2016; van Haren et al., 2016b). In the meantime, maternal immunization offers an attractive complement to newborn and infant immunization because it allows the transfer of high quality pathogen-specific immunoglobulin G (IgG) across the placenta, effectively protecting the newborn and young infant. Maternal immunization has already proven effective in reducing neonatal and infant disease due to tetanus, pertussis, and influenza, and might become a central component of the control of group B streptococcus (GBS) and RSV infections in young infants (Amirthalingam et al., 2014; Beigi et al., 2014; Dauby et al., 2012; Lindsey

et al., 2012; Lindsey et al., 2013; Niewiesk, 2014; Saso and Kampmann, 2016; Vidarsson et al., 2014). Further progress on this pathogen-specific focused avenue has however been limited by our current lack of understanding the mechanistic requirements to induce protective immunity (Iwasaki and Medzhitov, 2015; Koff and Schenkelberg, 2016).

It is increasingly appreciated that young infants can be protected from infectious pathogens through non pathogen-specific mechanisms. For example, vaccines have effects beyond inducing classic antigen-specific T and B cell-mediated adaptive immune responses targeting a specific pathogen (Aaby et al., 2014). Protective heterologous (“non-specific”) effects of vaccines have been demonstrated for live attenuated vaccines such as Bacillus Calmette–Guérin (BCG), oral polio, and measles vaccines, all of which reduce morbidity and mortality far beyond that attributable to prevention of the target disease (Aaby et al., 2014; de Castro et al., 2015; Goodridge et al., 2016; Lund et al., 2015; Sørup et al., 2014). Of note, BCG activates autophagy as part of CAI (Buffen et al.), iron sequestering as part of nutritional immunity (Kochan et al., 1969), epigenetic changes of

innate cells as part of trained innate immunity (Netea and van Crevel, 2014), and promotes T- and B cell responses to unrelated antigens (Kleinnijenhuis et al., 2014; Libraty et al., 2014; Ota et al., 2002). In mice, neonatal BCG also increases the number of Treg and IL-10 secreting cells, suggesting powerful anti-inflammatory effects (Li and Shen, 2009). Furthermore, while the impact of neonatal BCG on immune-mediated diseases in humans has not been sufficiently studied, existing data suggest a protection from immune pathology (Curtis, 2016; Rousseau et al., 2008). Such wide-ranging immune-modulatory, homeostatic activity of BCG might contribute to its broadly protective, heterologous effects against both infectious and immune mediated diseases.

And as mentioned above, multiple arms of the immune system are enhanced following microbial colonization, from CAI to barrier and innate immune function (Arrieta et al., 2014; Chu and Mazmanian, 2013; Khosravi et al., 2014; Renz et al., 2012). For example, in neonatal mice, microbiota regulate neutrophil homeostasis and host resistance to sepsis in a TLR4- and myeloid differentiation factor 88 (MyD88)-dependent pathway via IL-17 production in group 3 innate lymphoid cells (ILCs) (Deshmukh et al., 2014). And in humans, certain enteral probiotics reduce not only the risk of necrotizing enterocolitis in prematurely born infants but also infection-related mortality (AlFaleh and Anabrees, 2014; Denkel, 2015; Oncel et al., 2014; Panigrahi, 2013; Roy et al., 2014; Samanta et al., 2009; Strunk et al., 2015). Similarly, enhancing APP-mediated host innate immune defenses directly via supplementation with oral bovine lactoferrin reduces late onset sepsis in human preterm newborns (Pammi and Abrams, 2015). Taken together these data suggest that, in addition to interventions targeting antigen-specific immunity, non-pathogen-specific CAI, nutritional and/or innate immune functions can be harnessed to prevent infectious diseases in early life.

Concluding Remarks

The imbalance of environmental cues and demands in the context of the genetic constraints of a particular host determine the readily observed increase in clinical disease following infection in early life. However, the plasticity of the early life immune system makes it amenable to interventions aimed at preventing infection or clinical manifestation of infectious diseases. It is thus likely that protection from infection can safely yet effectively be enhanced by providing broadly active immune modulatory stimuli during ontogeny. Specifically, the human immune system develops early during fetal life and appears to be functionally programmed to promote tolerance to the maternal environment in utero and to commensal microbes, while tightly regulating perinatal inflammatory reactions. The efficacy of microbial stimuli—whether acquired within a commensal microbiome or as live attenuated vaccines—in safely enhancing host protection from infection or disease suggests these interventions do not bypass immune regulatory mechanisms but instead enhance them in the context of healthy immune homeostasis in early life. Boosting of CAI, nutritional, and other innate defense mechanisms may reduce the risk for infectious disease for a wide range of different pathogens that threaten the newborn and young infant. Such interventions might further benefit the young host by promoting immune regulation and homeostasis rather

than simply increasing effector functions. Elucidating the underlying molecular mechanisms is a key area for future research that will shed new light into immune ontogeny and inform development of age-specific immunomodulatory interventions. Together with the established pathogen-targeting approaches such as antigen-specific immunization of mother, newborn, and infant, efforts aimed at enhancing heterologous host resistance through optimized development and delivery of vaccines or probiotics will provide additional effective, practical, and affordable approaches for protecting newborns and young infants from the heavy burden of infectious diseases.

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