Clinical and Developmental Immunology

# Host Defense against Common Early Life-Threatening Infections

Guest Editors: Robert Bortolussi, Philipp Henneke, and Tobias Kollmann

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*Editorial*

# Host Defense against Common Early Life-Threatening Infections

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## 1. Introduction

Neonates suffer more severely and die more often than adults from a wide range of infections [1]. Although quantitative differences between neonatal and adult immune capacity are known, the molecular basis for the transition of immunologic function from fetal to postnatal life has remained a mystery. However, over the past decade, there has been an explosion of knowledge on immunity of the newborn and its importance to early host response to infection. Recent advances in developmental immunology now allow us to better understand the mechanisms underlying the susceptibility of neonates to many bacterial and viral infections [2, 3]. In addition, neonatal intestinal microbial ecology is now appreciated to play a role in host defenses and in the pathogenesis of neonatal necrotizing enterocolitis (NEC) [4]. In this special issue, we have collected reviews and papers to provide insight in immunologic adaptation of the neonate to its new environment and the consequences of this transition to newborn host defense mechanisms to bacterial and viral infection.

## 2. Immunologic Adaptation

The neonatal intestinal tract faces a particular challenge in adapting to the complex microbial ecosystem that develops immediately after birth. Host immunity and the microbiome develop through reciprocal interactions. The review by E. Tourneur and C. Chassin shows how aberrant development of the mucocutaneous intestinal barrier contributes to inflammatory disorders, such as NEC. This paper and that of D. J. Hackam et al. highlight the specific contribution of the receptor for bacterial endotoxin, Toll-like receptor 4 (TLR4), and related signaling events in normal development and NEC pathogenesis. Intestinal epithelial cells are innate immune competent and shape the development of intestinal immunity. Bacterial toxins have also been appreciated as critical effectors of several pathogens that threaten newborn infants. Their role in invasive neonatal infections is reviewed by A. F. Sonnen and P. Henneke. Given recent advances in toxin structure and membrane action, appreciating changes in immune cell signaling in newborns will be an essential step to understand pathogenic mechanisms of neonatal disease.

## 3. Bacterial Pathogenesis in the Newborn

While barriers such as skin and mucous membranes are more readily breached in the newborn compared to the adult, the infectious risk in early life is higher irrespective of route of infection [2, 3]. The clinically most relevant reason identified for these observations is the reduced ability of the newborn to prevent systemic spread of pathogens [5]. For example, A. M. Sherrid and T. R. Kollmann review how local cell-autonomous, as well as systemic innate and adaptive immunity of the newborn fails to restrict the spread of *L. monocytogenes*. And K. V. Driessche et al. highlight that the diminished proinflamatory cytokine capacity of the newborn following infection with *M. tuberculosis* allows bacterial spread. The reviews by M. R. P. Coombs et al.

for *Staphylococcus* species and E. A. Marchant et al. for coagulase-negative *Staphylococcus* emphasize that the inability for preterm and full-term newborns to localize infection leads to high morbidity and mortality early in life.

## 4. Viral Pathogenesis in the Newborn

The paper by S. Gantt and W. J. Muller reviews the immunologic basis for severe neonatal herpes disease and potential strategies for therapeutic intervention. They then explore differences between the immune system of newborns and those of older children and adults, which predispose them to severe infections. Two papers focus on another major viral pathogen, cytomegalovirus (CMV). In the first, M. Schleiss reviews the present state of knowledge on the immune responses of the fetus and newborn infant to CMV. With this as a background, the reader can better understand the rationale for the novel vaccine strategy proposed by M. Leviton et al. to prevent congenital infection, using an attenuated CMV vaccine.

It is our hope that this series of reviews and important new information will provide the reader with a clearer understanding of newborn host defense mechanisms and potential therapeutic strategies.

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*Review Article*

# Cytomegalovirus in the Neonate: Immune Correlates of Infection and Protection

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Fetal and neonatal infections caused by human cytomegalovirus (CMV) are important causes of morbidity and occasional mortality. Development of a vaccine against congenital CMV infection is a major public health priority. Vaccine design is currently focused on strategies that aim to elicit neutralizing antibody and T-cell responses, toward the goal of preventing primary or recurrent infection in women of child-bearing age. However, there has been relatively little attention given to understanding the mechanisms of immune protection against acquisition of CMV infection in the fetus and newborn and how this information might be exploited for vaccine design. There has similarly been an insufficient study of what deficits in the immune response to CMV, both for mother and fetus, may increase susceptibility to congenital infection and disease. Protection of the fetus against vertical transmission can likely be achieved by protection of the placenta, which has its own unique immunological milieu, further complicating the analysis of the correlates of protective immunity. In this review, the current state of knowledge about immune effectors of protection against CMV in the maternal, placental, and fetal compartments is reviewed. A better understanding of immune responses that prevent and/or predispose to infection will help in the development of novel vaccine strategies.

## 1. Introduction

Human cytomegalovirus (CMV) is the most common cause of congenital viral infection in the developed world, occurring in 0.5–2% of pregnancies in the United States and Europe [1, 2]. Congenital infections can cause severe sequelae among neonates including sensorineural hearing loss, cerebral palsy, microcephaly, cognitive impairments, and mental retardation [3–5]. During maternal primary infection, and to a lesser extent during recurrent infection, CMV can translocate the placental barrier and can cause infection of the developing fetus [6, 7]. Infection acquired *in utero* may have no clinical manifestations, or may manifest with hepatosplenomegaly, thrombocytopenia, cholestatic hepatitis, petechiae and purpura, central nervous system pathologies (including retinitis), viremia, and pneumonia [8]. In addition to being at risk for severe, occasionally life-threatening end-organ disease [9], infants with symptoms at birth also have an increased risk for long-term neurodevelopmental sequelae, including sensorineural hearing loss (SNHL). The long-term neurodevelopmental prognosis of a congenitally infected infant depends upon a number of factors, including the maternal immune status prior to the onset of pregnancy, whether or not she is reinfected with a new strain of CMV during pregnancy, and the timing of acquisition of fetal infection [10–12].

In addition to the impact of CMV infections acquired *in utero*, postnatal acquisition of CMV can also cause significant morbidity and occasional mortality. Disease is typically not observed in term infants, but can be a substantial problem for low birth weight premature infants [13, 14]. Because of the virtual elimination of transfusion-associated CMV heralded by the advent of leukofiltration of blood products [15], essentially all CMV infections in premature infants are acquired from maternal breast milk [16–18]. As is the case for congenital CMV infections, many breast milk-acquired infections in premature infants are asymptomatic, but a substantial percentage can produce severe, occasionally life-threatening disease, which can manifest as viremia, neutropenia, thrombocytopenia, hepatitis, pneumonia, enteritis,

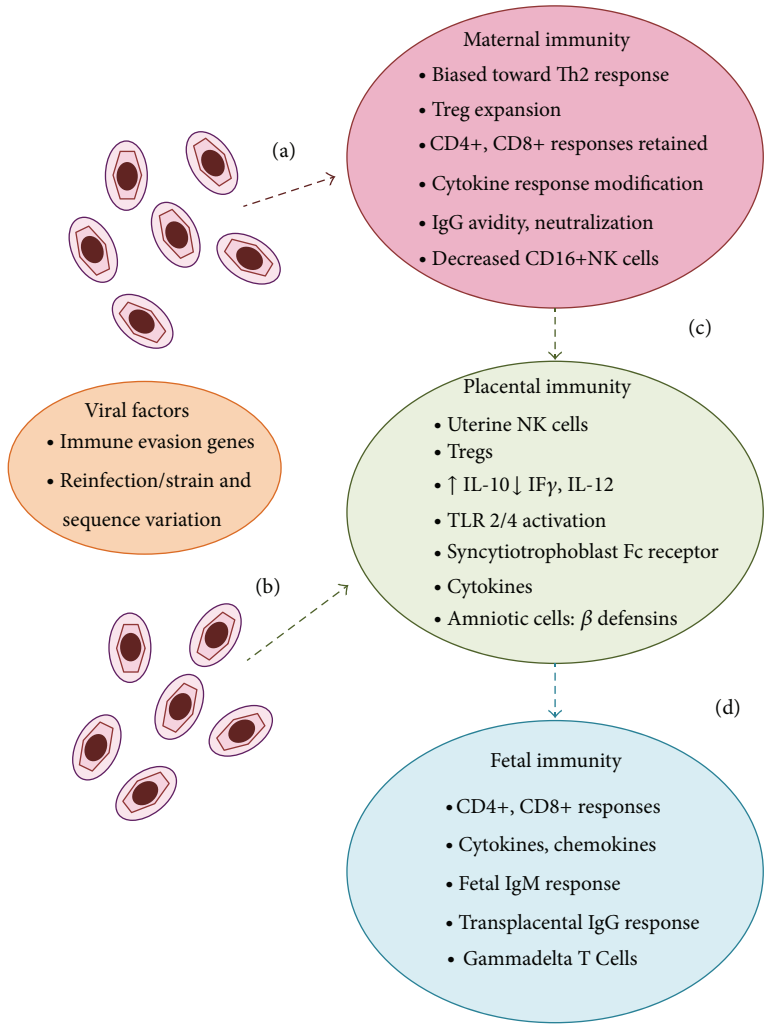


Figure 1: Schematic representation of pathways of CMV to the fetus and immune responses potentially important in transmission and prevention. The figure emphasizes the immunological milieu of pregnancy and some of the known immune adaptations associated with pregnancy. Left side of figure, CMV is known both to encode a plethora of immune evasion genes that subvert immune clearance of infection and to demonstrate substantial strain variation that can promote reinfection of already-immune hosts. Virus is believed to reach the placenta via the maternal compartment (a) or ascending infection via local extension in the reproductive tract (b). Although maternal antibody, CD4+, and CD8+ responses are generally intact in pregnancy, there are alterations in Th1/Th2 cytokine balance; alterations in NK cell subpopulations; increased Tregs; and modified cytokine responses. The uterine microenvironment in pregnancy may also play a role in direct local extension of CMV following virus exposure or reactivation (b), driven in part by increased localized IL-10 expression. Irrespective of the route of infection, the immunological profile of the placenta may either facilitate CMV transmission or inhibit it. Factors that may promote transmission include the less efficient killing potential of uNK cells; decreases in cytokines such as IL-12 and IF-$\gamma$; and the potential translocation of CMV particles across the syncytiotrophoblast if low avidity IgG is present. Factors that inhibit transmission include chemokines and $\beta$-defensins and, if present, high avidity neutralizing antibody, which may render virus noninfectious. Once virus enters the fetal compartment, the impaired capacity of fetal CD4+ to proliferate in response to CMV may impair immune control. The presence of transplacentally acquired IgG is believed to ameliorate the severity of disease. There is evidence that CD8+ cells, chemokines, and gammadelta T cells contribute to antiviral immunity in the fetal immune environment.

and a sepsis-like syndrome [14, 19]. It remains unclear whether such postnatal acquisition of infection poses any long-term risk for adverse neurodevelopmental outcomes [20–23].

Although the risks of CMV infection to the developing fetus and neonate are well recognized, the factors that dictate whether or not an infant has an asymptomatic infection or manifests with severe disease are not clear. Immune protection against congenital CMV infection is complex and requires consideration of immune responses in the mother, the fetus, and the placenta (Figure 1). Consideration must also be given to the burgeoning list of virally encoded immune modulation and immune evasion genes, which almost certainly exert a clinically relevant impact on the maternal and fetal immune responses to infection [24]. Another important issue is that of the problem of viral

strain variation and attendant reinfection, in light of the emerging recognition that maternal antiviral immunity to one strain of CMV may not protect against acquisition of, and subsequent fetal transmission with, a new strain [7, 25, 26]. Development of a vaccine against congenital CMV infection is a major public health priority [27], but most vaccine approaches to date have focused on what is probably an overly simplistic approach: namely, the prevention of primary infection in young women of child-bearing age. Although clearly infection of the fetus cannot occur if maternal infection is prevented by a successful vaccine [28, 29], current vaccine approaches have, by focusing almost exclusively on the prevention of maternal infection, failed to take into consideration the incompletely understood but critically important fetal and newborn immune responses to CMV that may play key roles in preventing CMV end-organ disease and sequelae. Understanding the immune response of the infected fetus may facilitate identification of correlates of protective immunity in the infant. Moreover, it is important to note that many of the CMV vaccines currently in clinical trials are focused on inducing immune responses known to be important in controlling CMV disease in solid organ and hematopoietic stem cell transplant patients, but these effectors of protection in transplant patients may or may not be relevant to the problem of prevention of maternal and fetal CMV infections [30].

Maternal-placental-fetal transmission of CMV occurs against the backdrop of the altered cytokine state of pregnancy, in which there is a functional immune suppression mediated via a shift from a Th1 response to a Th2 bias [31–33]. The altered immune state in pregnancy is likely to be highly relevant to the problem of sustaining vaccine-mediated protection against infection and transmission. Issues relevant to the study of the immunology of maternal-fetal CMV transmission are germane to the question of the key clinical and immunologic endpoints of vaccine trials, and may help define the most suitable patient population for ultimate administration of a licensed CMV vaccine against congenital CMV infection. A universal vaccine meant to provide a broad blanket of herd immunity on a population of young children may have different requirements than a vaccine selectively targeting young women of reproductive age. Acceptable attributes of a CMV vaccine in the latter scenario may include not only protection against transmission of infection, but also mediation of protection against CMV disease, even if transmission occurs. Congenital infection commonly occurs in the absence of symptoms or signs of illness, and asymptomatic infants generally have an excellent prognosis for normal neurodevelopment. Thus, better insight into those aspects of the immune response that may contribute to protection against disease in the asymptomatically infected infant might help guide future immunization approaches. This review summarizes recent observations gleaned from the study of the immune response to CMV, focusing on the pregnant woman and developing fetus. The potential application of these studies to immunotherapeutic interventions for prevention of congenital CMV infection and long-term disability is also discussed.

## 2. Innate Immunity

Innate immunity likely plays a crucial role in preventing acquisition of congenital and perinatal CMV infections; conversely, the failure of innate immunity, either due to host genetic factors, the immune tolerance state of pregnancy, and/or viral immune evasion, may contribute to an increased risk of acquisition of infection. Components of the innate immune response in the setting of congenital and perinatal infection include natural killer (NK) cells; toll-like receptors (TLRs); and cytokines. Available information about the role of each of these components in congenital and perinatal CMV infection and transmission is considered below.

*2.1. NK Cells.* NK cells are important effectors of innate immunity involved in control of viral infection. Human NK cells are typically characterized as CD3−CD56+ lymphocytes that make up about 15% of peripheral blood lymphocytes. They are further subdivided into $CD56^{bright}$ cells (lacking the expression of CD16 and the killer immunoglobulin-like receptor, KIR) and $CD56^{dim}$ cells, which express CD16 and KIR [34]. NK cells target virally infected cells through perforin and granzyme-mediated cell killing, Fas-ligand initiated apoptosis, and antibody dependent cellular cytotoxicity (ADCC). They also elaborate cytokines and modulate adaptive immune responses via interactions with plasmacytoid dendritic cells [35, 36]. CMV encodes a number of genes that interfere with the NK cell response. Some of these viral genes encode proteins that alter expression of NK cell receptor ligands, resulting in perturbation of function of the activating receptor, NKG2D; proteins that are homologs of MHC class I that bind the NK cell inhibitory receptor, LIR-1, with a higher affinity than host MHC class I; and proteins that decrease the expression of CD155, a ligand for NK cell activating receptors [24].

Modulation of the distribution of subtypes of NK cells during pregnancy may have an impact on the risk for acquisition of CMV infection by the placental-fetal unit. During pregnancy, the uterus contains cells known as "uterine NK cells (uNK)" or "decidual NK cells (dNK)". Although these differ from peripheral NK cells, they are in the $CD56^{bright}$ subset of NK cells, and hence have lower cytotoxic ability, similar to peripheral $CD56^{bright}$ NK cells [37]. This is one of many different manifestations of the "immune tolerance" state required during pregnancy to prevent rejection of the fetal allograft [38]. During the first trimester of pregnancy, uNK cells are the major population of maternal immune cells, accounting for ~70% immune cells in the decidua, with macrophages, T cells (CD8+, CD4+, and $\gamma\delta$T cells), and dendritic cells accounting for 20, 10, and 2%, respectively [39–41]. These uNK cells may compensate for their relative lack of cytotoxic potential by elaboration of antiviral cytokines, particularly interferon gamma, in the uterine microenvironment [42]. It has been reasonably speculated that the lack of an effector phenotype for the uNK cells may contribute to an increased risk of intrauterine CMV transmission [43]. However, it has also been noted that freshly isolated uNK cells can acquire major functional and phenotypic changes

and can become cytotoxic effectors following exposure to CMV-infected autologous decidual fibroblasts. NKG2D+ and CD94/NKG2C+ or 2E+ activating receptors are involved in the acquisition of cytotoxic function, and these cells in an *ex vivo* model of CMV-infected trophoblast colocalize with CMV-infected cells [44]. Hence, the cytotoxic potential of these cells following exposure to virus may be important in prevention of CMV transmission in early pregnancy [45].

In addition to the role NK cells play in the placental environment, a suboptimal or deficient NK cell response may play a role in modulating the clinical manifestations and severity of congenital CMV infection. A child with NK cell deficiency was noted to have severe herpesvirus infections, including CMV, although her CMV infection did not appear to be acquired in the perinatal period [46]. A deficiency in NK cell cytotoxic response to herpes simplex virus (HSV)-infected cells was proposed to be a predisposing factor influencing the severity of neonatal HSV infection [47]; whether such mechanisms are relevant for perinatally acquired CMV infection remains to be evaluated. A recent study demonstrated that increased proportions of NK cells expressing the activating killer lectin-like receptor, NKG2C+, were more frequently detected in children with congenital CMV infection. Strikingly, this immunophenotype was more common in symptomatic cases of congenital infection [48], suggesting this as an important correlate of disease outcome. Expansion of NKG2C+ cells also appeared more marked in children with postnatal infection (presumed to be acquired by breastfeeding) than in the group of infants with congenital asymptomatic infection. Based on analogy with studies performed in immune suppressed patients, the authors speculated that the magnitude of the NKG2C+ expansion might be inversely related to the effectiveness of the T-cell response to CMV infection; in other words, that NKG2C+ expansion might reflect inadequate T-cell immunity. Immunophenotyping of NK responses, therefore, might prove useful in assessing prognosis, or identifying infants that would be candidates for immunotherapies. Whether the expansion of NKG2C+ NK cells observed in the setting of symptomatic congenital or perinatal infection contributes to the immunopathogenesis, or conversely the long-term disease control of CMV infection, will require further study.

*2.2. Phagocytic Cells.* There is relatively little information about the role of phagocytic cells (neutrophils, macrophages) in protection against congenital infection or, in the setting of aberrant function, increased susceptibility to congenital infection. That neutrophils may be important in the first line of defense against vertical transmission of infection is suggested by pathologic studies of CMV-infected placentas demonstrating neutrophilic infiltrates in fetal blood vessels in the villus core [49]. In these studies, placentas with high levels of viral DNA were associated with neutrophilic infiltrations, whereas macrophages and dendritic cells were associated with low levels of DNA; hence, a response biased toward a phagocytic cellular response may be associated with less robust control of infection. Notably, congenital CMV infection does not appear to be associated with heritable abnormalities in neutrophilic oxidative burst, as seen in chronic granulomatous disease [50]. A case of congenital CMV was recently described in a patient with leukocyte adhesion defect type 1 [51], although this infant also had a natural killer cell deficiency, making it unclear to what extent the neutrophil defect contributed to the increased risk of CMV infection. In another case of fulminant congenital CMV, delayed fetal neutrophil differentiation was implicated as a possible contributing factor to the fatal outcome [52].

No cases of congenital CMV infection appear to have been correlated with aberrant macrophage function. However, macrophages do appear to play an as-yet incompletely defined role in modulating vertical transmission of CMV. In CMV infected placentas, marked hyperplasia of fetal-derived placental macrophages, termed Hofbauer cells, has been observed [53]. A serum proteomic comparison of infants with congenital CMV infection and controls demonstrated upregulation of a macrophage-derived chemokine in those infants with CMV infection [54]. It has been suggested that macrophages may potentiate CMV infection and spread in syncytiotrophoblasts. This is based on a cell coculture model system in which the presence of macrophages enhanced activation of CMV in syncytiotrophobast and promoted transmission of virus from cell-to-cell, an effect which was mediated by IL-8 and TGF-$\beta$ [55]. Macrophages are also themselves targets of CMV infection. Viral antigen was noted in macrophages in a study of term CMV-infected placentas [49]. The ability of CMV to infect macrophages was also demonstrated in a recently described decidual organ culture model [56].

*2.3. Toll-Like Receptors (TLRs).* There are ten toll-like receptors (TLRs) described in humans [57]. TLRs function to sense microbial pathogens through interactions of pathogen-associated molecular patterns (PAMPs) through their cognate pattern recognition receptors (PRRs), in the process signaling through MyD88-dependent and TRIF-dependent pathways, which in turn upregulates cytokine production [58]. There is evidence that interactions between some members of the TLR family with CMV influence both the immune response to, as well as the outcome of, infection.

TLR2, although typically considered in the context of PAMPs consisting of bacterial polysaccharides, is also a PRR for the CMV envelope glycoproteins, glycoprotein B (gB), and glycoprotein H (gH). Notably, a polymorphism in the TLR2 gene was shown to be associated with increased CMV replication and an increased risk of CMV disease in liver transplant recipients [59, 60]. No data exist, however, on whether this TLR2 polymorphism impacts the phenotype of fetal or perinatal CMV disease. CMV-mediated TLR2 signaling was noted to lead to an inflammatory response in a cell culture model utilizing syncytiotrophoblasts, suggesting that this pathway could have some influence on the manifestations of placental-fetal infection *in vivo* [61, 62]. This signaling occurred in the absence of viral replication, indicating that structural component(s) of the virion may be responsible. These structural components appear to be gB and gH, which have been shown to induce inflammatory cytokine

secretion in response to CMV exposure, independent of viral replication [63, 64]. This signaling appears to be mediated by a TLR heterodimer on the infected cell surface, consisting of TLR2 and TLR1 [63].

TLR3 interactions with CMV are related to the binding of double stranded RNA molecules produced during CMV replication [57]. The interrelationship between polymorphisms in TLR3 and susceptibility to herpesvirus disease was demonstrated in studies linking HSV encephalitis to a specific TLR3 polymorphism associated with diminished inflammatory cytokine production following stimulation with an agonist [65, 66]. This diminished cytokine responsiveness in turn correlated with an increased severity of herpes simplex encephalitis. Recently, an increased susceptibility to CMV infection was proposed for individuals with the L412F variant of TLR3 [67]. Peripheral blood mononuclear cells from these patients, who are known to have an increased risk for fungal infections and autoimmunity, were assayed for secretion of cytokines in response to TLR3 ligands and to CMV. Reduced IFN$\gamma$ and TNF$\alpha$ secretion were demonstrated when the L412F polymorphism was present. It was inferred that this TLR3 polymorphism conferred an increased risk of CMV disease [67]. On the other hand, no role for TLR3 signaling could be demonstrated during the early immune response of human monocyte-derived dendritic cells after infection with a wild-type isolate of CMV, strain TB40E. This study therefore failed to support a role for CMV-TLR3 interactions in the immunopathogenesis of infection [68].

TLR7 polymorphisms have been suggested to play a role in dictating the magnitude of the immune response to CMV glycoproteins. In a study genotyping 142 women who had been previously vaccinated with three doses of adjuvanted CMV gB vaccine, it was observed that homozygous carriers of single nucleotide polymorphisms in TLR7 demonstrated a higher vaccination-induced antibody response to gB than did heterozygotes or homozygotes for this allele [69]. Whether or not TLR7 polymorphisms impact the immune response to gB in the context of natural CMV infection, or transmission of CMV to the fetus, remains to be evaluated.

*2.4. Cytokines, Chemokines, and Defensins.* Many cytokines appear to be important in immune control of CMV infection, although defining cytokine(s) that may correlate either with protection or increased susceptibility to infection in the context of congenital or perinatal CMV infection has been difficult in studies reported to date. One difficulty in interpreting studies correlating altered cytokine profiles with an increased CMV infection risk is that such associations may not reflect isolated cytokine perturbations *per se*, but rather modulation of upstream events that trigger (or diminish) the elaboration of cytokines. These include the TLR polymorphisms already discussed, such as the TLR3 polymorphisms that are associated with decreased proinflammatory cytokine production [67]. Physiologic alterations in cytokine production during pregnancy may contribute to an increased risk of some viral infections. Normal pregnancy is also associated with increased production of IL-10 [70], an anti-inflammatory cytokine, and increased production of this cytokine during pregnancy has been proposed to increase susceptibility to fetal CMV infection [43]. Other cytokines proposed to be important in the context of perinatal viral transmission include TNF-$\alpha$, IL-12, IL-17, IL-18, IL-23, and IL-1$\beta$ [43, 71], although there has been no clear correlation between hereditary deficiencies in any of these cytokines with an increased risk of congenital infection. Similarly, therapeutic monoclonal antibodies targeting TNF-$\alpha$ have not been associated with an increased risk of congenital CMV. However, it has been pointed out that the use of TNF-$\alpha$ blocking agents during pregnancy may, by blocking the activity of the TNF superfamily members lymphotoxin-$\alpha$ and -$\beta$, negatively impact the development and organization of secondary lymphoid tissues [72]. A recent analysis of the safety of these agents during pregnancy and lactation suggested an increased risk of infection in infants exposed to these monoclonal antibodies [73], although CMV was not specifically mentioned.

An important consideration in the analysis of the cytokine profile of the congenitally CMV-infected infant is the fact that infant immunocytes produce smaller amounts of cytokines than do comparable adult cells [74, 75]. Hence, the fetus may be intrinsically at increased risk for CMV infection upon exposure to the virus. A study of cord and adult blood-derived myeloid dendritic cells, following infection with CMV, demonstrated significantly lower levels of IL-12, IFN-$\beta$, and IFN-lambda1 production in neonatal cells [76]. On the other hand, another study comparing immune responses between congenitally infected infants and their mothers (who all had serological evidence of primary infection) demonstrated that neonates had significantly higher levels of IL-8, but lower levels of IF-$\gamma$ [77]. Most of the infants in this report were asymptomatic, so the functional consequences of these differences in cytokine profiles with respect to the susceptibility, pathogenesis, and natural history of CMV infection are not clear.

The profile of chemokine and defensin production in the setting of congenital CMV infection has not been extensively studied. One study assayed cytokines and chemokines from midtrimester amniotic fluid in 8 patients giving birth to infants with congenital CMV; midtrimester sera from 12 pregnant women with primary CMV infection; and amniotic fluid and serum from uninfected pregnant controls. This analysis demonstrated that levels of chemokines CCL2, CCL4, and CXCL10 were significantly elevated in amniotic fluid from congenital CMV patients [71]. In this study, only CXCL10 was significantly elevated in the serum of CMV-infected pregnant women, compared to controls. This study did not comment on the chemokine profiles observed in the subset of women with primary CMV infections who did not go on to transmit virus to the fetus. Future studies of amniotic fluid comparing these subgroups would be of considerable interest in elucidating differences between transmitting and nontransmitting mothers. In another study of chemokine production in the CMV-infected placenta, expression of the chemokine MCP-1 was associated with fetal demise [78]. The effect was specific to CMV, insofar as other placental pathogens did not induce MCP-1 hyperexpression. These observations suggested that CMV-induced

chemokine dysregulation of placental function may be an important indirect contributor to fetal disease, leading to adverse pregnancy outcome even in the absence of fetal infection *per se*. An interesting study reported by Liu and colleagues used serum proteomic analyses in an attempt to compare protein biomarkers of potential interest in infants with congenital CMV and controls. This study had subgroups of congenitally infected infants who were asymptomatic at birth and compared their proteome profiles to those of symptomatic infants with hepatitis. Thus, this study had the potential to identify candidate biomarkers associated both with a heightened risk of CMV disease or, conversely, protection against disease. Intriguingly, two protein peaks were noted that were upregulated in asymptomatic infants. These protein peaks were interpreted by these investigators, based on molecular weight, as corresponding to $\beta$-defensins 31 and 8 [54]. Further studies to confirm this observation and to better define the role of $\beta$-defensins in protection against congenital CMV infection are warranted.

## 3. Adaptive Immunity

Adaptive immunity in the context of congenital and perinatal CMV infection has clearly been more extensively evaluated than innate immunity. This reflects to a substantial extent the fact that therapeutic interventions based on adaptive immune responses, such as vaccines, are in advanced stages in clinical trials [79]. Adaptive immunity conferred by passive transfer of therapeutic anti-CMV immunoglobulin is also an area of intense clinical research activity [80, 81]. A number of studies have attempted to elucidate the role of antibody in both protection against congenital CMV transmission and, paradoxically, in promoting transmission of CMV across the syncytiotrophoblast. Evaluation of cellular immune responses has suggested for many years that there is functional impairment of aspects of cell-mediated immunity in infants with CMV infection and their mothers that may be important in transmission and disease progression. These early studies included demonstration of diminished lymphocyte-mediated cytotoxicity in infants with congenital CMV infection and their mothers compared to controls [82] and diminished CMV-specific lymphocyte blastogenesis and interferon production in congenitally infected infants and their mothers [83]. More recent definitive analyses of specific T-cell populations and of immunoglobulins in the context of congenital infection have been undertaken. These studies are reviewed in the following section.

*3.1. CD4+ T Cells.* The magnitude of the maternal CD4+ T-cell response to CMV infection appears to play an important role in predicting whether virus is transmitted to the fetus. A study of 46 pregnant and 8 nonpregnant women, seropositive for CMV and actively shedding virus in urine, examined the frequency of CMV-specific CD4+ T cells in peripheral blood lymphocytes [84]. Intracellular cytokine staining for IF-$\gamma$ and TNF-$\alpha$ was also performed. There were no changes in the frequencies of CMV-specific CD4+ T cells in CMV-seropositive normal nonpregnant and pregnant women at any gestation, although the frequency of CMV-specific CD4+ T cells was increased in pregnant women with evidence of CMV reactivation or reinfection. There were no congenital CMV infections in these pregnancies, leading these authors to propose that the CD4+ T-cell response can contribute to protection against intrauterine transmission, particularly in the setting of exposure to either reactivated latent virus, or new strains of virus encountered in the setting of re-infection. Another prospective study examined CMV-specific lymphoproliferative response and intracellular cytokine (IFN-$\gamma$ and IL-2) production during the first year after primary infection in 49 pregnant women and 9 nonpregnant controls. During the first month after infection, IFN-$\gamma$ producing CD4+ and CD8+ T cells were uniformly present, whereas IL2-producing T cells were very rarely detected. Notably, a significantly delayed development of the CD4+ T-cell lymphoproliferative response was observed in infected mothers who transmitted virus to the fetus, compared with women who did not transmit [85].

Another study of 74 pregnant women and 29 nonpregnant controls with primary CMV infection enumerated CMV-specific CD4+ cells by cytokine flow cytometry and lymphoproliferative responses [86]. A significantly lower median stimulation index was observed in the 19 women who transmitted the virus than in the 21 women who did not. No other immunologic (IgM response, IgG antibody avidity) or virologic marker (magnitude of DNAemia) was predictive of transmission. Similar observations regarding the importance of the lymphoproliferative response to CMV have been noted by other investigators [87, 88]. These observations suggest that interventions designed to maximize the maternal CMV-specific lymphoproliferative CD4+ response may be useful in protection against congenital CMV infection. Other studies have examined the pattern of CMV-specific T-cell responses in pregnant women during the first year after acquisition of a primary infection, compared to those of pregnant women with prior preconception immunity to CMV. These analyses demonstrated that, in addition to the delayed lymphoproliferative response in CMV-transmitting mothers, there was also a significant delay in the reversion of CMV-specific effector memory T cells to the CD45RA+ phenotype [89, 90]. These investigators proposed that examination of CD45RA reexpression might be an important prognostic parameter in the setting of maternal-fetal transmission.

In addition to the importance of maternal CD4+ responses in CMV transmission, CD4+ responses in the fetus and newborn in the context of vertical transmission may also play a role in predicting the outcome of congenital infection. CD4+ responses to CMV infection in young children are of substantially diminished magnitude compared to adults. Young children have a selective and long-lived deficiency in CD4+ T cell immunity characterized by decreased IF-$\gamma$ and IL-2 production. It was postulated that this suboptimal CD4+ response might be responsible for the prolonged shedding of CMV observed in infants following acquisition of CMV infection [91]. In this study, these young children had no symptoms of CMV disease and had presumably acquired infection from breastfeeding or attendance in group day care.

In addition to the study of CD4+ responses in pregnant women and young children with post-natally acquired infections, the CD4+ responses of the CMV-infected fetus has been evaluated in several studies. These analyses suggest that the magnitude of the CD4+ response in the fetus may not play a significant role in protection, and in fact may correlate with the severity of CMV disease. In a study of perinatal CMV infection in a high seroprevalence population, the frequencies of CMV-specific CD4+ T cells detected by intracellular cytokine staining for IF-$\gamma$ and TNF-$\alpha$ were higher in infants with symptomatic congenital infection than in those infants with asymptomatic perinatal infection [92]. This could, of course, simply reflect a more intense infection with higher viral load and not a deleterious effect of the cytokine response *per se*. The authors suggested that monitoring these immunological markers could be useful in predicting the prognosis of congenital CMV infection.

Not all studies have readily demonstrated fetal/neonatal CD4+ responses in the setting of congenital infection. A study of seven patients with congenital CMV infection, six healthy infants who had acquired infection postnatally, and six CMV-seropositive adults found a striking paucity of CMV-specific IF-$\gamma$-producing CD4+ cells in congenitally infected infants, compared to the healthy infant and adult controls with CMV infection; however, the congenitally infected infants in this study were asymptomatic, so this study did not exclude a relationship between CD4+ response and symptomatic disease [93]. Another recent study compared T-cell responses in 24 children with congenital CMV infection (9 symptomatic), 19 children with postnatal CMV infection, and 8 adults with symptomatic primary CMV infection. Compared to adults, CMV-specific CD4+ T-cell responses in children younger than 2 years were low or undetectable, although they did appear to increase over time. No differences were noted with regard to CD8+ T-cell responses, and no differences were noted comparing symptomatic and asymptomatic children. These authors concluded that the inadequate response of CD4+ cells is a major factor responsible for lack of immune control of CMV infections in infants and young children [94]. It is of interest to reflect on these observations in light of a recent study demonstrating a striking inhibitory effect of CMV particles on CD4+ T-cell proliferation, concomitant with decreased levels of cytokines IL-4, IFN-$\gamma$, and TNF-$\alpha$ in cell culture [95].

An interesting subset of CD4+ cells are known as regulatory T cells (Tregs). These cells are critical to the maintenance of immune cell homeostasis by mediating a dominant negative regulation on other immune cells. These cells can be broadly classified into natural or adaptive (induced) Tregs. "Natural" Tregs are CD4+CD25+ T cells which develop in and emigrate from the thymus to maintain immune homeostasis, maintain tolerance to self-antigens, and abrogate autoimmune disease. "Adaptive" Tregs are nonregulatory CD4+ T cells which acquire CD25 expression outside of the thymus and are typically induced by inflammation and disease processes. Expansion of Tregs is important in the maintenance of normal pregnancy and contributes to the protection of the fetus from the maternal immune response [96, 97]. However, Tregs may also block beneficial immune responses by preventing development of sterilizing immunity to viruses [98, 99]. The role that Tregs play in susceptibility to or protection against fetal CMV infection has not been investigated. A recent analysis of Tregs during CMV replication in solid organ transplant recipients demonstrated that lower Tregs were observed in patients with spontaneous clearance of virus after transplantation and that the ratio of CMV-specific T cells to Tregs was highly predictive of relapse [100]. Treg-mediated suppression of anti-CMV responses was observed in a study in which Tregs were depleted from peripheral blood mononuclear cells prior to measurement of IF-$\gamma$ production. In this study, CD8+ T cells produced more IF-$\gamma$ in the absence of Tregs [101]. Following hematopoietic stem cell transplantation, Tregs do not appear to inhibit CMV clearance by conventional T cells [102]. The relevance of these observations to the role of Tregs in modulation of fetal and neonatal CMV infection remains to be examined.

*3.2. CD8+ T Cells.* Primary infection with CMV in immunocompetent hosts is accompanied by activation and differentiation of naïve CD8+ T cells, which become effector/memory cells capable of secreting IFN-$\gamma$ and attacking and lysing infected target cells [103]. Studies examining the specific virally-encoded targets of CD8+ T cells have demonstrated that there is a broad and diverse repertoire of responses to many viral peptides, although reactivity against CMV proteins pp65 (ppUL83) and IE-1 appear to be of the greatest importance in control of CMV disease [104–106]. Healthy CMV-seropositive individuals devote approximately 10% of the total memory T-cell pool in the peripheral blood to CD8+ cells specific for CMV antigens [107].

CD8+ responses are readily generated following CMV infection both in young children and by the fetus *in utero*. CD8+ responses in young infants, compared to adults, are known to demonstrate focused peptide specificity and lower peptide avidity, although the peptide specificity does broaden over time [108]. The development of CD8+ responses in the setting of perinatal/congenital CMV infection has been examined by a number of groups. To attempt to elucidate the role of CD8+ responses in protection against congenital CMV infection, Pédron et al. examined 16 transmitter mothers who underwent seroconversion during the first trimester of pregnancy and their fetuses (all were positive for CMV in amniotic fluid by PCR at 17–19 weeks of gestation). Fetal and maternal blood samples were collected between the 22nd and 39th week of gestation. Activation, effector, and memory phenotypes were compared, and IF-$\gamma$ secretion was examined. The responses were generally similar, although there was a smaller pp65-specific pool in the fetus, and fetal CTLs made less IF-$\gamma$ in response to stimulation with a CD3 monoclonal antibody [109]. Another study in 15 CMV-infected fetuses demonstrated CD8+ responses as early as the 22nd week of gestation. Compared with controls, CMV-infected fetuses demonstrated a dramatic increase in activated and terminally differentiated CD8+ T cells [110]. However, these authors noted that cellular immunity to CMV did not appear to be fully functional, evidenced by the fact that the number of T cells capable of secreting IFN-$\gamma$ was substantially

Table 1: Summary of innate immune responses and their proposed role in control of or susceptibility to congenital CMV infection.

| Innate immunity and susceptibility/protection in congenital CMV infection | | |
|---|---|---|
| Immune effector | Maternal/placental/fetal compartment | Proposed effect on CMV transmission/disease |
| NK cells-CD56$^{bright}$ | Maternal (pregnancy)<br>Uterine NK cells | (i) Decreased cytolytic potential<br>(ii) Increased risk of CMV transmission? |
| NK cells-NKG2C+ | Fetal compartment | (i) Expansion of this NK subset in congenital and perinatal CMV<br>(ii) Correlation with symptomatic CMV disease? |
| Phagocytic cells | Placental compartment | (i) Neutrophils: possible role in defense<br>(ii) Macrophage: potentiates spread to syncytiotrophoblasts? |
| Toll-like receptors | Maternal compartment<br>Placental compartment | (i) TLR2 polymorphism; ↓ signaling to CMV glycoproteins; ↑ risk of CMV disease in transplant patients; increased transmission risk?<br>(ii) TLR3 polymorphism; decreased signaling to CMV antigens<br>(iii) TLR7 polymorphism: decreased antibody response to glycoprotein B? |
| Cytokines<br>Chemokines<br>Defensins | Neonatal compartment<br>Maternal compartment<br>Placental compartment<br>Placental-fetal interface | (i) ↑ IL-8 ↓ IF-$\gamma$ may correlate with increased transmission risk<br>(ii) Increased maternal CCL-10 correlates with transmission<br>(iii) Increased placental MCP-1 expression correlates with fetal demise<br>(iv) Physiological increase in uterine IL-10 in pregnancy: increased risk of reactivation/transmission?<br>(v) Beta-defensins 8 and 31 proposed to be upregulated in amniotic fluid of asymptomatically congenitally infected infants |

lower after *in vitro* stimulation with CMV antigen than after exposure to stimulants such as phorbol myristate and ionomycin.

Although these studies raise questions about the functionality of CMV-specific CD8+ responses generated *in utero*, a study lead by Marchant and colleagues examining 8 infants with congenital infection and 15 uninfected controls demonstrated the expansion and the differentiation of mature CMV-specific CD8+ cells with similar characteristics to those detected in adults. These cells demonstrated potent perforin-dependent cytolytic activity and produced abundant amounts of antiviral cytokines, particularly IF-$\gamma$ [111]. These data support the concept of a potentially protective role for the development of fetal antiviral CD8+ T-cell responses in the control of CMV disease. These observations also suggest the provocative possibility of designing and developing prenatal vaccination strategies toward the goal of priming fetal immunity against CMV, as well as for other viral diseases [112, 113]. Further studies are clearly required to define the role of CD8+ cells—engendered both in the maternal and fetal compartments—in protection against congenital CMV infection.

*3.3. Gammadelta T Cells.* Gammadelta T cells are unconventional T cells that do not require antigen processing and major histocompatibility-complex presentation of peptide epitopes and, accordingly, can react rapidly upon activation [114]. These cells demonstrate features of both adaptive and innate cells and are described as a "bridge" between innate and adaptive immunity. A study of CMV-infected fetuses demonstrated that fetal gammadelta T cells are capable of expansion and differentiation [115]. Differentiated gammadelta T cells expressed high levels of IFN-$\gamma$, natural killer cell receptors, and other cytokines, and demonstrated antiviral activity. Differentiated gammadelta T cells could be detected as early as after 21 weeks of gestation. The extent to which this T-cell subset participates in antiviral defense *in utero* and in early life requires further investigation.

*3.4. Antibody.* Of all the immune effectors studied in the context of congenital and perinatal CMV transmission, perhaps the most studied are anti-CMV antibodies. This is driven in part by the intense interest in adjuvanted glycoprotein subunit vaccines engendered by recent clinical trials designed to elicit a protective antibody response against CMV gB. In both young women of childbearing age [116] as well as in solid organ transplant recipients [117], a CMV vaccine based on purified subunit gB demonstrated some degree of protection against acquisition of CMV infection. The fact that the gB vaccine was also capable of boosting antibody titers when administered to women who already had CMV antibody from previous infection suggests that a vaccine strategy of immunizing seropositives might be able to prevent reinfection with and subsequent transmission of new CMV strains in women with prior immunity [118, 119]. These successes notwithstanding, the role that anti-CMV antibody response plays in protection of the fetus remains incompletely understood. Notably, there is not known to be an increased incidence of congenital CMV infection in the setting of humoral immunodeficiencies, suggesting that antibody is not absolutely required for protection. However, a case of congenital CMV infection was recently reported in a woman receiving the anti-B cell monoclonal antibody, rituximab [120], suggesting that the inability to sustain a humoral response to CMV may confer an increased risk of transmission in some patients. Rituximab has also been associated with serious CMV disease in a patient on maintenance therapy [121].

Although antibody plays an important role in protection against CMV infection and disease, the level of protection

Table 2: Summary of adaptive immune responses and their proposed role in control of or susceptibility to congenital CMV infection.

| Adaptive immunity and susceptibility/protection in congenital CMV infection | | |
|---|---|---|
| Immune effector | Maternal/placental/fetal compartment | Proposed effect on CMV transmission/disease |
| CD4+ T cells | Maternal compartment<br>Fetal/neonatal compartment | (i) Delayed development of CD4+ T-cell lymphoproliferative response correlates with maternal-fetal transmission<br>(ii) Defective CD4+ immunity; diminished IF-$\gamma$ and IL-2 production in fetal and early childhood infection<br>(iii) Defective fetal CD4+ response may contribute to congenital CMV infection and disease |
| T-regs | Maternal compartment | (i) Treg expansion: normal response to pregnancy<br>(ii) $\downarrow$ Tregs: correlates with protection against CMV disease in transplant recipients<br>(iii) Relevance to congenital CMV unknown |
| CD8+ T cells | Maternal compartment<br>Fetal/neonatal compartment | (i) CD8+ response to infection appears unaltered in pregnancy<br>(ii) Fetal CD8+ response to CMV antigens noted as early as week 22 gestation<br>(iii) Exhibit cytolytic properties and elucidate IF-$\gamma$<br>(iv) Some studies raise questions about functionality? |
| Gammadelta T cells | Neonatal compartment | (i) Fetal gammadelta T cells differentiate and expand in setting of congenital CMV infection<br>(ii) Produce IF-$\gamma$ and other cytokines<br>(iii) Role in protection of fetus, control of virus not clear |
| Antibody | Maternal compartment<br>Placental compartment<br>Fetal compartment | (i) Variability in maternal antibody response based on viral strain variation, possibly TLR polymorphisms<br>(ii) Expression of neonatal Fc receptor may paradoxically promote transcytosis of CMV particles across syncytiotrophoblast by low-avidity antibody<br>(iii) High avidity antibody may neutralize CMV at placental interface<br>(iv) Transplacental transfer of therapeutic neutralizing antibody may improve outcome of infected fetus |

is clearly incomplete. Notably, CMV can readily infect the newborn infant, via ingestion of breast milk, even in the setting of passively acquired maternal antibody [17, 107]. Moreover, CMV reinfection of the pregnant woman with subsequent transmission to the fetus, as noted earlier, can occur even in the setting of preexisting maternal immunity [7, 122, 123]. These shortcomings aside, there is an emerging role for IgG as an immunotherapy for prevention and treatment of congenital CMV. A study of administration of CMV-specific hyperimmune globulin to pregnant women appeared to significantly lower the risk of congenital CMV infection and disease, although given the uncontrolled nature of the study, conclusions about the mechanism of protection could not be definitively drawn [124, 125]. The extent to which antibody therapy reverses established CMV disease in the infected fetus or prevents sequelae is uncertain, although some studies to date are very encouraging, suggesting both short-term [126] and long-term [127] benefits. The beneficial effect of immune globulin is proposed to be mediated by virus neutralization in the CMV-infected fetus, although it is possible that the benefit of IgG may be via another mechanism. The major target of the neutralizing antibody response in CMV hyperimmune globulin is directed at proteins in the gH/gL/UL128/UL130/UL131 complex [128], and if ongoing trials confirm a protective/therapeutic effect of hyperimmune globulin administration to pregnant women at high risk of CMV transmission to their fetuses [129], antibodies to this complex may emerge as an important serological correlate of protection.

In addition to providing a protective/therapeutic effect for the CMV-infected fetus, antibody appears to exert protection at the level of the placenta. In an ultrasonographic assessment of placental thickness, women with primary CMV infection who had a fetus or newborn with CMV disease had placentas that were significantly thicker than those of women with primary CMV infection who did not have a diseased fetus or newborn; moreover, receipt of hyperimmune globulin was associated with statistically significant reductions in placental thickness [130]. Immunohistochemical analyses also supported a benefit of IgG on placental health [131]. On the other hand, CMV antibody may, paradoxically, promote transmission of virus to the fetus, via the expression of the neonatal Fc receptor on syncytiotrophoblast. It has been shown that antibody-virus complexes can translocate the syncytial barrier via this receptor, allowing entry of virus into the fetal circulation [132]. In this model, virus transmission can be interrupted if the antibody is of sufficiently high neutralizing capacity and avidity. Viral transmission from mother to fetus may be increased if the maternal antibody response is of low avidity or of poor neutralizing activity.

There is relatively little information about the ability of the infected fetus to mount an independent antibody response to CMV infection. The infected fetus generates IgM antibodies to CMV but the antiviral activity, if any, of such antibodies has not been evaluated. CMV IgM antibodies can be measured in the newborn as an adjunct to other diagnostic studies [133] but it is unknown if they play any role in disease control.

## 4. Summary

In summary, there is limited information about the precise protective correlates of anti-CMV immunity in the setting of congenital and perinatal infection, although the increasing availability of cohorts of congenitally infected infants has enabled some research into the fetal and neonatal immune response in recent years. Protection of the fetus cannot be assured in the setting of maternal immunity, since reinfection with a novel strain of CMV can occur, with transmission to the fetus. Viral strain variation may contribute to reinfection, and low maternal IgG avidity may paradoxically promote transmission of virus across the syncytiotrophoblast, via the neonatal Fc receptor. Other immune mechanisms operating at the level of the placenta, particularly innate immunity, may play a more important role than antibody in limiting infection of the fetus. The physiological state of pregnancy may impair some aspects of the immune response to CMV, predisposing to placental and fetal infection. Tables 1 and 2 provide a summary of available information regarding the potential protective and predisposing parameters of innate and adaptive immune responses associated with CMV transmission. Importantly, the fetus can mount an immune response to CMV, although the role that this response plays in limiting the extent of CMV-associated disease or sequelae remains uncertain and requires further investigation. It is conceivable that novel vaccines and immunotherapies could exploit aspects of innate and adaptive immunity known to correlate with protection. These should be considered in ongoing vaccine design. It is also important to keep in perspective that not all congenital infections result in disease and/or sequelae. Prospective natural history studies that define the immune correlates associated with protection against transmission and/or protection of the newborn from progression to CMV disease are needed. Since most congenitally infected infants are asymptomatic and have a good prognosis for normal neurodevelopmental outcome, comparing the immunological profile of asymptomatic infants to those with disease and/or sequelae is a high-priority area for future research. These observations could in turn provide opportunities for the design and development of effective interventions to help address this unsolved public health problem.

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*Review Article*

# Neonatal Host Defense against Staphylococcal Infections

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Preterm infants are especially susceptible to late-onset sepsis that is often due to Gram-positive bacterial infections resulting in substantial morbidity and mortality. Herein, we will describe neonatal innate immunity to *Staphylococcus* spp. comparing differences between preterm and full-term newborns with adults. Newborn innate immunity is distinct demonstrating diminished skin integrity, impaired Th1-polarizing responses, low complement levels, and diminished expression of plasma antimicrobial proteins and peptides, especially in preterm newborns. Characterization of distinct aspects of the neonatal immune response is defining novel approaches to enhance host defense to prevent and/or treat staphylococcal infection in this vulnerable population.

## 1. Introduction

Over 30% of deaths in children under the age of 5 occur within the first 4 weeks of life [1]. In this context, understanding the immunologic mechanisms underlying neonatal susceptibility is essential for the development of novel approaches to prevent and/or treat bacterial infection. Newborns in neonatal intensive care units (NICUs) are at risk of infection. An improvement in practices regarding hand washing, nutrition, skin, and respiratory care decreases *Staphylococcus* spp. infections [2, 3]. Such NICU quality improvements also significantly decrease costs per patient [4]. Antibiotics are the primary treatment for staphylococcal infections, but the use of these agents is also associated with resistance and alteration of the host microbial flora. Herein, we review innate host defense against *Staphylococcus* spp. with an emphasis on *S. epidermidis* (SE) and *S. aureus* (SA). Accordingly, we searched PubMed, a computer-based literature search engine, using the following terms: "newborn" OR "neonate" OR "neonatal" AND "Staphylococcus" AND/OR "sepsis" OR "innate" OR "cytokine" OR "Toll-like receptor" OR "pattern recognition receptor" OR "antimicrobial peptide" OR "neutrophil" OR "monocyte." We then organized the resulting references grouping them into detector function, effector function, and translational efforts.

## 2. Neonatal Staphylococci Infections

SE colonizes newborns [5] and remains a part of the human normal flora [6, 7]. SE-induced sepsis in preterm newborns has been associated with an increased risk of adverse common outcomes, prolonged hospital stay, and increased costs [8–17]. SE is the most frequently isolated strain of coagulase-negative staphylococci (CoNS) and is identified diagnostically from SA because of its inability to produce coagulase [18, 19]. SE forms a biofilm on catheters and commonly infects immunocompromised patients [6, 20–22].

Invasive infections due to extracellular pathogens such as CoNS are largely restricted to premature infants. At the University Hospital of Patras in Greece, between 2006 and 2007, 8.5% of all NICU admissions had late-onset CoNS sepsis. SE was the most prevalent organism found, and biofilm production was identified as a determinant for persistent infection [23]. The majority of late-onset sepses (defined as 1 or more positive blood cultures obtained after 72 h of life) in very

low birth weight (VLBW) newborns were caused by Gram-positive organisms [19, 24–26]. CoNS were responsible for 48% of infections in a study that examined over 6000 NICU infants in the USA [24]. Risk factors identified included low gestational age, low birth weight, an increased duration of ventilator support, central vascular catheter, and prolonged parenteral nutrition [24]. Close to half of the newborns that were <25 weeks of gestation developed late-onset sepsis and had a longer length of hospital stay [24]. Although CoNS infections often rapidly resolve with a few days of intravenous antibiotics, they are responsible for significant healthcare costs and also induce inflammatory responses that can sometimes result in long-term harm to the newborn, including potential cerebral injury [8–13, 27].

SA is the second leading cause of late onset sepsis in neonates [24]. SA leads to more prolonged bacteremia, dissemination to additional anatomic sites (e.g., osteomyelitis), and substantially more sepsis-related deaths than CoNS infections [28, 29]. SA-associated neonatal sepsis is associated with increased antibiotic resistance [28, 30, 31]. Newborns are often colonized with SA from their mothers via horizontal rather than a vertical transfer [32]. Accordingly, a study examining over 400 mothers admitted for preterm labor and the newborns subsequently admitted to the NICU in the USA found that vertical transmission of methicillin-resistant SA (MRSA) at the time of delivery was unlikely [33]. These findings suggested that there was a horizontal transfer of MRSA from health care workers or from parents while taking care of their infants [33]. Indeed, community-based MRSA strains have been identified in some NICU infections in the USA [28].

## 3. Innate Immune System in Neonates

Given the "in-born" nature of the innate immune response, it has been surprising that the innate immune response actually develops with age [34]. As has been recently reviewed, the innate immune response in neonates is distinct from that of older individuals [35, 36]. Multiple cells mediate innate immune responses, including skin and mucosal epithelia, neutrophils, monocytes/macrophages, and dendritic cells [35, 36]. The innate immune system also influences the adaptive immune response, and therefore understanding neonatal innate immunity may also inform development of age-specific vaccines.

*3.1. Soluble Factors That Modulate Neonatal Immune Responses.* Newborn plasma contains multiple factors that modulate the immune response [37]. Neonatal cord blood plasma has significantly more adenosine, an endogenous purine metabolite that inhibits Toll-like receptor (TLR)-mediated Th1 responses, than adult plasma [38]. The neonatal adenosine system inhibits TLR2-induced tumor necrosis factor (TNF) production but not interleukin (IL)-6 [38]. Serum of human newborns in the first week of life demonstrates a higher basal IL-6/TNF ratio than that of adults [39]. Moreover, when compared to monocytes of adults, neonatal cord blood monocytes produce a high ratio of IL-6 to TNF in response to TLR stimulation [39]. IL-6 can impair neutrophil production, migration, and function during sepsis [39–42] possibly contributing to the susceptibility of newborns to bacterial infection.

*3.2. Antimicrobial Proteins and Peptides.* A key mechanism by which the innate immune systems kill microbes and neutralize microbial toxins is via expression and mobilization of antimicrobial proteins and peptides (APPs) [43–45]. APPs are typically cationic molecules that have membrane-active effects on bacteria. Some APPs have additional function such as lactoferrin, which binds iron, a key nutrient for many bacteria [46], and lysozyme, which has enzymatic activity by muramidase that damages bacterial cell walls [47]. Defensins are small cationic antimicrobial peptides produced by leukocytes and epithelial cells in humans [48, 49]. Of note, preterm human neonates demonstrate deficient expression of plasma APPs that may contribute to the ability of bacteria to proliferate rapidly in preterm bloodstream. Moreover, newborn neutrophils demonstrate impairment in production of nucleic acid-based neutrophil extracellular traps (NETs) that serve as scaffolds for APPs and are important for host defense [50]. Overall, reduced plasma levels of complement and APPs as well as impaired deployment of APPs on NETs may, in part, explain why neonates are more susceptible to infection [51, 52].

*3.3. Quantitative Differences in Phagocytes.* Premature neonates admitted to the NICU have a relatively high frequency of neutropenia that can reach up to 8% [53]. In full-term newborns, impaired function of phagocytes has been described at birth [54]. Newborn neutrophils demonstrate impaired chemotaxis, phagocytosis, and impaired respiratory burst [54–57] and an impaired ability to form extracellular traps important for capture and killing extracellular bacteria [50].

*3.4. Qualitative Differences in Leukocytes.* The neonatal immune response is skewed towards Th2 and anti-inflammatory cytokine production. This may be important for protection of the fetus in utero and to avoid excessive inflammation during colonization with normal flora during the first days of life. Preterm newborns demonstrate mostly an anti-inflammatory response characterized by high IL-10 production while production of other cytokines is relatively low [58].

Inhibitory immune receptors antagonize cell-activating signals. Several of these inhibitory immune receptors function through immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Newborn immune cells express a distinct pattern of inhibitory receptors compared to adult immune cells. Cord blood and 1-month-old newborn neutrophils express higher levels of the inhibitory receptors, leukocyte-associated immunoglobulin- (Ig-) like receptor-1 (LAIR-1), and siglec-9 than adults [59]. However, cord blood monocytes exhibited decreased expression of the immune receptor expressed on myeloid cells (IREM)-1, and 1-month-old newborn monocytes expressed lower levels of LAIR-1 compared to adults [59]. These observations suggest that neonatal neutrophils and monocytes are at a different basal set point from adult leukocytes.

## 4. Toll-Like Receptors (TLRs)

TLRs are pattern-recognition receptors (PRRs) of the innate immune system essential for early recognition of pathogen and also guide the adaptive immune response. There have been 10 TLRs identified in humans that signal through adaptor molecules such as myeloid differentiation factor-88 (MyD88) to activate transcription of immune mediators such as cytokines that direct the response to infection [60, 61]. While basal expression of TLRs is similar on full-term human newborn and adult monocytes [62–64], it can change with gestational age. Extremely low birth weight newborns (ELBW), <28 weeks of gestation, demonstrated lower expression of innate immune receptors TLR2, TLR4, CD14, and MD-2 on neutrophils [65]. Monocyte TLR4 mRNA and protein expression increase with gestational age [66]. In contrast, TLR2 expression is constitutively expressed on monocytes across gestational age and is therefore at similar levels in monocytes of preterms, full-term neonates, and adult monocytes [64]. Interestingly, Gram-positive bacteremia apparently induces increases in neonatal peripheral blood monocyte and granulocyte TLR2 expression in infected human newborns [67, 68].

Protein expression of MyD88, a cytosolic adaptor molecule essential TLR signaling, was decreased in newborn cord blood neutrophils [69] and monocytes [70] compared to those of adults. MyD88 mRNA levels increase in preterm infants cord blood mononuclear cells along gestational age. Preterm infants demonstrate lower MyD88 mRNA levels, but term infants are comparable to adults [64]; see Figure 1. Thus, there may be an inherent defect in newborns ability to make cytokine in response to infection due to a deficiency in this important signaling molecule.

A longitudinal study that examined TLR responses of individuals from birth to 2 years of age suggests that there is not a linear progression from an "immature" to "mature" innate immune response from newborns to adults [71]. The percentage of blood monocytes was higher in adults and newborns than 1- and 2-year-olds [71]. 2-year-olds demonstrated greater $PAM_3CSK_4$-(TLR2/1 agonist-) induced levels of intracellular cytokines than adults [71]. There was a higher percentage of 1- and 2-year-old classical (c)DCs making cytokine than adult cDCs [71]. Cytokines secreted from monocytes increased from birth to 2 years old for TNF and IL-1$\beta$; however, IL-6, IL-23, and IL-10 secretion decreased [71]. Preterm infants cord blood mononuclear cells have a significant defect in IL-12/IL-23p40 production in comparison to term infants after stimulation with TLR2/1 agonist $PAM_3CSK_4$, TLR2/6 agonist Fibroblast-stimulating lipopeptide (FSL), and TLR4 agonist LPS [58].

Micro- (mi-)RNAs involved in inhibiting the TLR4 signaling pathway are increased in newborn monocytes compared to adults and may contribute to decreased cytokine production [72]. Further investigation into the role miRNAs play into TLR2-signaling is warranted to gain further understanding of the potential role of miRNA in the neonatal innate immune response. Further research into other pattern-recognition receptors such as the NOD-like receptors and regulation of those receptors is warranted in the newborn to further understand neonatal staphylococcal-induced sepsis.

## 5. Staphylococcal Infections and Neonatal Host Immune Responses

TLR2 mediates innate immune responses to SE and is essential for clearance of SE in mice [73]. TLR2 also mediates the innate immune response to SA infection [74, 75]. Pretreatment of microglial cells with a TLR2 agonist decreased the inflammatory response to *S. aureus* but enhanced the microglial phagocytosis of this bacterium. Thus, TLR-modulation may be a useful treatment strategy to minimize inflammation in the eye [76].

When interpreting the literature of *in vitro* responses to staphylococci it is important to note that the immune response varies accordingly to whether the bacteria are heat-killed, ethanol-killed, or live [77]. Live SE induced significantly higher levels of cytokines compared to killed SE, including robust activation of the inflammasome for IL-1$\beta$ production, induction of type I interferon production, nuclear factor (NF)$\kappa$B, and signal transducers and activators of transcription (STAT)1 activation. In contrast, killed SE activated NF$\kappa$B but did not activate the other innate immune pathways [77].

In a novel model of intrajugular infection in mice less than 24 hours of life, newborn mice demonstrate impaired weight gain when injected intravenously with SE compared to saline-injected controls [78]. Similar to the pattern noted in the peripheral blood mononuclear cells of preterm human newborns during Gram-positive bacteremia [67, 68], TLR2 and MyD88 mRNA levels in the liver were significantly increased by injection of SE that induced inocula-dependent serum IL-6 and TNF concentrations [78].

SE-induced cytokine production from human neonatal mononuclear cells (MCs) *in vitro* is dependent on gestational age [79–82]. Monocytes of preterm newborns demonstrate reduced IL-1$\beta$, IL-6, IL-8, and TNF production in response to SE despite adult-level TLR2 expression [83]. Impaired TNF production may contribute to impaired neutrophil responses to *Staphylococcus* spp. as TNF activates neutrophils. SE-induced phosphorylation of cell-signaling molecules (e.g., phospho-p65, phospho-p38 and phospho-JNK) was similar between newborns and adults [83]. In contrast, treatment of preterm neonatal monocytes demonstrated decreased SA lipoteichoic-acid- (LTA-) induced/TLR-mediated phosphorylation of p38 and ERK in whole blood [64]. LTA-induced production of IL-1$\beta$, IL-6, and IL-8 increased with gestational age [64].

*Staphylococci* spp. evade clearance by the immune system in part by generating adenosine, an endogenous purine metabolite that acts via cognate seven-transmembrane receptors to induce immunomodulatory intracellular cyclic adenosine monophosphate (cAMP; Figure 1), and therefore modulate the immune response [84]. Among the effects of adenosine is to boost production of IL-6, which can inhibit neutrophil migration [41, 42, 85–88] while inhibiting production of TNF important to neutrophil activation [38, 89–91]. Neonatal

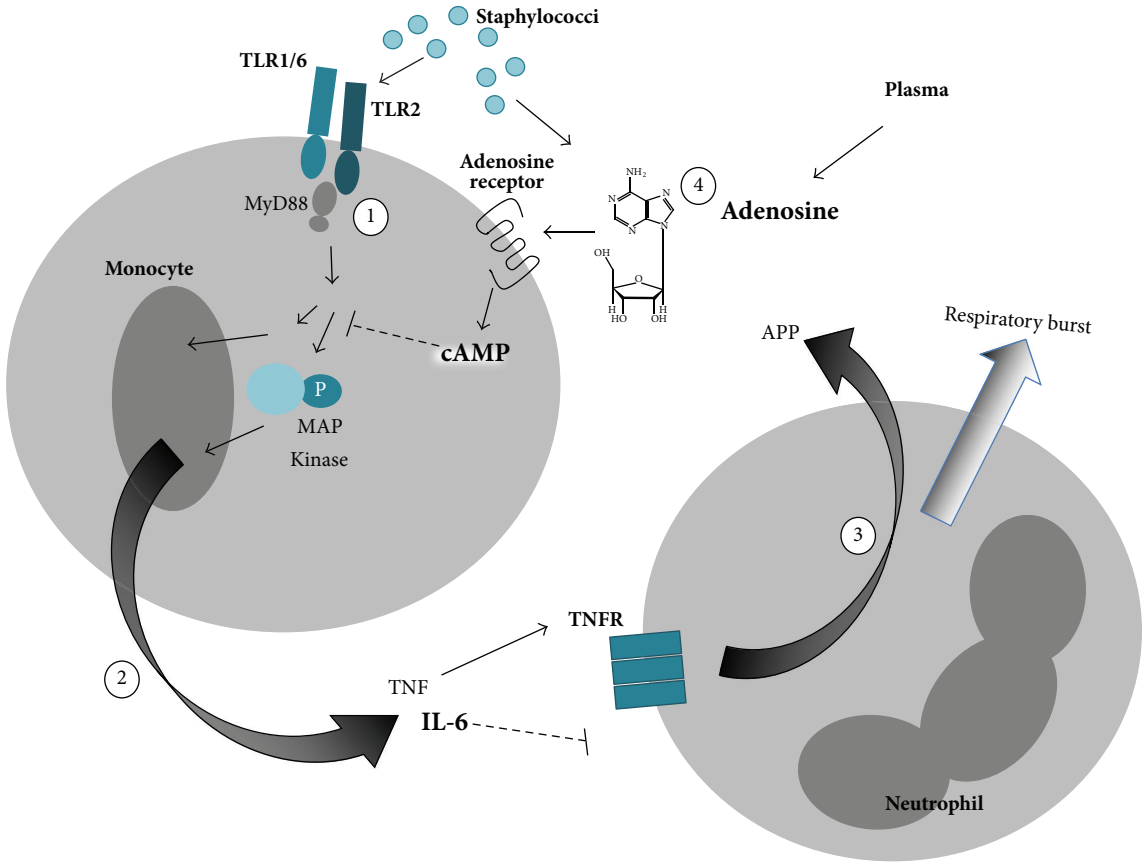


Figure 1: Sensor and effector function of neonatal innate immune system to *Staphylococci* spp. (1) Monocytes detect Staphylococci through TLR2 and (2) result in TLR-mediated production of proinflammatory cytokines such as TNF, and (3) TNF activates neutrophils to produce oxygen radicals and release APPs. (4) Both endogenous plasma and *Staphylococcus*-derived adenosine inhibit pro-inflammatory innate immune responses. Newborn monocytes are deficient in MyD88, activation of MAP kinases, and in TLR-mediated TNF but do produce robust amounts of IL-6, a proresolution cytokine that inhibits neutrophil migration. Overall, this pattern of response impairs neutrophil activation and migration and secretion of APPs. Deficiencies in neonatal responses to staphylococci are depicted by smaller font size, whereas agents that are elevated in newborns are indicated with a larger and bolded font.

mononuclear cells are particularly sensitive to the effects of adenosine [38]. Accordingly, this adenosine generating effect of *Staphylococci* spp. may be particularly effective at disarming neonatal innate defense.

*Opsonophagocytic Mechanisms*. Human newborn and adult monocytes demonstrate similar phagocytic capacity and intracellular killing of SE [83]. However, preterm neonatal neutrophils demonstrate impaired SE-induced neutrophil oxidative burst compared to term newborns [57]. The plasma of premature neonates, especially extremely low birth weight (ELBW) newborns, had a lower opsonophagocytic capacity than term neonates and adults for SA [65].

The impact of these differences on the innate immune responses depending on age to SE and SA is that lower gestational age has a significant impact on the susceptibility of the individual to infection (Table 1). Since neonates have impaired sepsis-induced cytokine production, replenishing cytokines or APPs in neonates may be particularly helpful in the treatment of the preterm newborn. Knowing the deficiencies in the innate immune response may provide specific avenues for developing new treatments.

## 6. Potential Therapeutics

Although SE infections are often cleared from the newborn bloodstream within a few days of intravenous antibiotics (e.g., vancomycin), these infections can recur and are associated with substantial morbidity and healthcare costs [92–94]. Moreover, vancomycin resistance may be emerging [95]. Accordingly, additional preventative and therapeutic strategies are needed.

Injection of the *S. simulans*-derived metalloendopeptidase lysostaphin that cleaves crosslinking pentaglycine bridges in staphylococcal cell walls to MRSA-infected 2-day-old mice reduced bacterial load, improved neonatal weight gain, and enhanced survival similarly to vancomycin [96].

Another approach to addressing staphylococcal infection is to boost host defense by enhancing the quality of phagocytic responses in early life. In a study examining leukocytes from extremely premature infants (24–32 weeks of gestation), treating their leukocytes *ex vivo* with interferon (IFN)-$\gamma$ reversed their innate immune deficiency [65]. Plasma from whole blood of ELBW newborns treated with IFN-$\gamma$ significantly increased the phagocytosis of SA and

Table 1: Differences in the innate immune response between preterm newborns, full-term newborns, infants, and adults in response to SE and SA.

| | Preterm newborns | Full-term newborns | Adults |
|---|---|---|---|
| Monocyte TLR2 expression | + | + | + |
| Monocyte MyD88 expression | ? | + | ++ |
| Phosphorylation of signaling molecules in response to G+ | + | ++ | ++ |
| Th1 cytokine expression | + | ++ | +++ |
| Neutrophil oxidative burst | + | ++ | ++ |
| Plasma opsonophagocytic capacity | + | ++ | ++ |
| Plasma antimicrobial proteins and peptides | + | ++ | +++ |

SE by HL-60 cells [65]. This suggests that further studies are warranted to explore any potential therapeutic benefits for newborns. Administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) to human newborns increased neutrophil production but had no impact on sepsis [97]. Treating septic very low birth weight infants with granulocyte (G)-CSF increased neutrophil phagocytic activity and oxidative burst but had no reported impact on sepsis due to the low number of sepsis patients in the study [98].

Since newborns have an increased susceptibility to sepsis, treating newborns with antibodies specific for SE and/or SA was examined. However, giving immunoglobulin intravenously from donors that had high titers of antibodies to SE and/or SA failed to significantly impact sepsis in preterm newborns [99–101]. However, the authors report a trend towards a decreased incidence ratio for SA infection in patients treated with antistaphylococcal antibodies suggesting that a higher-powered study would be required to examine efficacy more accurately [101].

## 7. Future Directions/Prospects

Many studies have documented late-onset sepsis in neonates due to staphylococcal infection. Current knowledge of the distinct immune system of preterm newborns provides at least three approaches to prevent and/or treat *Staphylococcus* spp. infections.

(1) *PRR Activation to Enhance Innate Antibacterial Defense.* Activation of PRRs can change the set point of the innate immune system resulting in enhanced host defense in response to subsequent challenge with a range of pathogens. This phenomenon is a form of innate memory, that is, demonstrable in many life forms, including plants and insects and has been called "trained immunity" [102]. For example, intraperitoneal administration of a TLR agonist 24 hours prior to a polymicrobial peritonitis challenge markedly enhances neonatal defense and survival after subsequent polymicrobial sepsis by boosting bacteria-induced cytokine production and phagocytic function [103].

(2) *Use of TLR Antagonists as Adjunctive Anti-infective Therapy*. In contrast to preexposure to TLR agonists to boost innate defense prior to an infection, a different strategy may be beneficial during an established infection. Antibiotic-killed bacteria are no longer viable but do continue to activate PRRs thereby inducing inflammation that can be harmful to multiple organ systems, including the brain [27]. Accordingly, adjunctive treatment with a TLR antagonist together with conventional antibiotics may help resolve infection-associated inflammation and reduce consequent morbidity of infection as has been demonstrated *in vivo* in other models and clinical settings [104, 105].

(3) *Use of APPs as Novel Anti-infective Agents.* APPs with activity against Gram-positive bacteria include defensins, cathelicidins, lactoferrin and secretory phospholipase A2 [106, 107]. Biopharmaceutical development of APPs as novel anti-infective agents is proceeding, and replenishing deficient levels in preterm newborns either by direct infusion of APPs or by administration of agents that induce their expression may represent a promising approach to reduce infection.

Overall, further research on unique aspects of the neonatal host/staphylococcal pathogen interaction is warranted to assess the safety and efficacy of the aforementioned approaches and to identify new ones.

## 8. Discussion

This review has summarized recent studies of the innate immune response in preterm and full-term neonates compared to adults in response to SE or SA infection. We highlight important progress in defining the distinct innate immune response of newborns to *Staphylococci* spp. As there are currently limited strategies to address disease caused by these pathogens, it is hoped that recent progress in defining relevant host defense and pathogenic factors [108, 109] will inform new approaches to prevent and treat late onset sepsis due to *Staphylococci* spp.

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*Research Article*

# An Attenuated Cytomegalovirus Vaccine with a Deletion of a Viral Chemokine Gene Is Protective against Congenital CMV Transmission in a Guinea Pig Model

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Development of a vaccine against congenital cytomegalovirus (CMV) infection is a public health priority, but CMVs encode immune evasion genes that complicate live virus vaccine design. To resolve this problem, this study employed guanosyl phosphoribosyl transferase (*gpt*) mutagenesis to generate a recombinant guinea pig CMV (GPCMV) with a knockout of a viral chemokine gene, GPCMV MIP (*gp1*). MIP deletion virus replicated with wild-type kinetics in cell culture but was attenuated in nonpregnant guinea pigs, demonstrating reduced viremia and reduced inflammation and histopathology (compared to a control virus with an intact GPCMV MIP gene) following footpad inoculation. In spite of attenuation, the vaccine was immunogenic, eliciting antibody responses comparable to those observed in natural infection. To assess its protective potential as a vaccine, either recombinant virus or placebo was used to immunize seronegative female guinea pigs. Dams were challenged in the early 3rd trimester with salivary gland-adapted GPCMV. Immunization protected against DNAemia (1/15 in vaccine group versus 12/13 in the control group, $P < 0.01$). Mean birth weights were significantly higher in pups born to vaccinated dams compared to controls (98.7 g versus 71.2 g, $P < 0.01$). Vaccination reduced pup mortality, from 35/50 (70%) in controls to 8/52 (15%) in the immunization group. Congenital GPCMV infection was also reduced, from 35/50 (70%) in controls to 9/52 (17%) in the vaccine group ($P < 0.0001$). We conclude that deletion of an immune modulation gene can attenuate the pathogenicity of GPCMV while resulting in a viral vaccine that retains immunogenicity and demonstrates efficacy against congenital infection and disease.

## 1. Introduction

Human cytomegalovirus (HCMV) is the most common cause of viral congenital infection in the developed world and is estimated to complicate approximately 0.5–2% of pregnancies in the United States and Europe. Congenital infections can cause severe sequelae among neonates including sensorineural hearing loss, cognitive impairments, and mental retardation [1–3]. In the setting of maternal primary infection or reinfection during pregnancy, HCMV can translocate the placental barrier and can cause infection of the developing fetus, with attendant morbidity and occasional mortality [4]. Unfortunately, animal models are of limited usefulness in the study of antiviral and vaccine strategies against HCMV, due to the extreme species specificity of CMVs. It is therefore necessary to study species-specific CMVs in animal models that mimic HCMV congenital infection, in order to evaluate therapeutic and preventive strategies that may ultimately be clinically useful. Among the CMVs of small mammals, the GPCMV has the unique advantage of crossing the placenta,

causing infection *in utero* [5, 6]. This feature of the biology of GPCMV makes it ideal for vaccine studies, since congenitally infected pups, like newborn infants, have virus-related morbidity and mortality.

All CMVs encode genes that confer immunomodulatory functions that may impact the efficiency of infection, dissemination, reactivation and persistence in the host (reviewed in [7]). Such immune evasion genes include homologs of chemokines (CKs), G protein-coupled receptors (GPCRs), and modulators of antigen processing and presentation [8–12]. In addition to contributing to modification of host immune responses, some of these viral proteins may play a role in promoting dissemination during acute infection. GPCMV, similar to other CMVs, encodes a number of potential immunomodulatory gene products [13, 14]. The *gp1* ORF encodes one such gene product that has been characterized in previous studies [13, 14]. The protein, GPCMV-MIP, is a member of the CC family of CKs and is most closely related to the macrophage inflammatory protein (MIP) 1$\alpha$ family. Previous studies with a recombinant form of this protein demonstrated that specific signaling could be mediated via the hCCR1 receptor and that this interaction could be blocked with human MIP 1$\alpha$ in competition experiments [14]. Moreover, migration assays revealed that GPCMV-MIP was able to induce chemotaxis in transfected hCCR1-L1.2 cells. Subsequent studies in a model of virus-induced labyrinthitis, comparing a recombinant GPCMV deleted of the GPCMV MIP gene with wild-type virus, indicated a potential role for this CK in pathogenesis, insofar as the "knock-out" virus demonstrated reduction both in the magnitude of hearing loss and in cochlear inflammation, following direct inoculation of the guinea pig cochlea via the round window [15, 16]. Thus, deletion of this gene appears to substantively attenuate the pathogenicity of the resulting recombinant virus *in vivo*.

Both purified protein subunit vaccines and live, attenuated vaccines have been proposed as strategies to prevent congenital CMV infection [17]. Significant concerns have been raised, however, regarding the deployment of live, attenuated HCMV vaccines in clinical practice. These include the concern that live virus vaccines might establish latency, theoretically putting the vaccine recipient at risk to develop as yet unproven long-term adverse consequences related to HCMV infection, including autoimmune disease, malignancy, and atherosclerosis [18]. In addition, any live virus vaccine must in principle be sufficiently attenuated such that it would not pose any untoward risks when administered to vaccinees, including the setting of inadvertent administration to a pregnant woman. A potential solution to the challenge of developing a safe live-virus vaccine for HCMV is to use recombinant technologies to engineer attenuated vaccines that, by virtue of targeted removal of genes that contribute to immune modulation and/or pathogenesis *in vivo*, could in principle retain immunogenicity while avoiding potential risks. This strategy has been successfully employed in the murine CMV (MCMV) model, where deletions of large segments of the viral genome encoding immune modulation genes result in a virus with essentially complete attenuation and an inability to establish latency, which nonetheless retains immunogenicity and provides protective efficacy as a vaccine [19–21]. However, a limitation of the MCMV model is that it does not allow testing of vaccines for prevention against congenital transmission, because of the inability of MCMV to cross the mouse placenta [22]. Therefore, we sought to test whether a live, attenuated GPCMV vaccine when administrated before conception could engender an immune response sufficient to protect against congenital viral transmission in the guinea pig model. Specifically, these studies were undertaken to test the hypothesis that a GPCMV (*gp1*) MIP deletion virus would, though attenuated, retain the ability to protect against congenital GPCMV infection and disease when used as a preconception vaccine.

## 2. Materials and Methods

*2.1. Cells and Viruses.* GPCMV (strain 22122, ATCC VR682), v545 [15, 16], and vAM403, an enhanced green-fluorescent-protein-(eGFP-) tagged GPCMV [23], were propagated on guinea pig fibroblast lung cells (GPL; ATCC CCL 158) in F-12 medium supplemented with 10% fetal calf serum (FCS; Gibco-BRl), 10,000 IU of penicillin/liter, 10 mg of streptomycin/liter (Gibco-BRL), and 7.5% $NaHCO_3$ (Gibco-BRL). For generation of the GPCMV MIP "knock-out" virus, guanosyl phosphoribosyl transferase (*gpt*) mutagenesis was employed, as previously described [23]. A 2.3 kb *EcoR* I fragment containing the GPCMV-MIP was subcloned into pBluescript (+) from a plasmid containing the *Hin*d III "D" fragment, yielding plasmid pKTS 107. A 320 bp *Stu* I collapse, deleting the GPCMV MIP gene, yielded pKTS 534. Following deletion of the vector *Xba* I site by digestion with *Xba* I, Klenow polymerase treatment, and religation (yielding pKTS 536), an *Xba* I linker was inserted into the unique *Stu* I site, yielding pKTS 540. Next, a 2.3 kb *Spe* I fragment from pQ106 [23] containing the *gpt* and enhanced green fluorescent protein (eGFP) cassette was inserted into the *Xba* I site of pKTS 540, yielding pKTS 545 (Figure 1(a)).

Generation of the MIP deletion virus was performed as described elsewhere [15, 16]. For generation of this recombinant virus, plasmid pKTS 545 was cotransfected with viral DNA into GPL cells, and selection was carried out using mycophenolic acid and xanthine as previously described [23]. Following limiting dilution, eGFP-positive wells were identified and 6 rounds of plaque purification were performed under selection, to ensure clonality of the recombinant viral stock. A clonal eGFP-positive recombinant virus was purified and designated as v545; this virus was used for subsequent pathogenesis and vaccine studies.

*2.2. Characterization of Recombinant Virus.* For characterization of recombinant virus, Southern blot analyses were performed. Viral DNA purified from v545 and wild-type (ATCC) GPCMV was subjected to restriction enzyme digestion with *Hin*d III and *Eco*R I, followed by agarose gel electrophoresis. Following transfer to Nytran membranes, DNA probes corresponding to the GPCMV-MIP gene [13] or to the *gpt*/eGFP cassette were labeled with [$^{32}$P] dCTP, using a High Prime kit (Boehringer Mannheim) according to

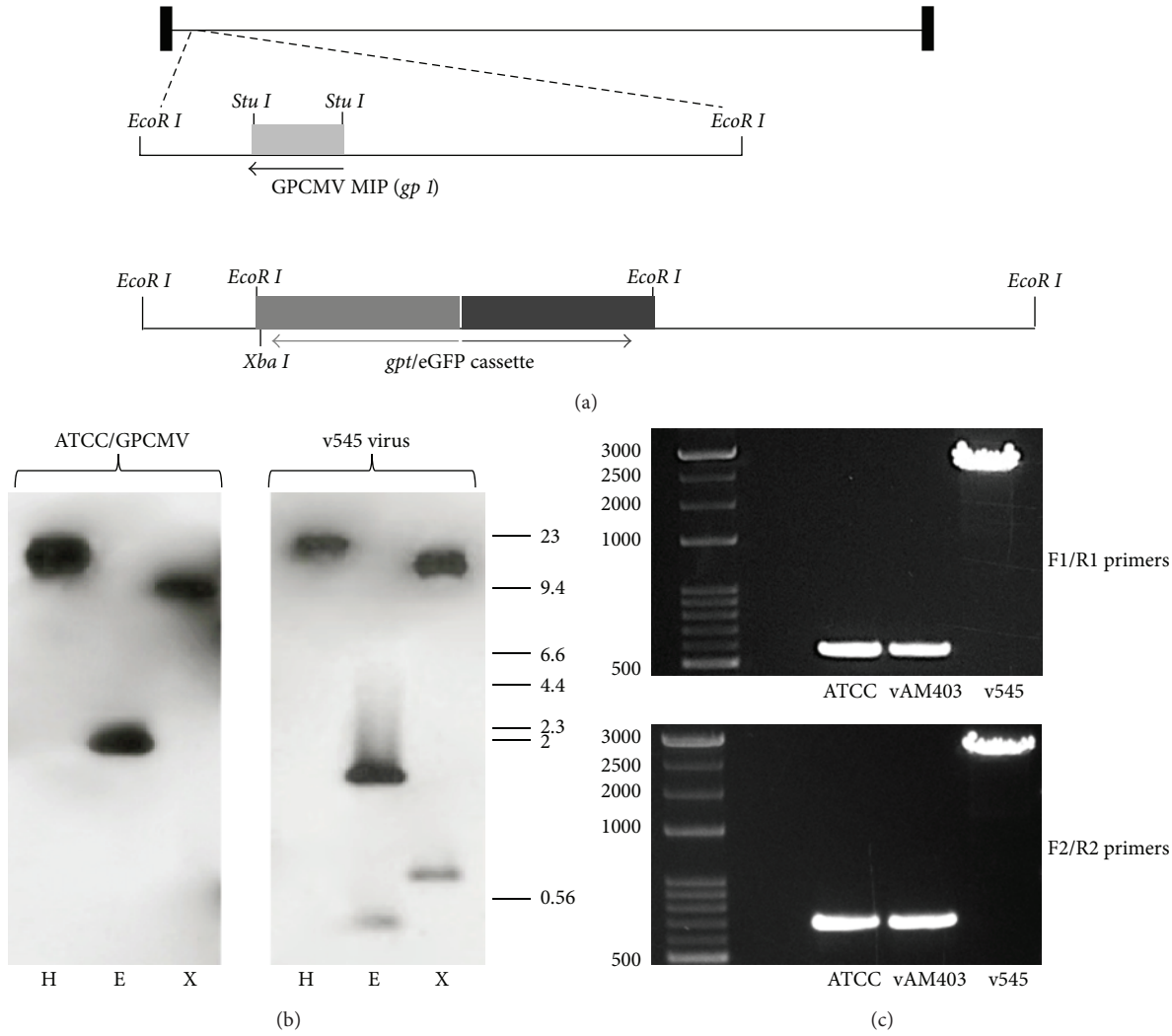



Figure 1: Schematic representation of the GPCMV MIP gene and generation of recombinant virus. (a) Map of GPCMV genome. GPCMV MIP (*gp1* gene) maps to 2.3 kb *EcoR* I fragment near left genome terminus. Knockout of *gp1* was achieved by introduction of *gpt*/eGFP cassette into a *Stu* I collapsed version of plasmid DNA. Following co-transfection of plasmid and viral DNA and *gpt* selection as described in text, a clonal recombinant virus was obtained by limiting dilution. (b) Southern blot analysis of wild-type (ATCC 22122; left panel) and v545 (recombinant) DNA. Probing with pKTS107 probe revealed presence of restriction polymorphisms demonstrating predicted configuration of recombinant virus. H, *Hin*d III digest; E, *EcoR* I digest; X, *Xba* I digest. Molecular weight markers, lamba/*Hin*d III ladder. (c) To further characterize the genome structure of the v545 mutant, viral DNA was analyzed by PCR. The PCR was done using primer pairs cassette F1/R1 (upper panel) and cassette F2/R2 (lower panel) which, respectively, amplify 552 bp and 671 bp products in wild-type GPCMV and vAM403. Insertion in v545 coupled with deletion of MIP gene results in overall shifts in bands to ~2.8-2.9 kb, as predicted. Subsequent sequence analysis of gel-purified products confirmed predicted insertion and genome structure of v545 vaccine virus.

manufacturer's specifications (Figure 1(b)). To further characterize the genome structure of the v545 mutant, viral DNA was analyzed by PCR. The PCR was done using primer pair cassette F1/R1 and cassette F2/R2 which, respectively, amplified 552 bp and 671 bp products in wild-type GPCMV. Primer sequences were cassette F1, 5′-GACCCTCTAACATATCGGAG-3′; cassette R1 5′-AAGAACATGGCTGTCCGCTA-3′; cassette F2 5′-TTCTCTCACGTTGAGCGCAT-3′ cassette R2 5′-CCTATCGATACGTGGATACG-3′. The PCR reaction was performed in a total volume of 50 $\mu$l using GoTaq long PCR Master Mix (Promega laboratories) and 1.0 $\mu$M primers. The conditions for the PCR were initial denaturation at 95°C for 2 min, followed by 95°C for 30 s, 56°C for 30 s, 72°C for 3 min for a total of 35 cycles, and elongation at 72°C for 10 min. The PCR product (9 $\mu$L) was subjected to electrophoresis in a 0.7% agarose gel (Figure 1(c)).

Rescue virus was generated as previously described [24]. Briefly, 1 $\mu$g of v545 DNA was cotransfected with 20 $\mu$g of rescue plasmid (pKTS 107) onto GPL cells. Rescued, eGFP-negative viral plaques were picked and subject to further rounds of plaque purification by limiting dilution. Rescue of GPCMV-MIP was confirmed by PCR and by restriction profile analysis (data not shown).

One-step growth curve analyses were conducted to compare wild-type (GPCMV ATCC, 22122), recombinant, and rescued viruses. Clonal stocks were used to infect confluent

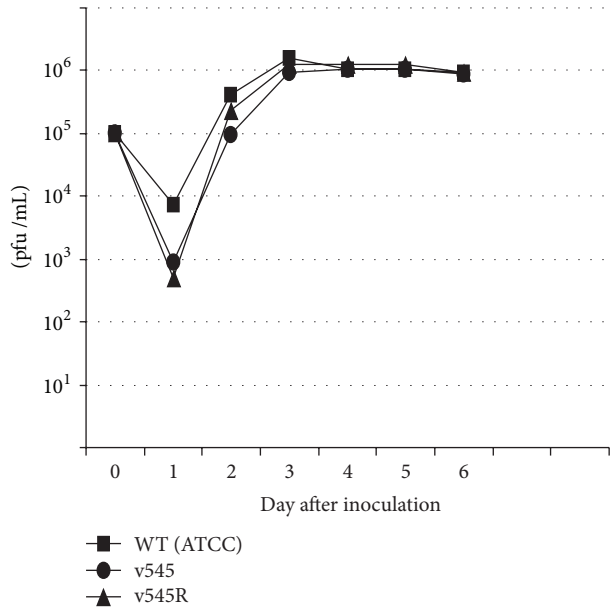



Figure 2: One-step growth curve analysis of recombinant virus v545. Wild-type (ATCC 22122), recombinant (v545) and rescuant (v545R) viruses were used to infect confluent cell culture monolayers at an m.o.i. of 0.5 pfu/cell. After absorption for 1 h at 37°C, the cells were washed with media and a zero time point was harvested. The remainder of the sample was incubated at 37°C with additional time points taken at indicated times. The viral titer of each time point was determined by plaque titration assay on GPL cells.

monolayers at an m.o.i. of 0.5 pfu/cell. After adsorption for 1 h at 37°C, the cells were washed with media and a zero time point was harvested. Additional time points were obtained at 24, 48, 72, 96, 120, and 144 h after inoculation, and the viral titer of cell culture supernatant obtained at each time point was determined by plaque titration assay on GPL cells (Figure 2).

*2.3. Animal Studies of Recombinant Viruses following Systemic and Footpad Inoculation.* Hartley guinea pigs were purchased from Elm Hill Laboratories (Chelmsford, MA, USA). Strain 2 guinea pigs were maintained in the vivarium of the University of Minnesota Medical School. Animals were housed under conditions approved by the American Association of Accreditation of Laboratory Animal Care, in accordance with and following approval from the Institutional Animal Care and Use Committee at the University of Minnesota.

The v545 recombinant had been previously demonstrated to have an altered pathogenesis in guinea pigs compared to a similar recombinant, vAM403 (which has the eGFP/*gpt* cassette inserted into a noncoding region of the GPCMV genome), following intracochlear inoculation [15, 16]. To further characterize the impact of deletion of the GPCMV MIP on the *in vivo* pathogenesis of infection, viremia (Figure 3) and footpad inoculation studies (Figure 4) were conducted in nonpregnant, weanling animals. For viremia studies, young strain 2 guinea pigs ($n = 6$/group) were inoculated by intraperitoneal route with $1 \times 10^7$ pfu of either vAM403 or v545 virus (Figure 3(a)). The vAM403 virus was used as a control, rather than the v545 rescue virus, since it (like vAM403) expressed eGFP and also had a similar insertion into the viral genome. Animals were then monitored every other day for nine days following infection for weight loss or gain. On day 10, animals were bled for analysis of systemic viral load by PCR (Figure 3(b)). To evaluate inflammation at a local site of primary infection, additional adult animals ($n = 6$) were inoculated in the footpad with $5 \times 10^4$ pfu of either vAM403 virus or v545 virus. For each group, media alone was inoculated into the contralateral paw. Foot thickness was measured using a digital caliper as described elsewhere [25], daily for 6 days (Figure 4(a)), in a coded, blinded fashion. On day 7, animals were sacrificed and tissues harvested, formalin fixed, and decalcified; after embedding in paraffin, 5 $\mu$m sections were cut and stained with hematoxylin and eosin to evaluate for histopathology and evidence of inflammation (Figure 4(b)).

*2.4. Vaccine/Challenge Studies.* Hartley strain guinea pigs were purchased, as previously noted, from Elm Hill laboratories. All animals ($n = 15$/group) were determined to be GPCMV seronegative prior to vaccination by ELISA. Animals were immunized twice, with an interval of 3 weeks between doses, with $5 \times 10^4$ pfu of v545 vaccine, by subcutaneous route in a total volume of 1 mL. Control animals received an identical volume of phosphate-buffered saline. Bleeds were performed once a week for 6 weeks on all guinea pigs following vaccination for both quantitative PCR analysis of viral load and for serum analysis. After 6 weeks, bleeds were performed every other week until the conclusion of the study.

Three weeks following the second immunization, animals were mated with seronegative male Hartley guinea pigs. Mating persisted until abdominal palpitation confirmed pregnancy. Approximately 4 weeks before delivery, pregnant dams were challenged with a subcutaneous injection of $1 \times 10^6$ pfu/mL of a virulent, salivary gland-derived GPCMV workpool. Bleeds were performed in infected dams on days 5, 10, and 15 after challenge for quantitative PCR analysis of viral load and for plasma analysis. Upon delivery, pups were weighed and live-born pups sacrificed within 96 hours of birth and blood obtained for quantitative PCR analysis of viral load. Lung, liver, spleen, and placenta were also extracted for evaluation of organ pathology and quantitative PCR analysis of viral load.

*2.5. Immunologic and Virologic Studies.* ELISA analysis (Figure 5(a)) was performed as previously described [26], with titer determined by limiting dilution assay (initial dilution, 1 : 80). A positive result was determined by calculating the reciprocal of the highest dilution that produced an absorbance of at least 0.1 and twice the absorbance observed using a negative control antigen. Plates were read at a wavelength of 450 nm using the SpectraMax M2 Spectrophotometer (Molecular Devices) and the ScanMax Pro program. Western blot analysis was performed on serum collected from a subset of guinea pigs from the vaccine and control groups

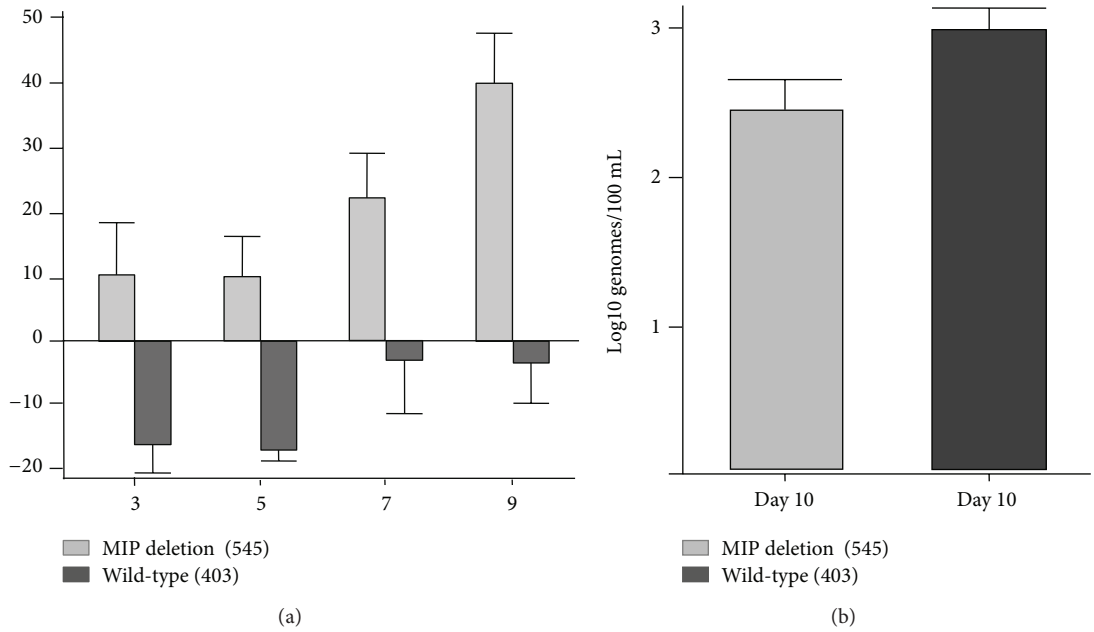
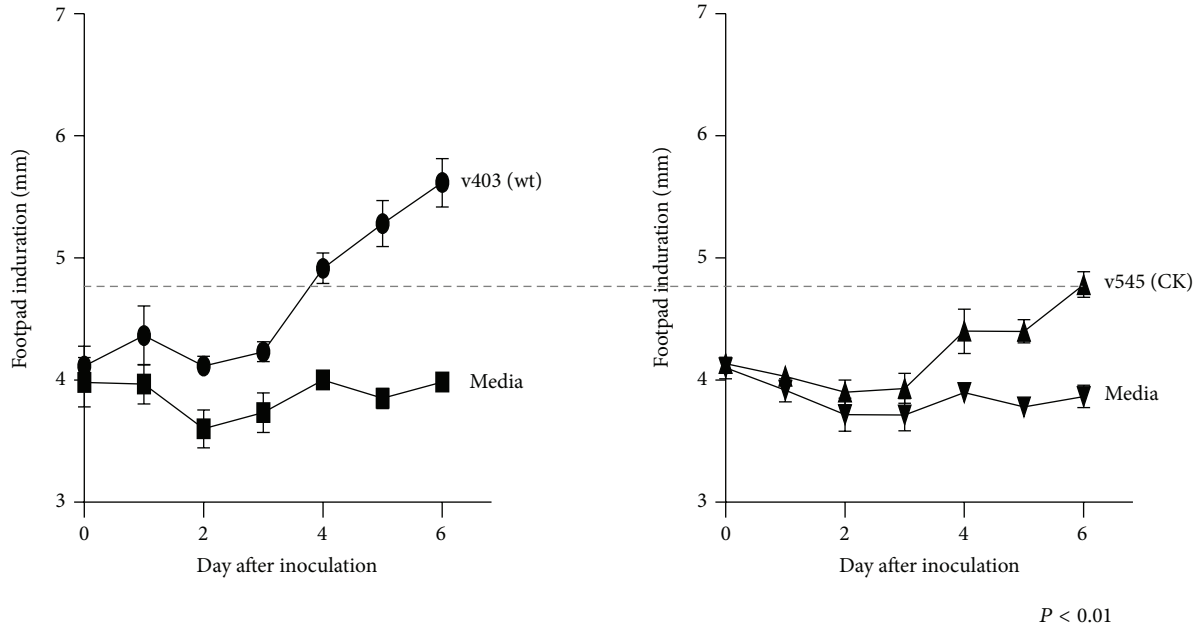



Figure 3: The v545 virus is attenuated in strain 2 guinea pigs. (a) Weight loss and DNAemia profiles were compared following inoculation of young, nonpregnant guinea pigs with v545 and control vAM403 virus. Animals inoculated with v545 gained weight throughout this postinoculation time period (mean weight gain at day 9, 40 grams) compared to animals infected with vAM403 (weight loss of 5 grams; $P < 0.01$). (b) Animals challenged with v545 virus demonstrated reduced DNAemia measured at day 10 (mean viral load, 2.5 $\log_{10}$ genomes/mL versus 3.0 $\log_{10}$ genomes/mL in vAM403 group; $P < 0.05$).

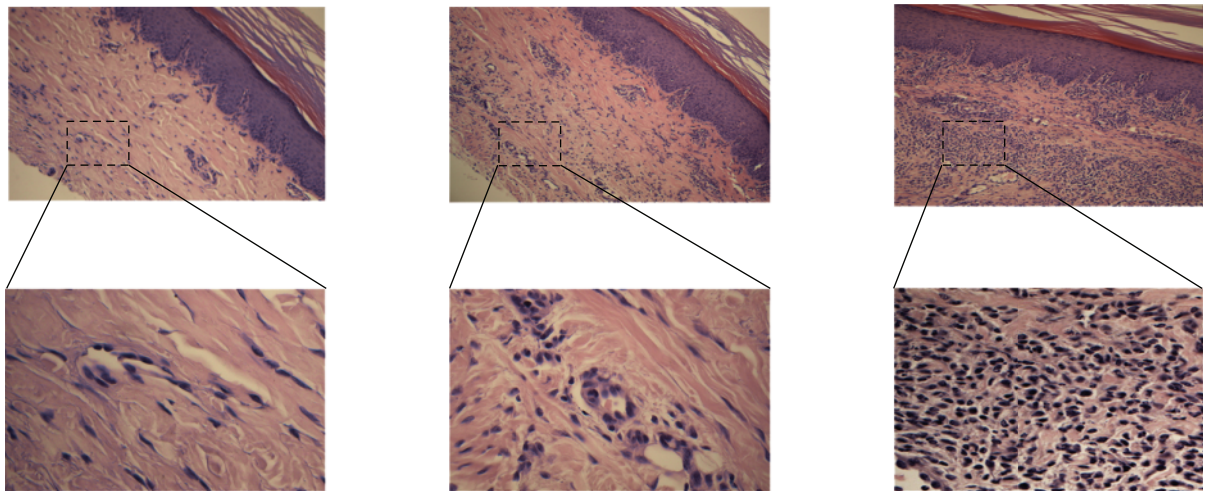
(Figure 5(b)). Serum was collected before vaccination from each experimental group (preimmune) and at 2, 3, 5, 7, 9 and 11 weeks, as outlined schematically in Figure 5. For western blot studies, virus particles were subjected to SDS-PAGE and were then transferred onto nitrocellulose membranes by electroblotting. Membranes were blocked using membrane blocking agent (GE Healthcare) resuspended in Tris-buffered saline plus 0.5% Tween (TBST) and then incubated for 3.5 hours with the aforementioned collected sera (1 : 800). Along with serum samples obtained from the above time points, a gB-specific polyclonal rabbit antiserum (1 : 500) and a high-titer guinea pig polyclonal antiserum (1 : 10,000) were used as controls [27]. After washing in TBST, blots were incubated with either a horseradish peroxidase-conjugated rabbit anti-guinea pig antiserum (1 : 10,000; Santa Cruz Biotechnology) or a horseradish peroxidase-conjugated donkey anti-rabbit antiserum (1 : 10,000; Santa Cruz Biotechnology) for 2 hours at 27°C. Antibody binding was then detected using the ECL Western Blot Detection Kit (GE Healthcare), followed by autoradiography (Hyblot CL; Denville Scientific, Inc.).

For detection of viremia following intraperitoneal inoculation of strain 2 guinea pigs (Figure 3), a quantitative, competitive PCR assay was employed, as described elsewhere [28]. For congenital transmission comparisons and for assessment of viral load in pregnant animals, real-time PCR was performed. DNA was extracted from 200 $\mu$L citrated whole blood using the MagNA Pure LC System (Roche) or 150 $\mu$L of 10% tissue homogenate using the QIAxtractor (QIAGEN). The GPCMV gB-specific primer pair LCF1 (5′-CTTCGTGGTTGAACGGG-3′) and LCR1 (5′-GTAGTCGAAAGGACGTTGC-3′) were utilized for the real-time PCR assay. The PCR reaction was performed in a 20 $\mu$L volume reaction as specified by the LightCycler FastStart DNA Master HybProbe reaction mix (Roche Diagnostics). GPCMV gB-specific hybridization probes were used for detection (LCPG 5′-TGGTGACCTTCGTTACCAATCCGTTTGGA-F; LCPR 5′R640-CTTCGTGGTGTTCCTGTTCTGCGT-P). PCR was performed using the LightCycler 480 real-time PCR System (Roche) under the following conditions: initial denaturation at 95°C for 10 min, followed by 95°C for 10 s, 54°C for 15 s, 72°C for 15 s for a total of 50 cycles, followed by melting curve analysis at 95°C for 1 min and 45°C for 1 min and ending at 85°C, and then a final hold step at 40°C. Data were analyzed with the Light-Cycler Data Analysis Software (version 1.0; Roche) using standard curves generated using serial dilutions of plasmid and viral DNA at known concentrations. The magnitude of DNAemia was expressed as the total number of genome copies per mL of blood or total number of genome copies per $\mu$g of tissue.

*2.6. Statistical Comparisons.* Antibody titers were compared using the paired Student's $t$-test. Parametric variables were compared by ANOVA. Nonparametric variables were compared using the Mann-Whitney $U$ test or the paired Wilcoxon test. The proportion of live-born pups, infected pups, and the birth weights were compared/calculated using generalized linear mixed models (GLMM) to account for within-litter variability [29]. Statistics were analyzed using the Prism 5.0 software package (GraphPad Software, San Diego) and R v2.13.0.




(a)




(b)

Figure 4: The v545 virus is attenuated in a footpad inoculation model. Using calipers, differences between wild-type (vAM403) and CK deletion virus (v545) were noted following footpad inoculation, beginning at day 3 and progressing through day 6. The level of swelling produced by the vAM403 virus was noted to be ~20% above preinoculation levels, compared to the results obtained with v545 ($P < 0.01$). All swelling resulted from the effects of virus in the inoculum because inoculation of an identical volume of tissue culture medium alone failed to elicit any response (controls, Figure 4(a)). Feet were collected from sacrificed guinea pigs at day 6 after inoculation, and midline longitudinal sections were prepared. Examination by light microscopy at both low and high power (×10 and ×60; Figure 4(b)) revealed substantially larger amounts of both cellularity and edema in animals inoculated with vAM403 virus (Figure 4(b), right panel), containing the GPCMV MIP ORF, than in v545 virus-inoculated animals (Figure 4(b), middle panel) or in media-inoculated control (Figure 4(b), left panel).

## 3. Results

*3.1. MIP Gene Deletion Does Not Modify Viral Replication in Cell Culture but Attenuates GPCMV Infection and Dissemination in Guinea Pigs.* The GPCMV MIP gene was deleted from the viral genome using a well-established model of selection for recombinant virus, *gpt* selection. The genome structure of the recombinant virus, v545, was verified by Southern blot study (Figure 1(b)). A GPCMV-MIP-specific probe was found to hybridize with viral DNA purified from GPCMV (ATCC) DNA, but not DNA purified from v545. However, a *gpt*/eGFP-specific probe was found to hybridize with DNA purified from v545, but not ATCC DNA. Moreover, insertion of this cassette into the GPCMV genome

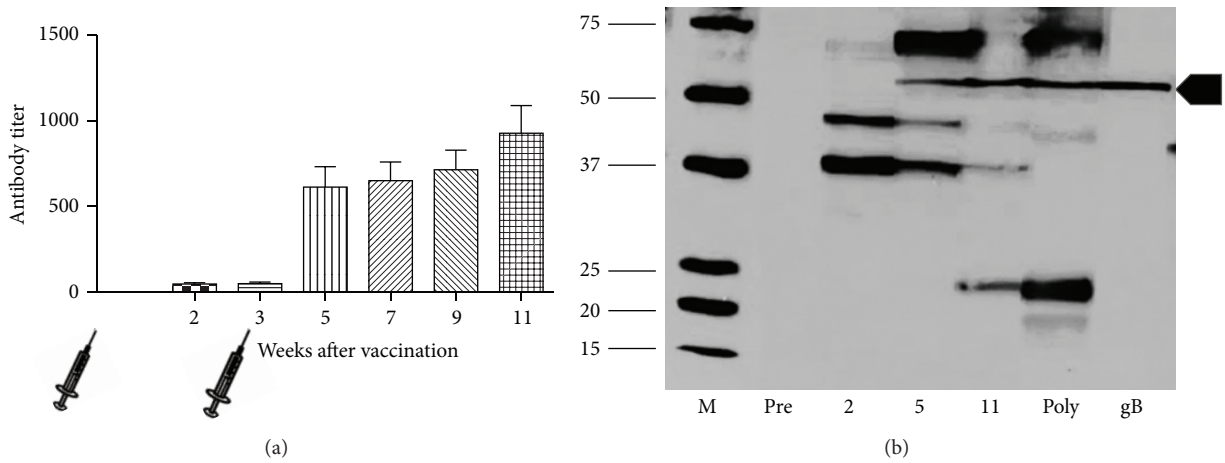



Figure 5: Immunogenicity of v545 attenuated vaccine. (a) ELISA profile following vaccination. Animals were immunized twice, with an interval of 3 weeks between doses, with $5 \times 10^4$ pfu of v545 vaccine, by subcutaneous route of inoculation. Control animals received an identical volume of phosphate-buffered saline. Bleeds were performed at regularly intervals, as described in the text, on all guinea pigs following vaccination for serum ELISA analysis. Selected sera were also used in western blot assay as indicated in panel (b). Data shown represents the mean ± SD reciprocal ELISA titer for the vaccine group at each time point. (b) Representative profile of response from vaccinated dam using viral particles in western assay. Marker lane is indicated (M). Lanes 1–4, preimmune sera and sera from weeks 2, 5, and 11 following vaccine. Lane 5, western profile of high-titer anti-GPCMV antisera. Lane 6, western blot with anti-GPCMV gB polyclonal antibody. Immunized animals demonstrate anti-gB antibodies (arrowhead) in addition to antibody to other GPCMV polypeptides. Initial response targets virion proteins of ~35 and 45 kDa noted at 2 weeks after vaccination, followed by responses to gB and a ~70 kDa protein following second immunization at 5-week time point. Last serological responses to appear target proteins of ~22 kDa, noted at 11-week time point.

generated novel *EcoR* I restriction polymorphisms, which could be demonstrated both with GPCMV-specific probes and *gpt*/eGFP-specific probes. To confirm the Southern blot observations, and also to confirm that potential adjacent ORFs were not modified, PCR was performed (Figure 1(c)), followed by sequence analysis. This confirmed the predicted orientation and insertion into the GPCMV genome. During the course of these studies, it was noted by Inoue and colleagues that two types of strains of GPCMV are present in virus stocks obtained from ATCC: GPCMV/full, and GPCMV/del, containing a 1.6 kb deletion in a locus encoding the GPCMV homologs of the HCMV UL128-131 complex [30]. We noted by PCR that both v545 and vAM403 had a GPCMV/del genome variant, presumably due to prolonged passage of ATCC virus in fibroblast cells (data not shown). We conclude based on these analyses that v545 has a targeted deletion of GPCMV MIP, but otherwise an intact genome structure, compared to the GPCMV/del ATCC variant.

Replication kinetics of v545 in cell culture were compared to that of wild-type (ATCC) GPCMV and another eGFP-tagged recombinant. In these experiments, one-step growth curve analyses were performed with v545; ATCC virus; a v545 rescuant; and a previously described recombinant GPCMV with the *gpt*/eGFP cassette inserted in a noncoding region of the viral genome, vAM403 [23]. These comparisons revealed that deletion of the GPCMV MIP had no impact on the replication of GPCMV in cell culture (Figure 2).

Previously, we reported on the reduced pathogenesis of the v545 mutant virus in a direct inoculation model of cochlear pathogenesis and hearing loss [15, 16]. It was therefore of interest to further examine the impact of deletion of the GPCMV MIP ORF on the pathogenesis of infection. Weight loss and DNAemia were compared following inoculation of young, nonpregnant guinea pigs with v545 (MIP deletion virus; Figure 3). Animals inoculated with v545 gained weight throughout this postinoculation time period (mean weight gain at day 9, 40 grams) compared to animals infected with vAM403 (weight loss of 5 grams; $P < 0.01$). In addition, animals challenged with v545 virus demonstrated a significant reduction in the magnitude of DNAemia measured at day 10 (mean viral load, 2.5 $\log_{10}$ genomes/mL versus 3.0 $\log_{10}$ genomes/mL in vAM403 group; $P < 0.05$).

To further characterize the biology of wild-type and deletion virus in guinea pigs, a footpad inoculation model was employed, as previously described for analyses of other rodent CMVs [25, 31]. Using this model, differences between wild-type (vAM403) and CK deletion virus (v545) were noted beginning at day 3, and progressing through day 6, when the level of swelling produced by the vAM403 virus was noted to be ~20% above preinoculation levels, compared to the results obtained with v545. All swelling resulted from the effects of virus in the inoculum because inoculation of an identical volume of tissue culture medium alone failed to elicit any response (controls, Figure 4(a)). In order to further investigate cellular infiltrates in response to GPCMV MIP, feet were collected from sacrificed guinea pigs at day 6 after inoculation, and midline longitudinal sections were prepared from formalin-fixed, paraffin-embedded blocks. Examination by light microscopy at both low and high

power (×10 and ×60; Figure 4(b)) revealed substantially larger amounts of both cellularity and edema in animals inoculated with vAM403 virus, containing the GPCMV MIP ORF, than in v545 virus-inoculated animals. All areas of inoculated feet (dorsal, internal, and ventral) appeared less inflamed, with differences in foot thickness measured grossly using calipers correlating with the histopathological findings. At low power, the presence of the GPCMV MIP gene correlated with a much more intense local inflammatory response, and differences in cellularity with an increased mononuclear cell infiltrate were readily discernable.

*3.2. Immunogenicity and Safety of v545 Employed as an Attenuated Vaccine Candidate.* Based on its favorable attenuation profile in nonpregnant animals, we studied the MIP deletion virus as a candidate live, attenuated vaccine. A two-dose immunization series ($5 \times 10^4$ pfu) was administered subcutaneously to 15 young, GPCMV-seronegative guinea pigs, at three-week intervals. A similar group of 15 guinea pigs were immunized with phosphate-buffered saline (negative control group). Analysis of ELISA response indicated that only 2/15 animals (13%) demonstrated a response within three weeks of the first vaccination; however, within two weeks of the second vaccination, all animals were GPCMV-seropositive (mean reciprocal ELISA titer, 616; Figure 5(a)). Western blotting was performed in order to further characterize the humoral response following vaccination with v545 virus. These analyses demonstrated (Figure 5(b)) similar patterns of response to those noted using a high-titer polyclonal, anti-GPCMV antisera obtained from an animal infected with wild-type virus.

*3.3. Maternal and Fetal Outcomes in a Pregnancy/Challenge Study Using v545 Vaccine.* The effect of the 545 attenuated virus vaccine on pup mortality due to disseminated GPCMV in Hartley guinea pigs (two doses of vaccine) was analyzed. All 15 dams in the vaccine group completed pregnancy. In the control group, 13 animals became pregnant and had evaluable pregnancy outcomes. In the control group, pup mortality was 35/50 (70%). In contrast, pup mortality in litters born to v545-vaccinated dams was 8/52 (14%; $P < 0.0001$ compared to vaccine group). Mean birth weights of the pups delivered in each group were also compared. Among pups born in the control group, live-born pups ($n = 15$) had a mean weight of 87.1 g (4.25 SE), while dead pups ($n = 35$) had a mean weight of 62.2 g (8.70 SE). In contrast, in the v545 vaccine group, live-born pups ($n = 44$) had an average weight of 97.4 g (2.16 SE), while stillborn pups had a mean weight of 107.6 g (3.97 SE) ($n = 7$; one stillborn pup was not weighed). Overall, mean pup weight was 71.2 g (6.13, SE) in the control group, and 98.7 g (1.86, SE) in the vaccine group ($P < 0.0001$; Table 1). Of 15 litters born to vaccinated dams, 3 (5%) had at least one dead pup, while 12/13 litters born to dams in the control group had at least one dead pup ($P < 0.001$ compared to control group, Fisher's exact test).

The v545 vaccine had a substantial impact on maternal viremia and congenital GPCMV infection. All maternal blood samples taken before SG virus challenge were negative by qPCR. Following SG virus challenge, in control dams, 12/13 demonstrated DNAemia at day 5 postinfection, with a mean viral load of 4.4 ± 0.4 $\log_{10}$ genomes/mL. In contrast, only 1 of 15 dams immunized with v545 vaccine demonstrated DNAemia on day 5 after salivary gland virus challenge, with the sole positive sample having a viral concentration of 3.5 $\log_{10}$ genomes/mL. PCR of DNA purified from pup organs demonstrated congenital GPCMV infection in 24/49 liver homogenates; 19/46 spleen homogenates; and 28/46 lung homogenates. One dead pup in this group was homogenized en bloc (PCR negative). Overall, 35/50 pups (10/15 among live-born pups, and 25/35 among dead pups) in the control group had congenital infection as evidenced by at least one positive tissue PCR (70% overall congenital infection rate) (Table 2). A total of twenty placentas could be retrieved from this group, and all were positive for GPCMV DNA by PCR. In the v545 vaccine group, PCR of DNA purified from pup organs demonstrated congenital GPCMV infection in 6/52

Table 1: Pregnancy outcomes (pup mortality) after challenge with SG-passaged GPCMV in vaccinated and control dams.

| Litter | Dead/total |
|---|---|
| Control | |
| 1 | 3/3 |
| 2 | 3/4 |
| 3 | 4/4 |
| 4 | 2/5 |
| 5 | 0/2 |
| 6 | 4/4 |
| 7 | 3/3 |
| 8 | 1/4 |
| 9 | 2/3 |
| 10 | 3/4 |
| 11 | 5/5 |
| 12 | 3/6 |
| 13 | 2/3 |
| Total | 35/50 (70%) |
| V545 vaccine | |
| 1 | 0/3 |
| 2 | 0/3 |
| 3 | 0/4 |
| 4 | 5/5 |
| 5 | 0/4 |
| 6 | 0/3 |
| 7 | 0/4 |
| 8 | 1/1 |
| 9 | 0/4 |
| 10 | 1/5 |
| 11 | 0/4 |
| 12 | 0/3 |
| 13 | 0/2 |
| 14 | 1/4 |
| 15 | 0/3 |
| Total | 8/52 (15%) |

Table 2: Summary of litters and presence or absence of congenital GPCMV infection in vaccine and control groups.

| Litter | PCR positives |
|---|---|
| Control | |
| 1 | 3/3 |
| 2 | 4/4 |
| 3 | 4/4 |
| 4 | 4/5 |
| 5 | 2/2 |
| 6 | 2/4 |
| 7 | 3/3 |
| 8 | 1/4 |
| 9 | 2/3 |
| 10 | 4/4 |
| 11 | 1/5 |
| 12 | 4/6 |
| 13 | 1/3 |
| Total | 35/50 (70%) |
| V545 vaccine | |
| 1 | 0/3 |
| 2 | 0/3 |
| 3 | 1/4 |
| 4 | 2/5 |
| 5 | 0/4 |
| 6 | 0/3 |
| 7 | 0/4 |
| 8 | 0/1 |
| 9 | 1/4 |
| 10 | 0/5 |
| 11 | 0/4 |
| 12 | 0/3 |
| 13 | 1/2 |
| 14 | 1/4 |
| 15 | 3/3 |
| Total | 9/52 (17%) |

liver homogenates; 2/52 spleen homogenates; and 5/52 lung homogenates. Overall, congenital GPCMV transmission was identified in 9/52 pups (7/44 live-born pups and 2/8 dead pups) for an overall 11% congenital infection rate ($P < 0.0001$ compared to control). A total of 8/34 retrieved placentas were positive for GPCMV DNA (24%; $P < 0.0001$ versus control group). In PCR-positive placentas from the vaccine group, the mean viral load was $2.2 \pm 0.3$ $\log_{10}$ genomes/mg DNA, compared to $5.7 \pm 1.0$ $\log_{10}$ genomes/mg in placentas from control (unvaccinated) animals ($P < 0.0001$, Mann-Whitney test).

## 4. Discussion

In this study, a live, attenuated CMV vaccine was generated, based on deletion of a functional CCCK gene from the GPCMV genome [13, 14], using a *gpt*-based mutagenesis approach. This recombinant virus was highly attenuated for replication in guinea pigs, both in terms of its capacity to elicit systemic infection as well as in its ability to elicit localized inflammation and histopathology following footpad inoculation. In spite of this attenuation, the virus was capable, when administered as a vaccine to nonpregnant female guinea pigs, of eliciting high-titer ELISA antibody responses. Unfortunately, neutralizing titers could not be performed, due to a limitation in available serum, but the magnitude of the ELISA response was comparable to that observed in natural infection, and the ELISA titer has correlated with the neutralizing response in past studies in this model [32]. Vaccinated animals, following establishment of pregnancy, were protected against DNAemia after virulent salivary-gland virus challenge, and their pups were protected both against GPCMV-associated mortality and GPCMV infection. These observations provide further evidence both for a role of virally encoded immune modulation genes in the pathogenesis of infection *in vivo*, as well as for the highly attenuating effect of genetic manipulations designed to delete such genes in the design of live, attenuated vaccines.

The presence of virally encoded mimics of host CCCK genes has been noted for other rodent CMVs. The most extensively studied of these CKs has been the CCCK encoded by the MCMV MCK-1 gene. Mice infected with recombinant MCMVs with mutations in this gene developed less inflammation at the site of inoculation, demonstrated reduced secondary viremia, and had lower viral titers in the salivary glands [25, 33]. Rat CMV (RCMV) encodes a CCCK with similarity to the MCK-1 gene product, and rats infected with deletion mutants had reduced viral loads in the spleen and salivary glands, as well as reduced swelling and macrophage infiltration at the site of virus inoculation [31]. Previous study of the GPCMV CCCK, GPCMV-MIP [13], demonstrated that a mutant deleted of the CK coding sequences was attenuated for its ability to elicit inflammation and hearing loss following direct intracochlear inoculation in guinea pigs [15, 16]. In additional comparisons of the CK deletion virus, v545, with a virus containing the intact CK gene, vAM403, we observed both reduced DNAemia and weight loss following systemic viral challenge (Figure 3) and reduced footpad swelling and histopathology following localized infection (Figure 4) with the deletion virus compared to the CK-intact virus. This suggests that, as with other rodent CMVs, there is an important role for this CK in the pathogenesis of GPCMV infection *in vivo*.

The concept of using molecular genetic approaches to engineer recombinant CMVs, toward the goal of creating less pathogenic and/or more immunogenic live, attenuated vaccine candidates, has been previously described for both GPCMV [26] and MCMV [19–21, 34]. The targeting of immune modulation genes in vaccine design is of particular appeal given that there are substantial concerns about the potential long-term risks of live, attenuated HCMV vaccines, which could theoretically include latency, oncogenesis, autoimmune disease, and atherosclerosis [35]. In a previous report, the deletion of 3 GPCMV genes with homology to host MHC-I genes, using a bacterial artificial chromosome recombinatorial approach, resulted in a vaccine virus that was rapidly cleared in animals but was nonetheless highly

immunogenic and protective in the congenital infection model. The mechanism of immune evasion mediated by the class I gene family is currently under investigation but may be related to impairment of NK cell clearance. In the present study, *gpt* mutagenesis was chosen to generate recombinant virus, since the location of the GPCMV MIP gene was near the site of the BAC insertion in the viral genome in the BAC construct [23], making additional insertions and modifications in this region more challenging. Presumably, the mechanism of attenuation of the v545 (CCCK deletion) virus described in this study stems from its decreased propensity to mediate an acute inflammatory response. Since the ability of virus to disseminate and establish latency in the salivary gland may be impaired by deletion of viral CKs [25, 31], vaccine strains generated with such targeted deletions may have an improved safety profile.

One limitation of the analyses of the GPCMV MIP knockout and wild-type viruses performed in these studies is that the deletion of the MIP gene was superimposed on a tissue culture-derived (ATCC) viral stock of GPCMV. Subsequent to the initiation of our studies, it was demonstrated that the ATCC stock of GPCMV has a 1.6 kb deletion that removes several GPCMV genes, including *GP129*, *GP131,* and *GP133* [30, 36]. These genes encode homologs to HCMV UL128, UL130, and UL131, respectively, proteins which play a critical role in formation of the pentameric complex (PC), along with gpUL75 [gH] and gpUL115 [gL], essential for HCMV for the endocytic entry pathway required for infection of endothelial and epithelial cells [37–40]. Recently it has been shown that the GPCMV homologs similarly encode a PC that plays a role in virus entry [41]. Viruses lacking this 1.6 kb region are impaired for replication *in vivo* following experimental challenge of guinea pigs compared to those that retain these gene products [30, 36]. Since our recombinant viruses were generated against an ATCC background lacking this 1.6 kb region [23, 42], the deletion of GPCMV MIP in the recombinant virus, v545, is therefore superimposed upon a virus already lacking in genes that play an important role in the pathogenesis of infection. However, it is clear that the deletion of GPCMV MIP confers additional attenuation, above that already conferred by deletion of the 1.6 kb pathogenicity locus. Importantly, the attenuation of v545 pathogenicity reported in this and other studies [15, 16] was based upon comparisons to vAM403 virus [23], a GPCMV recombinant that is also lacking in this 1.6 kb locus. The vAM403 virus, also engendered by *gpt* mutagenesis, is similar to v545, except that (1) it retains the GPCMV MIP gene; (2) it has the *gpt*/eGFP cassette inserted in a noncoding region of the GPMCV genome. Therefore, the clear pattern of attenuation conferred by deletion of the GPCMV MIP gene in the v545 virus, compared to vAM403, provides reassurance regarding the role of this CCCK in pathogenesis. Efforts are in progress to reengineer the MIP deletion against the backdrop of a full-length GPCMV genome, using bacterial artificial chromosome-based approaches [42].

It was of interest to note that the v545 vaccine was capable of eliciting a highly protective immune response, in spite of the fact that it lacks the GP129-133 PC proteins. This observation suggests that the PC is not required for a successful CMV vaccine. Recently, it was shown that, in the absence of the rhesus CMV genes *Rh157.5*, *Rh157.4*, and *Rh157.6* (homologs of HCMV *UL128*, *130*, and *131*), CD8+ T cells are generated against unusual, diverse, and highly "promiscuous" epitopes [43]. The ability of UL128, 130, and 131 proteins to divert CD8+ T cell targeting away from unconventional epitopes could suggest that deletion of these genes might confer a more diverse T cell response to a CMV vaccine. If similar mechanisms are at play in GPCMV, this could contribute to the efficacy of a live, attenuated vaccine with a deletion in this region of the genome, the lack of an antibody response to the PC notwithstanding. Additional studies of recombinant GPCMVs deleted of key immune evasion and/or pathogenesis genes may shed light on attractive live, attenuated vaccine strategies for consideration for future development in human clinical trials.

## Conflict of Interests

None of the authors report any conflict of interests regarding commercial entities or products or other financial conflicts regarding the work reported in this paper.

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*Review Article*

# Innate Immune Signaling in the Pathogenesis of Necrotizing Enterocolitis

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Necrotizing enterocolitis (NEC) is a challenging disease to treat, and caring for patients afflicted by it remains both frustrating and difficult. While NEC may develop quickly and without warning, it may also develop slowly, insidiously, and appear to take the caregiver by surprise. In seeking to understand the molecular and cellular processes that lead to NEC development, we have identified a critical role for the receptor for bacterial lipopolysaccharide (LPS) toll like receptor 4 (TLR4) in the pathogenesis of NEC, as its activation within the intestinal epithelium of the premature infant leads to mucosal injury and reduced epithelial repair. The expression and function of TLR4 were found to be particularly elevated within the intestinal mucosa of the premature as compared with the full-term infant, predisposing to NEC development. Importantly, factors within both the enterocyte itself, such as heat shock protein 70 (Hsp70), and in the extracellular environment, such as amniotic fluid, can curtail the extent of TLR4 signaling and reduce the propensity for NEC development. This review will highlight the critical TLR4-mediated steps that lead to NEC development, with a focus on the proinflammatory responses of TLR4 signaling that have such devastating consequences in the premature host.

## 1. Introduction

Necrotizing enterocolitis (NEC) is a challenging disease to treat. While NEC may develop quickly and without warning, it may also develop slowly, insidiously, and to take the caregiver by surprise. In its most severe and extreme form, NEC is not particularly difficult to recognize; the premature infant with a grossly distended abdomen, bilious nasogastric aspirates, and maroon-colored stools can be readily considered to have NEC as a unifying explanation for his/her constellation of symptoms. In earlier stages of the disease, however, where the only symptoms may be feeding intolerance and mild hemodynamic instability, the diagnosis of NEC often cannot be made with high confidence or reliability, reflective perhaps of the fact that a variety of septic states can share features that overlap with NEC. Yet despite the inability to reliably diagnose NEC in these early stages, it is precisely at the earliest stages of the disease where an accurate diagnosis is most critical, as it is here that the ability to intervene may be expected to have the greatest potential for benefit before irreversible intestinal necrosis and overwhelming systemic sepsis occur. Given the relative imprecise nature of the diagnostic approaches for early stage NEC, scientists who investigate the molecular underpinnings of this disease and clinicians who take care of patients who suffer from it have focused their attention on gaining a greater understanding of the events that lead to its early development. In this regard, we and others have identified a necessary role for the innate immune lipopolysaccharide receptor toll like receptor 4 (TLR4) in the pathogenesis of NEC. In this review, we

will investigate the evidence that points to a role for TLR4 signaling in the pathogenesis of NEC, through its ability to promote intestinal injury and through its deleterious effects on intestinal healing in the premature host that together lead to the development of NEC.

## 2. Injury and Repair in the Intestinal Tract of the Premature Infant

The intestinal mucosa of the premature infant exists in a state of constant injury and repair, which must be perfectly balanced in order to maintain homeostasis. Injury to the intestinal mucosa occurs during a variety of settings that may be present within the setting of prematurity, including hypoxia [1, 2], remote infection [3], and the administration of nonbreast milk infant formula [4]. We [5] and others [6] have shown that the initial injury to the small intestine primarily involves the loss of epithelial villi through apoptosis, which subsequently leads to the development of necrosis, a process that is consistent yet only incompletely explained. Loss of the epithelial barrier through apoptosis permits the translocation of bacteria and other antigens that are present within the lumen of the intestine, and which must normally be appropriately shielded from the immune system of the premature host in order to prevent the exaggerated inflammatory response, that is, typical of intestinal inflammatory conditions such as NEC [7, 8]. In response to the loss of epithelial continuity (which may be reflective of primary apoptosis or the early events that migh culminate in apoptosis), a multipronged healing program is initiated. Healing of the intestinal mucosa occurs initially through the process of enterocyte migration, which involves the movement of healthy enterocytes into the wounded area in order to provide a rapid seal, which limits the degree of bacterial translocation that can occur [9]. While enterocyte migration can facilitate the early steps that lead to intestinal restitution, these events are short lived and unlikely to have a long-lasting effect without the generation of new enterocytes, a process that occurs within the Crypts of Luberkuhn [10, 11]. The steps that regulate the proliferation of enterocytes from existing precursors have been well characterized in a series of thorough recent reviews [12, 13]. We have described that under conditions of prematurity, NEC is associated with a marked inhibition in both enterocyte migration and proliferation, which renders the host uniquely susceptible to further injury through the combined loss of both of the important reparative processes that are normally present within the intestine [5]. In the subsequent sections, the mechanisms by which enterocyte migration and proliferation are each impaired in the pathogenesis of NEC will be reviewed in further detail.

## 3. Activation of the Innate Immune Receptor Toll Like Receptor 4 (TLR4) Inhibits Enterocyte Migration

As described above, the earliest reparative event that occurs in response to epithelial injury involves the migration of healthy enterocytes from uninjured areas to sites of epithelial disruption. We [5, 14, 15] and others [16] have shown that necrotizing enterocolitis is characterized by a marked impairment in enterocyte migration, and, as a result, severe mucosal defects are present. In seeking to identify the mechanisms by which the impairment in enterocyte migration occurs, we have shown that TLR4 is expressed within the intestinal epithelium, and that activation of TLR4 leads to an abrupt inhibition in enterocyte migration both in vivo and in vitro [14, 15]. We have further demonstrated that the reduction in enterocyte migration in response to TLR4 activation occurs via an increase in actin-mediated stress fibers, leading to an increase in the degree of adhesiveness with which the enterocyte is anchored to the underlying basement membrane [17]. The increase in stress fibers occurs due to a TLR4-dependent increase in the activity of the small GTP-ase RhoA, which catalyzes the formation of stress fibers within the cytoplasm, and through the activation of focal adhesion kinase (FAK), which further strengthens the degree of anchoring of the enterocyte to the underlying membrane [5, 15]. TLR4 signaling on enterocytes also leads to an efflux of beta-1 integrins from the cytoplasm towards the cell membrane, resulting in enhanced cell-matrix contacts that together serve to limit the degree of cell movement that can occur [18]. In support of the physiological relevance of these findings, the treatment of cells with either antibodies to beta integrins or with reagents that inhibit FAK activation can reverse the deleterious effects of TLR4 activation on enterocyte migration and promote cell movement, even in the face of tonic TLR4 signaling [5, 15, 18]. In further studies, we have shown that intercellular communication via gap junctions is required for effective enterocyte migration to occur, and that NEC is characterized by a marked reduction in the expression of gap junctions on the surface of enterocytes that is mediated through the release of the proinflammatory cytokine interferon gamma [19]. In additional studies, we showed that interferon gamma inhibited enterocyte migration through the displacement of Connexin43 from lipid rafts, which are discrete and highly organized areas of the plasma membrane that represents important sites of cellular signaling [20]. In addition, the proinflammatory signaling molecule nitric oxide was shown to play an important role in the regulation of enterocyte migration in NEC as exposure of enterocytes to nitric oxide was shown to impair cell movement both in vitro and in vivo, in part through the activation of RhoA and enhancement of cell-matrix adhesiveness [14]. Consistent with these studies, Besner and colleagues have examined the role of E-cadherin and integrins in NEC, and have determined that the growth factor heparin bound epidermal growth factor (HB-EGF) can promote intestinal restitution in NEC through effects on integrin-extracellular matrix interactions and intercellular adhesions [16]. These studies are particularly relevant, as they support an earlier observation in human patients, in which a decrease in trefoil peptides that are known to play a key role in intestinal regeneration was found to be reduced in NEC [21]. Taken together, these findings illustrate important role of impaired enterocyte migration in the pathogenesis of NEC and identify important mechanistic events in mediating the impaired migration mediating these events.

## 4. TLR4 Activation on the Intestinal Epithelium Leads to Enterocyte Apoptosis

One of the earliest changes that are observed within the intestinal mucosa in the setting of experimental NEC is a marked increase in the loss of enterocytes, which die through the process of exaggerated apoptosis [7, 21–26], leading to the transluminal passage of indigenous microbes and an unbridled activation of the host immune system. We have recently identified that the activation of toll like receptor 4 (TLR4) within the intestinal epithelium plays a critical role in the early initiation of the steps that lead to enterocyte loss [5, 27–29], a finding that is supportive of studies by Jilling and colleagues showing that mice deficient in TLR4 were protected from NEC development [30]. TLR4 signaling in enterocytes both in vitro and in vivo leads to enterocyte apoptosis, while the inhibition of TLR4 signaling in the newborn intestinal epithelium prevents NEC development and attenuates the degree of enterocyte apoptosis [5, 27, 28]. While these studies have placed the spotlight on the role of TLR4 in the pathogenesis of NEC, the observation that most premature infants do not develop NEC despite the seemingly tonic activation of TLR4 within the intestinal epithelium and elsewhere raises the important possibility that TLR4 signaling must somehow be curtailed within the newborn intestinal epithelium, in order to limit the propensity for spontaneous NEC development. This concept will be explored in detail below.

## 5. Cellular Strategies That Limit the Extent of TLR4 Signaling in the Intestinal Epithelium

In seeking to define whether negative regulatory strategies for TLR4 within the newborn intestinal epithelium could participate in the pathogenesis of NEC, we focused on the intracellular chaperone heat shock protein 70 (Hsp70), to determine whether perhaps Hsp70 could negatively regulate TLR4 signaling within enterocytes and by extension, whether a loss of Hsp70 could lead to NEC development through uninhibited TLR4 activation. The heat shock proteins—of which Hsp70 is a predominant member—represent a family of intracellular proteins that are activated by a variety of stressors and that can assist in the delivery of target proteins to the ubiquitin-proteosome system for degradation through cochaperone molecules such as CHIP, which stands for C-terminus of Hsp70 interacting protein [31]. In support of a possibility for Hsp70 in the regulation of enterocyte apoptosis, we note that TLR4 has previously been shown to play an important role in the modulation of apoptosis after various forms of stress [32–35], and Hsp70 has been shown to serve a protective role in the intestine as demonstrated by Tao and colleagues [36, 37]. Through its combined roles of both clearing proteins and modulating cell death, the net effect of Hsp70 induction within cells is to restore the host to a nonstressed environment [38–40]. We have recently determined that the expression of TLR4 is significantly reduced in mice and humans with NEC [22], suggesting but not proving that Hsp70 may negatively regulate TLR4 signaling and that a reduction in Hsp70 may result in the progression to NEC. In support of this possibility, using enterocytes that either lack or are induced to express Hsp70 as well as by examining mice that either lack Hsp70 or that overexpress Hsp70 within the intestinal epithelium, we have determined that intracellular Hsp70 limits TLR4 signaling in enterocytes and, moreover, that Hsp70 plays a central role in the pathogenesis of NEC [22]. The mechanism by which Hsp70 limits TLR4 signaling in the gut involves an increase in CHIP-mediated ubiquitination and degradation of TLR4 via the ubiquitin-proteosomal pathway [22]. Importantly, pharmacologic upregulation of Hsp70 within the intestinal mucosa led to a reduction in TLR4 signaling and a decrease in enterocyte apoptosis, leading to an attenuation in NEC severity [22]. Taken together, these findings illustrate a novel pathway linking the regulation of Hsp70 with the negative control of TLR4 signaling within the gut and provide evidence that the development of NEC results in part from exaggerated TLR4-induced enterocyte apoptosis due in part to reduced Hsp70 activity [22]. Moreover, these results suggest that pharmacologic upregulation of Hsp70 could provide a novel approach to the prevention and/or treatment of NEC through the inhibition of TLR4 signaling in the newborn intestine [22, 41].

It is important to note that the findings in which cytoplasmic Hsp70 serves to curtail the signaling of TLR4 within the intestinal epithelium are distinct from other studies that have focused on the extracellular role of Hsp70 and other heat shock proteins in activating the innate immune system via TLR4. For instance, Retzlaff et al. showed that the exogenous administration of Hsp70 could increase IL-1, IL-6, and TNF in cultured macrophages [42], while Wheeler et al. have shown that the extracellular exposure of Hsp70 to neutrophils from wild-type mice leads to the release of IL-8, yet this effect is not observed in neutrophils from C3H/HeJ mice that have inhibitory mutations in TLR4 [43]. Whether these results reflect the effects of heat shock proteins themselves or whether the signaling responses may be due to contaminants such as LPS which could inadvertently be present within the protein preparations as some have suggested [44–47] is beyond the scope of the current discussion although this has been carefully and extensively reviewed recently [48, 49]. In contrast to studies in the field of extracellular Hsp70 biology, the novelty and importance of the current findings lie in the newly discovered link between TLR4 and Hsp70 within the enterocyte both in vitro and in vivo, and the potential etiological relevance to the development of NEC.

## 6. TLR4 Activation on the Intestinal Epithelium Inhibits Enterocyte Proliferation in the Pathogenesis of NEC

As described above, the early events that occur in the pathogenesis of NEC involve a loss of enterocytes through apoptosis, which leads to bacterial translocation and systemic sepsis. In response to the loss of enterocytes, the host initiates a reparative process that commences with migration of

enterocytes from healthy areas towards the sites of injury and subsequently involves the proliferation of enterocytes *de novo* from precursor stem cells that are located within the intestinal crypts. We and others have examined the processes that regulate enterocyte proliferation in the newborn mucosa and in particular, have examined the pathways by which enterocyte proliferation is reduced in NEC [28, 50, 51]. Previous authors have identified a critical role for the $\beta$-catenin-signaling pathway in enterocyte regulation, which occurs via the upstream inhibitor GSK3$\beta$ [52]. Importantly, we recently established a link between TLR4 and $\beta$-catenin, which could provide a novel explanation for the initiation and propagation of the mucosal injury seen in NEC. Specifically, we demonstrated that TLR4 activation significantly impaired enterocyte proliferation in the ileum in newborn mice as well as in cultured enterocytes [28] via the inhibition of $\beta$-catenin signaling. These effects were specific for newborn mice and for the ileum and were not seen in the colon or in adult mice, providing additional relevance to the pathogenesis of necrotizing enterocolitis which tends to favor the ileum. To determine the mechanisms involved, TLR4 inhibited the phosphorylation of the upstream inhibitory kinase GSK3$\beta$, causing $\beta$-catenin degradation and subsequently a decrease in proliferation and repair. Strikingly, the inhibition of enterocyte $\beta$-catenin signaling in NEC resulted in increased enterocyte proliferation restored and attenuated NEC severity, suggesting that strategies that can promote or enhance proliferation may have a potential therapeutic role in either the prevention or treatment of NEC [28, 53].

## 7. TLR4 Activation on Intestinal Stem Cells Leads to Their Loss through Apoptosis

As described above, the ability of the intestinal epithelium to undergo regular and rapid turnover in the face of injury is determined primarily by the activity of a discrete population of stem or progenitor cells located at the base of the intestinal crypts [54–56]. Various authors have recently identified precise and reliable markers for intestinal stem cells, which have allowed for a careful evaluation of their individual capacities to divide and differentiate, including the markers Bmi1 [57–61] and Lgr5 [62–65]. Given that the intestinal stem cells exist in close proximity to the microbial flora, it stands to reason that signaling receptors that recognize components of the flora may be present on and have effects on intestinal stem cells. Given our recent findings that TLR4 can regulate enterocyte proliferation [5], we recently explored whether TLR4 itself may be expressed on the intestinal stem cells and thus regulate their function. In support of this possibility, using flow cytometry and fluorescent in situ hybridization for the intestinal stem cell marker Lgr5, we determined that TLR4 is indeed expressed on the Lgr5-positive intestinal stem cells and that the activation of TLR4 leads to a reduced proliferation and an increase in apoptosis of the intestinal stem cells both in vivo and in vitro. This finding was not observed in mice in which we had specifically removed TLR4 from the Lgr5-positive cells, confirming the in vivo significance of this effect. To define the potential molecular mechanisms involved, TLR4 was found to inhibit intestinal stem cell proliferation and increase apoptosis via the p53-upregulated modulator of apoptosis (PUMA), as TLR4 did not affect crypt proliferation or apoptosis in intestinal stem cell cultures or in mice lacking PUMA. Furthermore, the effects of TLR4 on intestinal stem cells in vivo required TIR-domain-containing adapter-inducing interferon-$\beta$ (TRIF) but were independent of myeloid-differentiation primary response-gene (88) (MYD88) and TNF$\alpha$. Importantly, the inhibition of PUMA in vivo restored intestinal stem cell proliferation and reduced apoptosis in NEC, which itself was associated with reduced intestinal stem cell function [66]. The findings that NEC is associated with reduced intestinal stem cells is supportive of earlier work by Besner and colleagues, who not only showed that the intestinal stem cells and all subsequent lineages were reduced in experimental NEC, but also demonstrated that the administration of the heparin-binding epidermal growth factor (HB-EGF) could restore intestinal stem cells and therefore attenuate the severity of NEC [67]. Taken together, these findings reveal therefore that the development of NEC may reflect in part a reduction in crypt progenitor cells due to exaggerated TLR4 signaling in this compartment and raise the possibility that strategies that enhance mucosal healing through effects on the now identified TLR4-PUMA axis may be harnessed therapeutically.

## 8. TLR4 Activation in Necrotizing Enterocolitis versus Inflammatory Bowel Disease

It should be noted that the role of TLR4 in the pathogenesis of NEC in its effects on promoting injury in the small intestine may be quite distinct from the role of TLR4 in other diseases of intestinal inflammation including ulcerative colitis and Crohn's disease, in which TLR4 signaling may play a lesser or perhaps even opposite role. Various authors have carefully and convincingly demonstrated that mice lacking TLR4 have increased susceptibility to the development of colitis, suggesting that TLR4 plays a protective role in this disease [68, 69]. As we have recently discussed [8, 22], there may be several reasons to account for this apparent discrepancy. For instance, TLR4 activation leads to intestinal injury in a well-defined and physiologically relevant context, namely, the newborn small intestine. In support of this concept, we have recently demonstrated that TLR4 activation with LPS leads to increased enterocyte apoptosis in the terminal ileum of newborn mice but not adult mice, and in the small intestine but not the newborn colon [27]. Further, reports that demonstrate a protective role for TLR4 in models of colitis have typically been based upon the use of global TLR4 knockout mice, in which TLR4 signaling is disrupted in enterocytes as well as T-cells and myeloid cells. We have recently shown that TLR4 signaling within the enterocyte itself is important for the induction of intestinal injury leading to NEC, using enterally administered adenoviral constructs that bear inhibitory mutations in TLR4 whose expression is largely favored within the small bowel mucosa [28, 70]. It is therefore reasonable to conclude that the protectiveeffects attributed to TLR4 signaling in the gut by previous authors

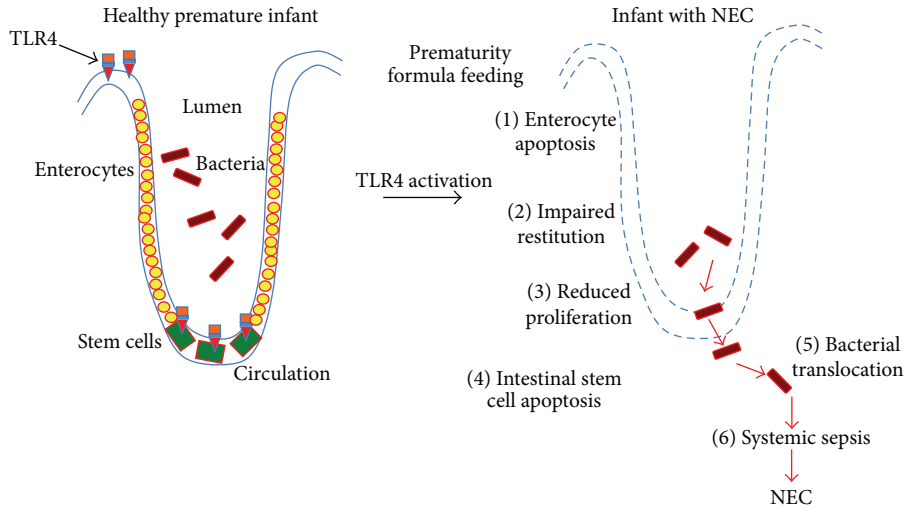


Figure 1: Mechanisms to explain how TLR4 activation in the premature intestinal epithelium is required in the pathogenesis of necrotizing enterocolitis. As shown, colonization of the intestine of the premature infant leads to the activation of TLR4 within the intestinal epithelium as well as on the intestinal stem cells, due in part to the elevated expression levels of TLR4 in the setting of prematurity. TLR4 activation leads to enterocyte apoptosis, impaired restitution and reduced proliferation as well as stem cell apoptosis, leading to bacterial translocation, systemic sepsis, and the development of NEC. See text for further details.

may reflect in part the mitigating effects of TLR4 signaling on other cells. In support of this possibility, we note that Fukata and colleagues have recently shown in an elegant study using chimeric mice that TLR4 signaling in colonic epithelial cells worsened intestinal inflammation [71]. These findings argue that the effects of TLR4 in the development of intestinal inflammation are strongly influenced by a variety of factors, including the effector cells involved, developmental factors, and involved region of the intestine.

## 9. Prematurity Is Associated with Increased TLR4 Expression and Activation in the Fetal Intestine

Given our findings regarding the critical importance of TLR4 signaling in enterocytes in the pathogenesis of NEC via effects on mucosal injury and repair, we next considered why the premature infant is at uniquely increased risk for the development of NEC in the first place. To do so, we considered whether the expression of TLR4 may be higher in the premature infant compared with the full-term infant and, further, whether the increased elevation of TLR4 could contribute to the development of NEC. To test this possibility directly, using quantitative RT-PCR, we determined that the expression of TLR4 is significantly greater in the premature mouse and human compared with the full-term state and moreover, the expression of TLR4 is significantly elevated under conditions that are particularly relevant to the pathogenesis of NEC, namely, the presence of hypoxia and exogenous bacteria or LPS [72, 73]. We further demonstrated that TLR4 is not only expressed at high levels within the premature intestine, but it is also functionally active [70], as the delivery of the TLR4 agonist LPS directly into the intestinal lumen of the developing mouse using ultrasound guided microinjection showed a marked increase in the induction of TLR4-dependent cytokines that matched the expression of TLR4. These findings lead us to propose that, in the setting of prematurity, TLR4 expression is very high and yet must normally decrease shortly after birth to levels that allow for the normal adaptation of the intestinal mucosa to bacteria. However, in the setting of prematurity, levels of TLR4 remain high, as we and others have shown and remain at high levels after birth in the premature state [70, 74–76]. When the premature intestine with accompanying elevated expression levels of TLR4 becomes colonized, in the setting of the correct environment that favors exaggerated TLR4 signaling, TLR4-mediated enterocyte apoptosis and delayed mucosal repair ensue, which together favor bacterial translocation and the development of systemic sepsis and NEC (see Figure 1). These findings provide additional mechanistic insights into NEC development and also provide additional insights into the increased susceptibility of the premature infant to NEC compared to full-term infants.

## 10. TLR4 Signaling in the Intestinal Epithelium Regulates the Normal Differentiation of the Intestinal Epithelium and Is Required for Goblet Cell Formation

We have recently examined the reasons for which TLR4 is significantly elevated within the intestine of the fetal mouse and human and in particular have considered the possibility that TLR4 could exert a role in the normal development of the intestine itself. To do so, we generated a novel strain of mice in which TLR4 had been selectively deleted from the intestinal epithelium and examined the differentiation of the small intestine in these mice [29]. To our surprise, we noted that

mice lacking TLR4 showed an unusual phenotype that was characterized by a marked increase in goblet cells [29]. In seeking the mechanism by which TLR4 could regulate goblet cell differentiation, we further determined that deletion of TLR4 was associated with a marked decrease in signaling through the Notch pathway, which is a major determinant of differentiation within the intestinal epithelium, and that this phenotype could be reversed by overexpression of TLR4 in vitro [29]. These findings are consistent with earlier studies that had shown that Notch signaling could be activated by TLR stimulation [77] in macrophages. We also determined that exposure of bile acids to TLR4-deficient IEC-6 cells as well as to crypts from TLR4-deficient mice reversed the goblet cell phenotype, a finding that is particularly relevant given that bile acids can activate Notch in the gastrointestinal epithelium [78, 79], and that increased bile acids were associated with reduced goblet cells in newborn rats and increased NEC severity [80]. These findings together indicate that TLR4 can regulate goblet cell differentiation and suggest the possibility that bile acids may serve as intermediates in the regulation of Notch. In seeking to understand the potential teleological explanation of the regulation of intestinal differentiation by an innate immune receptor, it is apparent that TLR4 activation in the regulation of intestinal differentiation is likely distinct from its signature role in host defense. It is possible that in the postnatal environment to which the premature infant is exposed, in which the expression of intestinal TLR4 remains persistently elevated—the *developmental* role for TLR4 switches to a *proinflammatory* role upon its interaction with colonizing microbes, leading to NEC development. Indeed, we have shown a separate role for TRIF and MyD88, with the former being required for goblet cell differentiation and the latter playing a key role in proinflammatory cytokine production; the future identification of molecules that can selectively activate TRIF and not MyD88 may reveal important clues into the developmental role for TLR4 within the developing gut.

## 11. Amniotic Fluid: An Elixir That Can Regulate TLR4 Signaling within the Gut of the Developing Fetus?

The developing fetus exists in a state of persistently exaggerated TLR4 expression, due in part to our recent identification of the role for TLR4 in regulating the normal differentiation of the intestinal epithelium [29]. However, the persistently elevated expression of TLR4 during development renders the fetus at risk for potentially deleterious effects; should in fact the normally sterile environment of the womb in fact be breached by microbial pathogens. Several reports have shown that the microbial DNA may be detected within the amniotic fluid itself, raising the possibility that bacterial colonization of this normally sterile environment may occur [81, 82]. It would seem therefore that in order to limit the consequencesof exaggerated TLR4 signaling in that may occur in the fetus upon exposure to microbes, there must be a counterregulatory mechanism that could limit TLR4 activation. In addressing what the potential anti-TLR4 signaling effects could be, we focused on the fact that the fetus is continuously swallowing amniotic fluid and, as a result, the fetal intestine becomes exposed to amniotic fluid as a natural consequence of the in utero environment. This raised the possibility that perhaps amniotic fluid itself could serve to limit the extent of TLR4 signaling within the intestine. In support of this possibility, we demonstrated recently that amniotic fluid reduces TLR4 signaling in the fetal intestinal mucosa, as well as in cultured enterocytes that had been exposed to bacterial products [83], resulting in a marked reduction in the degree of proinflammatory cytokine release. In seeking the molecular mechanism involved, we focused on the fact that amniotic fluid is extremely rich in epidermal growth factor (EGF), which we showed to be required for its inhibitory effects on TLR4 signaling via its ability to activate the transcription factor peroxisome proliferator-activated receptor gamma (PPAR$\gamma$) and, indeed, amniotic fluid did not inhibit TLR4 signaling in enterocytes that were deficient in either the EGF receptor or in PPAR$\gamma$, nor in mice lacking EGFR within the intestinal epithelium [83]. Further evidence that EGF in amniotic fluid was responsible for its inhibitory effects on TLR4 signaling was found in the observation that purified EGF attenuated the exaggerated intestinal mucosal TLR4 signaling, while depletion of EGF from amniotic fluid reversed the protective effects [83]. Strikingly, the development of NEC in both mice and humans was associated with reduced expression of EGFR within the intestinal epithelium that was restored upon the administration of amniotic fluid to mice and, moreover, the administration of amniotic fluid significantly attenuated NEC severity in mice. These findings may explain how premature infants may be at increased risk for NEC development due to the lack of exposure to the protective effects of amniotic fluid in the setting of elevated TLR4 expression [83]. Given that several groups have reported that the administration of EGF or its homologue HB-EGF can treat experimental NEC [50, 84], it is tempting to speculate that these prior studies may be explained through an inhibitory effect of these growth factors on TLR4 signaling, in addition to their restorative and reparative effects on the intestinal mucosa.

## 12. Other Factors That Are Important in the Pathogenesis of Necrotizing Enterocolitis

Despite the focus on TLR4, we readily acknowledge that there are other important factors that are important in NEC pathogenesis. For instance, Besner and colleagues have shown that heparin binding epidermal growth factor (HB-EGF) is important in regulating the pathogenesis of NEC, in part through determining the extent of enterocyte migration [85]. This work is in agreement with the findings of Clark and colleagues as well as Sheng and colleagues, who have demonstrated that EGF administration can attenuate NEC severity by enhancing the healing response to mucosal injury [86, 87]. Soliman and colleagues have demonstrated that platelet-activating factor (PAF) plays an important role in NEC pathogenesis and have shown that TLR4 signaling can upregulate PAF expression and therefore increase injury in experimental NEC [88, 89]. Cherrington and colleagues

have shown that the accumulation of ileal bile acids causes significant injury in the small intestine in NEC pathogenesis [90], of which we showed to act in concert with TLR4-mediated impairment of enterocyte function [29]. In a parallel line of investigation, we showed that intact intestinal restitution requires intercellular connectivity that is mediated through small channels termed gap junctions that are rich in the protein connexin 43 [19, 20]. Importantly, we have shown that proinflammatory cytokines including interferon gamma cause the internalization of connexin 43, thereby impairing intercellular connectivity and reducing the extent of intestinal restitution in the pathogenesis of NEC [19, 20]. Taken together, these lines of evidence indicate that, in addition to TLR4, there are other important factors that also play significant roles in the pathogenesis of NEC, which should be taken into account when seeking a full and complete understanding of the pathogenesis of this complex and interesting disease.

## 13. Model: TLR4 Activation Plays a Critical Role in the Pathogenesis of NEC

The studies described above lead us to propose the following model to explain how NEC develops part to exaggerated TLR4 in the intestinal mucosa of the premature infant (Figure 1). As we have recently described [91], under normal conditions, the full-term infant is characterized by low levels of TLR expression, which in the setting of microbial colonization does not elicit a particular proinflammatory response and also allows for the normal adaptation of the small intestine to the colonizing microbes. By contrast, under conditions of prematurity, TLR4 expression remains high, as a consequence in part of the developmental role of TLR4 in utero, in which TLR4 is important for the normal differentiation of the intestinal epithelium. When the premature infant intestine is colonized by flora within the neonatal intensive care unit and in the absence of host-derived factors that normally serve to restrict TLR4 signaling such as Hsp70, TLR4 signaling within the gut becomes particularly exaggerated, leading to increased mucosal injury and decreased mucosal repair. The net effect of these TLR4-mediated responses leads to gut barrier failure, bacterial translocation, the development of systemic sepsis, and the clinical and pathologic features of necrotizing enterocolitis. Based upon these findings, we propose that an understanding of the early TLR4-mediated signaling events will not only allow for a greater understanding of the pathogenesis of NEC, but also offer new and innovative approaches to its treatment based upon their capacity to inhibit TLR4 signaling within the gut. Through these efforts, it is our hope and the hope of all those involved in the care of infants with NEC that these tiny infants may one day be seen as they have never been seen before—as entirely curable.

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*Review Article*

# Neonatal Sepsis due to Coagulase-Negative Staphylococci

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Neonates, especially those born prematurely, are at high risk of morbidity and mortality from sepsis. Multiple factors, including prematurity, invasive life-saving medical interventions, and immaturity of the innate immune system, put these infants at greater risk of developing infection. Although advanced neonatal care enables us to save even the most preterm neonates, the very interventions sustaining those who are hospitalized concurrently expose them to serious infections due to common nosocomial pathogens, particularly coagulase-negative staphylococci bacteria (CoNS). Moreover, the health burden from infection in these infants remains unacceptably high despite continuing efforts. In this paper, we review the epidemiology, immunological risk factors, diagnosis, prevention, treatment, and outcomes of neonatal infection due to the predominant neonatal pathogen CoNS.

## 1. Epidemiology of Neonatal Sepsis

Neonatal sepsis is defined as infection in the first 28 days of life, or up to 4 weeks after the expected due date for preterm infants [1]. Epidemiologists defined two types of infections in neonates: early-onset neonatal sepsis (EONS), which manifests in the first 72 hours of life (up to 7 days) and late-onset neonatal sepsis (LONS), whose incidence peaks in the 2nd to 3rd week of postnatal life [1]. The mortality from neonatal sepsis has dramatically decreased over the last century, because of medical advances. In the preantibiotic era (<1940), the case fatality rate of neonatal sepsis was extremely high, exceeding 80% [2]. By the late 1960s, the introduction of antibiotics and the development of modern perinatal care had lowered this case fatality rate to less than 20% overall [2]. The composition of pathogens causing neonatal sepsis has also changed dramatically over the last century [2–6]. In the early 1930s, *Streptococcus pneumoniae* and group A streptococci were responsible for almost half of the cases of LONS [2, 3]. By the 1960s, gram-negative bacilli had become major pathogens [3], along with the emergence of group B streptococci (GBS) as a predominant cause of EONS [2]. In North America, gram-positive organisms account for the majority of neonatal sepsis cases (up to 70%). Sepsis due to gram-negative organisms (~15 to 20%) and fungi (~10%) is less common, and polymicrobial bloodstream infections contribute to less than 15% of cases [2, 7, 8]. Coagulase-negative staphylococci (CoNS) are the major pathogen involved in LONS, particularly in infants born at a lower gestational age. According to more recent data from the National Institutes of Child Health and Development (NICHD), infection-related mortality in very low-birth-weight (VLBW) infants (birth weight < 1500 grams) averages 10% [7] but can reach 40% depending on the pathogen involved [9–11]. Preterm neonates have a high risk of developing neonatal infections, resulting in high mortality and serious long-term morbidities [5, 7, 12]. In North America, it is estimated that each episode of sepsis prolongs the duration of a neonate's hospital stay by about 2 weeks, resulting in an incremental cost of USD$25,000 per episode [13]. In a more recent study, authors

estimated that nosocomial bloodstream infections increase the neonatal hospitalization cost for VLBW infants in the lowest birth weight group (401–750 grams) by 26%, and that of the highest birth weight group (1251–1500 grams) by 80% [14]. This study also estimated that the duration of hospital stay increased by four to seven days in all VLBW categories with a nosocomial bloodstream infection [14].

*1.1. Burden of Neonatal Sepsis in Developing Countries.* In developed countries, advances in medical care have enabled a greater proportion of premature infants to survive, albeit with an increased risk of infection [15]. However, because the greatest burden of neonatal sepsis falls on low-resource developing countries, the global economic impact is difficult to estimate [16]. Globally, infections still cause an estimated 1.6 million neonatal deaths annually, representing 40% of all neonatal deaths [16–18]. About 12% of children are born prematurely worldwide, including about 2% of VLBW. Together, prematurity and neonatal infections account for the greatest burden of neonatal deaths overall [16]. The limited access to medical resources combined with geographical comorbidities (e.g., severe malnutrition) can lead to mortality from neonatal sepsis remaining unacceptably high in developing countries [16].

## 2. Pathogenesis

Within the first week of life, neonates become rapidly colonized by microorganisms originating from the environment [19–22]. During this period, the risk of CoNS infection increases substantially with the use of central venous catheters (CVC), mechanical ventilation, and parenteral nutrition, and with exposure to other invasive skin- or mucosa-breaching procedures [8, 15, 23–28]. CoNS are common inhabitants of the skin and mucous membranes; although a small proportion of neonates acquire CoNS by vertical transmission, acquisition primarily occurs horizontally [22, 29]. Consequently, infants admitted to a hospital obtain most of their microorganisms from the hospital environment, their parents, and staff [30, 31]. Transmission via the hands of hospital staff can lead to endemic strains circulating for extended periods [29, 32–35]. Because CoNS is a ubiquitous skin commensal, authors have assumed that colonizations of the skin and of indwelling catheters are important sources of sepsis [34, 36]. However, recent studies suggest that epithelial loci other than the skin, such as the nares, may be important access points of infection [34, 36]. Antibiotic resistance in skin-residing strains has been found to be low at birth but to increase rapidly during the first week of hospitalization [37]. Selective pressure as a result of perinatal antibiotic exposure, therefore, is an additional major factor influencing the spectrum and antibiotic resistance pattern of microorganisms isolated from neonates.

*2.1. Host Immunological Factors.* Some components of the immune response are particularly important in preventing sepsis due to CoNS (reviewed in [38]). The immune system is traditionally described in terms of the innate and the adaptive immune systems. The innate immune system is responsible for the "naïve," more rapid, first-line response to infection. At birth, the neonate's own adaptive immune system is largely uneducated. To protect against infection, neonates must therefore rely heavily on innate immune responses and on passive adaptive immune mechanisms acquired from the mother (e.g., transplacental transfer of antibodies), which are deficient in preterm neonates [39–43]. Specific host innate immune factors have been studied in the context of neonatal CoNS infections: mucosal barriers, including antimicrobial peptides (AMP), cells (neutrophils), and pattern recognition receptors (PRR, e.g., Toll-like receptors), as detailed below.

*2.1.1. Mucosal Barriers.* The outermost layer of the skin (*stratum corneum*) acts as a physical barrier and first line of defense against bacterial invasion. The skin secretes AMP, which are early-response factors creating a microbicidal shield particularly effective against CoNS [43–48]. In preterm neonates, the immature *stratum corneum* only fully matures at one to two weeks after birth [42, 43, 46]. The vernix caseosa, a waxy coating on neonates' skin, provides additional antimicrobial protection in mature neonates. It is mainly formed during the last trimester of gestation, leaving extremely premature neonates far more vulnerable to infection [49–51]. Immunity against CoNS is also limited in other mucosal surfaces in preterm neonates, for example, because of a thinner glycocalyx layer coating the intestinal epithelium [52, 53], lower secretory IgA [53], and reduced AMP production by Paneth cells [54–56]. Necrotizing enterocolitis (NEC) is a progressive ischemic necrosis of the neonatal intestine that occurs in preterm infants [57]. The cause of NEC is unclear but is believed to develop as a result of gut injury, with a key role for bacteria in its pathogenesis [57–59]. Frequent isolation of enterotoxin-producing CoNS from the intestinal flora of infants with NEC has led authors to propose that overgrowth of CoNS plays a role in this complication [60–62]. A poor barrier function and an overall immaturity of the premature gastrointestinal immune system [63, 64] contribute largely to the development of NEC, possibly by favoring bacterial overgrowth and translocation [57, 63, 65].

*2.1.2. Cells.* Neutrophils also play a major role in protection against neonatal sepsis, including CoNS, as first-responder leukocytes in the blood [66–69]. Certain characteristics of neonatal neutrophils have been proposed as mechanisms of increased susceptibility to CoNS sepsis [70]: their relatively inefficient recruitment and extravasation to the site of infection [69]; their reduced bacterial killing capacity, in part due to the failure to upregulate their oxidative burst response [71]; and the reduced ability of neonatal neutrophils to form "extracellular traps" [50].

*2.1.3. Pattern Recognition Receptors.* PRR detect the presence of microorganisms in the tissue through the recognition of conserved molecular structures specific to microbes (known as pathogen-associated molecular patterns: PAMP). To date, the best characterized PRR are the Toll-like receptors (TLR), which include ten receptors in humans [72–75]. Recent studies in mice have suggested that Toll-like receptor 2 (TLR2), an

extracellular member of the TLR family, plays an important role in the immune recognition of CoNS [76]. Additionally, *S. epidermidis* induces an upregulation of TLR2 and MyD88, and a systemic increase in proinflammatory cytokines (e.g., interleukin (IL)-6) [77]. As inflammatory stimuli, the PAMP produced by the gram-positive CoNS are less potent than PAMP expressed at the surface of gram-negative bacteria (e.g., lipopolysaccharide, LPS). However, the most prevalent clinical isolate of CoNS, *S. epidermidis*, is known to produce a complex of bacterial peptides called phenol-soluble modulins, which induce a considerable proinflammatory response through TLR2 [78–80]. Interestingly, activation of TLR2 by a yet unidentified product of *S. epidermidis* triggers the enhanced production of the human AMP family of $\beta$-defensins from keratinocytes and underscores a potential role of AMP in the control of staphylococcal infections [81, 82]. Reliance on TLR-induced CoNS immunity has important implications, since preterm neonates exhibit marked defects in TLR signaling cascades and cytokine responses [39, 83]. Indeed, monocytes of premature neonates display a gestational age-dependent reduction in TLR-induced production of proinflammatory cytokines [84], whereas other monocyte functions related to phagocytosis and intracellular bacterial killing develop earlier, well before 30 weeks of gestation [85].

*2.2. Bacterial Virulence Factors.* CoNS lacks several of the virulence factors shared with the closely related species *S. aureus* [31]. Compared with *S. aureus, S. epidermidis* produces lower levels of cytolytic toxins [86]. Therefore, *S. epidermidis* must rely on other mechanisms, such as biofilms and the anionic polymer poly-$\gamma$-DL-glutamic acid (PGA) to evade hosts' immune responses.

Biofilm formation serves as the primary mode of immune evasion of CoNS [87]. These multilayered bacterial aggregates strongly adhere to inanimate objects such as indwelling medical devices. CoNS are particularly adept at biofilm formation, and this capacity is a key mechanism of their pathogenesis, particularly in relation to catheter-related infections [88, 89]. Biofilms act as nonselective physical barriers that obstruct antibiotic diffusion and hinder the cellular and humoral host immune responses [86, 90–93]. In addition, biofilms provide protection from antimicrobial therapy [30, 31, 94, 95]. Poly-*N*-acetylglucosamine surface polysaccharide, also termed polysaccharide intercellular adhesin (PIA), is crucial in facilitating cellular aggregation during biofilm formation and is the most extensively studied biofilm molecule [31, 90]. In rat models, PIA defective mutants have been shown to exhibit decreased virulence [31]. Lack of PIA in *S. epidermidis* results in mutants susceptible to phagocytosis and killing by human neutrophils as well as enhanced AMP susceptibility [92]. Additionally, the expression of an ATP-binding cassette transporter allows for the export of AMP out of the bacterial cell, thus contributing to AMP resistance [86, 96]. Other components help CoNS evade immune defenses; for example, a glutamyl endopeptidase from *S. epidermidis* is expressed specifically in biofilms and degrades the complement-derived chemoattractant C35 [31].

The secreted anionic extracellular polymer PGA also plays an important role in immune evasion of *S. epidermidis* [91]. However, PGA is not specific to *S. epidermidis*, and is also secreted by other staphylococcal species and *Bacillus* strains [91, 97]. PGA appears to play an important role in the persistence of *S. epidermidis* colonization on medical devices [91]. Moreover, PGA contributes to resistance against phagocytosis and microbicidal action of AMP like LL-37 and human $\beta$-defensin 3, as demonstrated by increased susceptibility to neutrophils and AMP activity; however, the precise mechanisms of this PGA-mediated resistance remain unclear [91]. To avoid antistaphylococcal human AMP, *S. epidermidis* is also equipped with resistance mechanisms such as the Aps (antimicrobial peptide sensing) system and the AMP-degrading protease SepA [86, 96].

Finally, bacteria have multiple creative antibiotic resistance mechanisms, including modification of target structures (e.g., altered penicillin-binding proteins in staphylococci) and production of antibiotic-inactivating enzymes (e.g., beta-lactamases to hydrolyze penicillins, cephalosporins and/or carbapenems). Genes encoding proteins responsible for these mechanisms often reside on mobile genetic elements, enabling transfer of resistance between bacteria of the same or different species**.** In a recent study, authors proposed that CoNS may be a significant reservoir of methicillin resistance genes that can be transferred horizontally to other common related neonatal pathogens such as *S. aureus* [98].

## 3. Diagnosis

Neonatal sepsis is clinically diagnosed by a combination of clinical signs, nonspecific laboratory tests and microbiologically confirmed by detection of bacteria in blood by culture. Clinical signs of sepsis in neonates are usually nonspecific and often inconspicuous. They include the presence of fever or hypothermia (in the preterm neonate, this is more commonly seen as a general disturbance in thermoregulation); lethargy; poor feeding; respiratory distress or apnea; pallor; jaundice; tachycardia or bradycardia; hypotension; disturbances in gastrointestinal function (diarrhea, bloody stools, abdominal distention, and ileus); and thrombocytopenia [30, 31, 99, 100]. With CoNS, such clinical signs are often more subtle because of the low virulence of these organisms. However more serious, often persistent illness due to more virulent strains can occur in a considerable minority of cases, in association with severe thrombocytopenia [101].

The gold standard for diagnosis of neonatal sepsis remains blood culture. However, in many situations this test is fraught with practical problems, including the small blood volumes obtainable, especially in the smallest of preterm neonates. Indeed, this volume is often below the recommended 1 mL lower limit of detection, leading to a high proportion of false negative test results [102–105]. Conversely, the nonspecific nature of clinical signs in neonates probably leads to frequent overuse of broad-spectrum antibiotics with the potential to select for resistant bacteria and fungi, especially in preterm neonates. Therefore, there is a great need for better rapid diagnostic tests to differentiate infants with sepsis from those who are sick from other causes.

Hematological indices (e.g., numbers of white blood cells, neutrophils, platelets) [106] and biochemical markers of

inflammation, such as C-reactive protein [107], and procalcitonin [108] are routinely used in clinical practice and can aid in the diagnosis of neonatal sepsis. This is particularly useful in cases of persisting clinical symptoms and in the absence of a confirmatory positive blood culture, or in situations where localized sources of infection are being considered [105]. Furthermore, the abundance of CoNS as a natural skin commensal often leads to blood culture contamination and a subsequent overestimation of neonatal sepsis cases [109, 110].

CVC, which are often used in smallest preterm neonates, provide a sanctuary for CoNS, leading to persistence of an infection. A number of methods to determine if the CVC is the source of an infection have been suggested, including observing a positive culture from the CVC but not from a peripheral site [111, 112] and reduced "time to positivity" of a CVC culture (as opposed to a peripheral site). A higher bacterial load in the CVC [113, 114] and a three- to fivefold differential magnitude of colony-forming units between a quantitative CVC and peripheral culture are indicative of a CVC as the primary focus of infection [99, 115]. However, these methods are impractical when applied to neonates. The small lumen size of the CVC makes removal of blood, and therefore CVC culture, impossible in most cases. Furthermore, any comparison of CVC and peripheral cultures would rely on identical sample volumes from both sites being taken and processed at exactly the same time, which is often not feasible.

In the future, new diagnostic technologies involving microfluidics may considerably reduce the amount of blood volumes required for diagnosis [116]. At present, the relatively high cost of this technique limits its routine use in the clinical setting [117]. In some instances, polymerase chain reaction (PCR) can be useful to characterize subspecies [118, 119]. Adjunctive use of nucleic acid-based technologies with blood cultures can facilitate a faster diagnostic turnaround time and easier antibiotic susceptibility profile identification. Molecular typing techniques, such as pulsed field gel electrophoresis and multilocus sequence typing [99], are also useful in subspecies differentiation [120]. PCR-based diagnostic methods may be most useful clinically in the short term by providing clinicians with the ability to detect the presence of genetic markers of antibiotic resistance [118].

## 4. Prevention and Treatment

*4.1. Prevention.* In the hospital setting, the mainstay of prevention against neonatal sepsis includes strict hand-washing practices; careful aseptic procedures in the management of intravenous lines; skin care; judicious use of antibiotics; promoting early enteral (as opposed to parenteral) nutrition, preferably using breast milk (i.e., to enhance the infant's own gastrointestinal immune defenses); and minimizing invasive interventions (e.g., prompt removal of CVCs, reducing mechanical ventilation) [7, 121–123]. Hand washing is a widely accepted and cost-effective measure to decrease the occurrence of nosocomial infections including CoNS [15, 124–127]; yet universal compliance is difficult to achieve [3]. Minimizing the indwelling time and number of CVCs decreases the risk of CoNS and other pathogens of LONS [15, 128]. In some studies, more than half of all cases of CoNS sepsis occurred while indwelling CVCs were in place [129]. The number of central lines experienced by the neonate from birth, rather than the duration of insertion, was an important predictor of CoNS sepsis [28]. Some authors have proposed the use of prophylactic antibiotics immediately before and for 12 hours after removal of a CVC in preterm neonates [129].

Clinical trials of vancomycin added to parenteral nutrition solutions have demonstrated decreases in the incidence of CoNS sepsis in preterm neonates [130, 131], without reduction in mortality or duration of hospital stay [132]. Others have proposed using antibiotic-coated devices for CVC [133–135]. However, these measures carry a risk of increasing antimicrobial resistance and have not been universally adopted. Antimicrobial "locks," that is, leaving a microbicidal substance within the catheters in between administration of other drugs represents another proposed solution to decrease bacterial colonization. Antiseptics (e.g., alcohol, taurolidine), anticoagulants (e.g., heparin, EDTA), and antibiotics (e.g., vancomycin, rifamycin) have all been studied [105, 136–139]. Two studies reported a reduction in catheter-related sepsis in critically ill neonates through the use of either fusidic acid and heparin, or vancomycin locks [140, 141]. The benefit of antibiotic lock over prophylactic antibiotic administration is the avoidance of systemic effects of antibiotics in the patient, since the solution remains within the catheter. A similar measure incorporates antiseptic-impregnated catheters to decrease cutaneous bacterial load and catheter colonization [134, 135, 139, 142]. However, clinical experience with these methods is very limited in VLBW infants. In the absence of more definitive evidence, the standard of care is to use strict hand hygiene and skin antisepsis protocols prior to, during, and after catheter insertion [8, 143].

*4.2. Treatment.* The subtle, nonspecific nature of clinical signs and the rapid progression of neonatal sepsis make prompt diagnosis and antibiotic treatment crucial. Any delay in antimicrobial therapy places a neonate with sepsis at greater risk of mortality. Empirical antibiotic therapy should be based on knowledge of local epidemiology and antibiotic resistance patterns of neonatal sepsis, since geographic variation can be influential. Because colonization of infants with CoNS is unusual in the first 48 hours after birth, the preferred empirical treatment of EONS is mainly based on the use of ampicillin and gentamicin to cover more predominant GBS and gram-negative bacilli, and, to a lesser extent, *L. monocytogenes*. For LONS, administration of antistaphylococcal penicillin (e.g., oxacillin) or an alternative agent such as vancomycin is indicated. The advantage of a penicillin is the low toxicity and potent *in vivo* bactericidal activity, even in difficult infections such as endocarditis [30, 31]. In areas with widespread beta-lactam resistance in CoNS and/or a high prevalence of methicillin-resistant *S. aureus*, vancomycin is often preferred [144]. Although not as bactericidal as oxacillin, little resistance has been reported to vancomycin. Considerable rates of gram-negative organisms in LONS dictate that empirical treatment cannot consist solely of antistaphylococcal antibiotics. Therefore, aminoglycosides are frequently used in addition, as in EONS,

and may have a synergistic antistaphylococcal effect when administered with penicillins and vancomycin, although the *in vivo* significance of this is not entirely clear [145–147]. Linezolid, another class of antibiotics, possesses potent antistaphylococcal activity comparable to that of vancomycin, with little reported resistance [148, 149]. Once culture results are available, antibiotics can be modified to specifically target the isolated pathogen according to the results of susceptibility testing.

The presence of a CVC or other indwelling foreign material is highly associated with persistence of infection despite appropriate antibiotic therapy, because of biofilm formation. *In vivo* antibiotic action is also antagonized by the neutralization of pharmaceuticals like vancomycin by the polysaccharides of CoNS biofilms [150]. In addition, the low metabolic activity of biofilms limits the activity of many antibiotics which require rapid metabolism of growing bacteria to exert their microbicidal effect [151]. Antibiotic resistance and biofilm formation are among selective factors for the persistence of endemic nosocomial strains and probably contribute to the predominance of *S. epidermidis* and *S. haemolyticus* as clinical isolates on NICU infants [37, 100, 152]. In such cases, it may be imperative to remove the CVC.

## 5. Long-Term Sequelae

Multiple studies show that neonatal sepsis has major long-term neurodevelopmental consequences in survivors, particularly in preterm infants [153]. In modern intensive care, about half of extremely preterm neonates born at 24 weeks' gestation and the majority of neonates over 25 weeks' gestation generally survive [154]. The risk of such morbidity in extremely premature neonates is inversely proportional to their gestational age [4, 155–158]. In VLBW infants, neonatal sepsis dramatically increases the long-term risk of motor, cognitive, neurosensory and visual impairments [157–159]. The risk of adverse neurodevelopmental outcome in VLBW neonates with sepsis is further increased with other comorbidities such as bronchopulmonary dysplasia [157, 160]. This increased risk of neurodevelopmental impairment in preterm infants with sepsis has several reasons, including a high risk of meningitis; heightened adverse effect of sepsis-associated cardiovascular instability during a vulnerable period for the developing brain; and increased neurotoxic effects of inflammatory mediators [153]. Surprisingly, the risk of adverse neurodevelopmental outcome in VLBW infants surviving from neonatal sepsis does not appear to depend on the infecting organism [157], although in some studies extremely premature infants who experienced sepsis had a greater risk of a hearing impairment when the infection involved gram-negative, fungal, or combined infections [157].

## 6. Future Therapies

Despite limited natural antibody immune protection in preterm neonates, meta-analyses of intravenous immunoglobulin administration have so far failed to demonstrate sufficient therapeutic benefits [31, 161–163]. Other immunomodulatory therapies designed to improve neonatal immune deficits, such as granulocyte transfusions, or administration of granulocyte-macrophage colony stimulating factor which increases neutrophils and enhances their antimicrobial activity, have also not yet translated into concrete benefits in clinical trials [161]. Finally, lactoferrin, an antimicrobial glycoprotein that sequesters iron, may be useful in reducing the incidence of late-onset sepsis in low-birth-weight neonates [164]. The future of antistaphylococcal immunotherapy and immunoprophylaxis requires more research. This may require a combined use of adjunctive immunomodulatory treatments to enhance the innate immune system of neonates while disabling virulence factors that enable resistance to conventional antibiotic treatment of CoNS.

## 7. Conclusion

The 20th century saw CoNS emerge as the foremost pathogen of neonatal sepsis in developed countries. VLBW neonates contribute disproportionately to CoNS-related morbidity and mortality, in stark contrast to their full-term counterparts who usually suffer milder symptoms. Several reasons make prematurity the single most important factor for neonatal sepsis: innate immunological deficiencies; prolonged stays in the NICU; and, notably, the higher use of indispensable but invasive medical interventions in these developmentally immature neonates. Advances in medical technology have dramatically increased the survival rate of premature neonates. This corresponds to a growing burden of both short- and long-term problems associated with neonatal sepsis. Effective prophylactic measures, prompt and accurate diagnoses, and subsequent administration of targeted therapy are vital to curb the excessive burden of disease that CoNS infection imposes upon this highly vulnerable age group.

## Authors' Contribution

E. A. Marchant and G. K. Boyce contributed equally to this paper.

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*Review Article*

# Immune Vulnerability of Infants to Tuberculosis

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One of the challenges faced by the infant immune system is learning to distinguish the myriad of foreign but nonthreatening antigens encountered from those expressed by true pathogens. This balance is reflected in the diminished production of proinflammatory cytokines by both innate and adaptive immune cells in the infant. A downside of this bias is that several factors critical for controlling *Mycobacterium tuberculosis* infection are significantly restricted in infants, including TNF, IL-1, and IL-12. Furthermore, infant T cells are inherently less capable of differentiating into IFN-$\gamma$-producing T cells. As a result, infected infants are 5–10 times more likely than adults to develop active tuberculosis (TB) and have higher rates of severe disseminated disease, including miliary TB and meningitis. Infant TB is a fundamentally different disease than TB in immune competent adults. Immunotherapeutics, therefore, should be specifically evaluated in infants before they are routinely employed to treat TB in this age group. Modalities aimed at reducing inflammation, which may be beneficial for adjunctive therapy of some forms of TB in older children and adults, may be of no benefit or even harmful in infants who manifest much less inflammatory disease.

## 1. Introduction

It is believed that one third of the world population is infected with *Mycobacterium tuberculosis* (*M.tb*) [1]. However, the majority of adults have immune systems capable of containing *M.tb* without developing active disease even if they are unable to completely eradicate the organism from their bodies. These individuals are said to be latently infected although "latent infection" is now appreciated to be a highly dynamic state. Latently infected individuals are asymptomatic, but harbor a 5–10% lifetime risk of developing active disease [2]. Of those individuals who eventually progress to active tuberculosis (TB), approximately half will do so within 2 years of acquiring the infection [3] although reactivation can occur even 30 or more years after primary infection [4]. In endemic countries, TB acquired later in life is more often due to reinfection with another *M.tb* strain than to reactivation of latent infection [5, 6]. Overall, no bacterial organism in the world claims more casualties than *M.tb*, with an estimated 8.7 million new cases and 1.4 million deaths in 2011 [7].

The number of pediatric deaths attributable to TB is harder to estimate because TB in children is difficult to diagnose, especially in resource-limited settings carrying the greatest burden of disease [8]. This is likely the explanation why UNICEF does not include TB in its under-five mortality reports [9]. It is apparent from natural history of disease studies conducted in the prechemotherapy era that *M.tb*-infected infants (children less than one year of age) are at much greater

risk for progression to active TB than are immunocompetent adults. A recent nosocomial TB outbreak in a Kangaroo care unit (where mothers nurse their premature babies) in Cape Town, South Africa confirmed their vulnerability [10]. Four out of six newborns developed pulmonary TB within 6 months after spending multiple days in the same room as a mother with undiagnosed pulmonary TB. Three of the four children had extensive disease at the time of diagnosis. In the absence of preventive measures ~50% of infants, even those delivered at term, developed active TB after infection [11].

In addition to their increased rate of progression to TB, infants are more likely to develop severe disseminated forms of TB associated with high morbidity and mortality. Before the availability of TB treatment, mortality varied from 55% in infants less than 6 months of age to 30% in those 1 to 2 years of age [12], and the death rate remains higher in infants than in other age groups even in the era of TB chemotherapy. Most lethal is congenital infection, which can occur either in utero or during birth; about one third of congenitally-infected children do not survive [13]. Fortunately, congenital TB infection is relatively rare; Schaaf reported that congenital TB constituted 1% of the childhood TB caseload in Tygerberg Children's Hospital in Cape Town [13]. More commonly, infants are infected postnatally; 8% of the pediatric TB caseload in Schaaf's report was comprised of such cases. Infants whose mothers have active TB are particularly at risk. The outcome of isolated pulmonary TB in infants is usually good if treatment is started early [13, 14], but they have increased susceptibility to disseminated (miliary) TB. Furthermore, TB meningitis becomes increasingly problematic after the first months of life. It has been estimated that, without prophylaxis, 30–40% of infants develop pulmonary disease following exposure, and a further 10–20% develop TB meningitis or disseminated (miliary) disease [11], which has near universal mortality in this age group [15, 16]. Most children who survive TB meningitis suffer from long-term sequelae including mental, motor, vision, and hearing impairment [17–19].

This paper provides an overview of immune factors that are likely to underlie the TB vulnerability of infants. Data from human infant studies will be discussed when available, and data from animal models will be utilized to provide deeper mechanistic insights. As the human immune system exhibits profound heterogeneity, probably only a subset of genetically susceptible individuals develop active TB. However, because of the age-specific maturation of the immune response, discussed further in this review, a larger proportion of infants seem to be susceptible. Environmental factors such as BCG vaccination and exposure to environmental mycobacteria may shape the infant immune response to *M.tb*, but knowledge in this area is limited, and we will restrict our discussion to factors intrinsic to the infant immune response. General reviews of immunity against *M.tb* and the immune status of infants are available [20–22]; we focus specifically on the intersection of these areas.

## 2. Immune Vulnerability of Infants to TB

The infant's immune system is shaped by past and present challenges encountered during life in the uterus and in the brave new postnatal world. Within the womb, detrimental immune responses between the mother and fetus are prevented by an intrauterine environment that restricts the development of cells that promote proinflammatory responses [23–25]. Birth represents a great transition from the sterile environment of the womb to a world full of bacteria; colonization of the newborn starts at the time of delivery. Several recent reviews describe the synergy between humans and bacteria [26–30], and the growing consensus is that the microbiome represents an essential part of our immune defense. The magnitude of colonization is impressive; a human adult is colonized with one kilogram of bacteria and in order for this beneficial process to occur, the immune system has to be tuned to allow it. The downside of this choice of nature is an infant's immune system that is susceptible to infections, and TB is arguably foremost among these. In the following section, we will first summarize the role of each relevant molecule or cell type in the immune response to TB, and then discuss how alterations during infancy may shape this response (summarized in Table 1).

### 2.1. Macrophages

*2.1.1. Role in TB.* *M.tb* is transmitted by airborne particles when a person with pulmonary TB coughs [31]. Infection probably occurs in the distal alveoli where the bacteria are first ingested by alveolar macrophages. Macrophages have been shown to play a central role in the phagocytosis, growth arrest, and intracellular killing of *M.tb*. Combination of receptors and corresponding ligands is utilized for phagocytic entry into macrophages, and it has been hypothesized that the fate of *M.tb* depends on the initial receptors involved in the process [32, 33]. Once inside the phagosome, delivery of bactericidal lysosomal contents to the compartment will lead to the demise of the bacterium within. To avoid this fate, *M.tb* has the capacity to sustain the phagosome in its early, immature state [34, 35]. In addition to modulating the phagosome, recent studies suggest that *M.tb* can evade it all together by escaping into the cytoplasm [36, 37]. Ultimately, to kill or at least curb *M.tb* replication, macrophages must be activated by TNF and IFN-$\gamma$ derived from antigen-specific T cells and perhaps innate cells. As the first responder to *M.tb* infection, the response of the alveolar macrophage is critical, not only for directly controlling TB, but also in setting the stage for the subsequent innate and adaptive immune responses [38]. Although T cell-mediated immunity is essential for immune control during later stages of disease, *M.tb*-specific T cells operate in large part by activating and equipping macrophages to control intracellular bacteria. Mouse studies have shown that during later stages of disease, monocyte-derived lung macrophage populations harbor a large percentage of *M.tb* ([39] and K. Urdahl, unpublished data).

*2.1.2. Status in Infants.* Although the concentration of blood monocytes (precursors to tissue macrophages) in the fetus and neonate is on par with that in adults [40, 41], autopsy studies have revealed that full-term infants have very few detectable alveolar macrophages at birth [42]. In monkeys, these numbers increase to adult levels rapidly, within 1-2 days

Table 1: Key elements of the immune response to TB with their activity in infants.

| | Role in TB | Relative activity in infants |
|---|---|---|
| Macrophages | Intracellular *M.tb* killing and growth arrest [38, 210, 211]; alveolar macrophages initiate innate response [212] | Diminished chemotaxis [45] and intracellular killing [56]; reduced numbers of alveolar macrophages [40] |
| Neutrophils | Possible role in intracellular *M.tb* killing [62, 213–215]; promote T cell priming by facilitating *M.tb* uptake by DCs [63] | Diminished chemotaxis [65] and intracellular killing [64]; limited neutrophil storage pool [64] |
| Dendritic cells | Primary producers of IL-1 and IL-12 [73, 74]; initiate, regulate, and maintain T cell responses [39, 75–77, 216] | Low circulating number [78]; diminished capacity to produce TNF, IL-1, and IL-12 [80, 96, 217]; diminished capacity for priming Th1 cells |
| Cell death pathways | Regulate *M.tb* replication and dissemination [20, 21, 200, 218, 219] | Unknown, but necrotizing granulomas are unusual in infants |
| Pattern-recognition receptors | Phagocyte activation [88–90]; trigger cytokine production including TNF, IL-1, IL-12, and IL-10 [95] | Similar expression [98, 99], but altered signaling [100]; reduced triggering of proinflammatory and increased anti-inflammatory cytokines [97, 220] |
| TNF | Macrophage activation [20, 21, 103]; promotes immune cell recruitment [105, 106]; regulates cell death pathways [108, 109, 221] | Reduced levels and production capacity [68, 96, 110] |
| IL-1 | Intracellular *M.tb* killing and/or growth arrest [118, 119]; T cell costimulation [125] | Reduced levels and production capacity [96, 222, 223] |
| IL-12 | Induction and maintenance of IFN-$\gamma$ producing T cells [20, 74, 154, 224, 225] | Reduced levels and production capacity [80, 139, 140] |
| IL-10 | Restricts Th1 development and impairs IFN-$\gamma$-mediated signal transduction [133–135] | Increased levels and production capacity [96, 110, 140] |
| Antimicrobial peptides | Direct mycobactericidal activity [142, 143, 226] | Reduced levels and production capacity [152] |
| $CD4^+$ T cells | Primary cellular source of IFN-$\gamma$ and other factors that equip macrophages to restrict intracellular *M.tb* replication [20, 154, 227, 228]; provide help to maintain $CD8^+$ effector T cells [169] | Bias against Th1 differentiation [172] and for Th2 and Treg induction [176] |
| $CD8^+$ T cells | Cytolysis and production of IFN-$\gamma$ [155, 190, 227] | Diminished IFN-$\gamma$ production [193] and cytolytic function; bias towards short-lived effectors [192] |
| Delayed adaptive immune response | Facilitates prolonged mycobacterial replication and niche establishment in the lung [20, 196] | Infant immune status could even further delay the response |

after birth [43], and cell recoveries from neonatal bronchoalveolar lavages suggest that a similar rapid increase takes place in human newborns [44]. Furthermore, chemotaxis of neonatal blood monocytes is slower than that of adult monocytes, which may further exacerbate the response to infection when lung resident macrophage numbers are low [45]. In addition to absolute numbers, the function of alveolar macrophages seems to be diminished in newborns. While neonatal blood monocytes demonstrate good phagocytic and microbicidal activity to a range of pathogens, neonatal alveolar macrophages perform these functions poorly [46–55]. For intracellular killing of *M.tb*, the ability to produce superoxide anion in response to IFN-$\gamma$ is particularly important. Neonatal macrophages have a reduced capacity to perform this function, apparently due to decreased IFN-$\gamma$ receptor signaling and diminished STAT-1 phosphorylation, despite comparable IFN-$\gamma$ receptor expression [56]. The extent to which the paucity of alveolar macrophages and their limited functional capacity compromises immunity against TB during early infancy is unknown and deserves further study.

### 2.2. Neutrophils

*2.2.1. Role in TB.* In the mouse model, neutrophils represent a transiently dominant population of infected lung cells in the second week of pulmonary TB infection [57], suggesting that neutrophils are the major population of lung phagocytes to acquire *M.tb* immediately after bacilli escape from alveolar macrophages. Whether or not neutrophils contribute to immunity by directly killing *M.tb* has not been well established. However, patients with chronic granulomatous disease that have impaired neutrophilic intracellular killing are susceptible to mycobacterial infections [58–61]. This correlation suggests that neutrophils may have a direct role in lysing *M.tb*, and a recent report in the zebrafish model of TB supports this idea [62]. Interestingly, in the mouse

model, apoptotic neutrophils were recently shown to facilitate the uptake of *M.tb* by dendritic cells (DCs)—the third major phagocytic population to acquire *M.tb* in the lung [63]. Thus, neutrophils appear to be an important bridge between innate and adaptive immunity, as the acquisition of *M.tb* by DCs is essential for the initiation of the T cell response. Elimination of neutrophils slowed both DC acquisition of bacteria and *M.tb*-specific T cell priming [63].

*2.2.2. Status in Infants.* Neonates, and in particular preterm neonates, have a decreased capacity to mobilize neutrophils in response to infection. This deficiency is in large part due to a limited neutrophil storage pool [64]. Further, neonatal neutrophils express low levels of integrins and selectins and perform poorly in functional assays for chemotaxis, rolling adhesion, transmigration, and lamellipodia formation, all of which are crucial for timely neutrophil recruitment to the site of infection [65–67]. The continuous production by neonatal monocytes of IL-6 (an effective neutrophil migration inhibitor) further depresses neutrophil function [68, 69]. Even the weaponry neutrophils have against microbial invaders is restricted, illustrated by reduced amounts of lactoferrin and diminished oxidase activity [64]. Although more investigation into the role of neutrophils in TB is needed, the profound deficiencies in neutrophil number and function during infancy have the potential to impair both innate and adaptive immunity to TB.

### 2.3. Dendritic Cells

*2.3.1. Role in TB.* DCs comprise the third major phagocytic population to acquire *M.tb* in the lung. However, in contrast to neutrophils, the predominance of *M.tb*-infected DCs is long lived. In mice, *M.tb*-infected DCs first appear during the second week of infection and are abundant throughout the course of disease [39, 70]. In humans, DCs are also abundant in the tuberculous granuloma [71, 72]. Although the extent to which they kill or restrict intracellular growth of *M.tb* is unknown, mouse studies have shown that DCs play a critical role in the innate response to *M.tb* by producing cytokines essential for host defense. These cytokines include TNF, IL-1, and IL-12, and DCs are primary producers of the latter two [73, 74]. DCs are the consummate professional antigen-presenting cells and are essential for initiating and regulating the T cell response. To trigger *M.tb*-specific T cell responses, migratory DCs must first acquire *M.tb* in the lung, traffic to the lung draining lymph node, and present *M.tb* antigens to naïve antigen-specific T cells in the lymph node [39, 75–77]. DC populations that reside in lung granulomas are likely to be important for maintaining and regulating *M.tb*-specific T cells in the lung although this is less well studied than their role in the lymph node.

*2.3.2. Status in Infants.* Although the number of resident DCs in the infant lung is unknown, neonates appear to have fewer circulating DCs than adults [78]. Furthermore, since most DCs in the lung that respond to *M.tb* infection are derived from blood monocytes, which have a diminished capacity for chemotaxis in neonates [45], it seems likely that the number of DCs responding to TB in the lung is lower in infants than in adults. As will be discussed further in a forthcoming section on pattern-recognition receptors in TB, infant DCs (as well as macrophages) also produce substantially lower levels of proinflammatory cytokines, including TNF, IL-1, and IL-12. The critical role of each of these cytokines in immunity against TB will be discussed in greater detail below. Studies examining the potential of neonatal DCs to process and present antigen to T cells provide conflicting results [22]. Although adult and neonatal blood DCs express similar cell surface levels of the MHC class II molecule HLA-DR as well as the costimulatory molecules CD40 and CD80, expression by neonatal DCs increases less in response to stimulation via toll-like receptors (TLRs) [79]. The diminished capacity of neonatal DCs to produce IL-12 may restrict their ability to prime Th1 cells, at least in some settings [22, 80]. As will be further discussed below, priming a rapid and robust Th1 response in the lung appears to be essential for immune control.

### 2.4. Cell Death Pathways

*2.4.1. Role in TB.* Another way that *M.tb* shapes innate immunity is by manipulating macrophage death pathways. Necrosis and apoptosis can be observed simultaneously in tuberculous granulomas. Furthermore, both can be promoted by host and mycobacterial factors, and both can either promote or restrict bacterial replication, depending on the circumstances [20, 21]. During the early innate immune response, direct visualization of infected macrophages in the zebrafish embryo has shown that *M.tb* may orchestrate its own dissemination by inducing apoptosis through expression of virulence genes encoded by the RD1 region [81, 82]. The inflammatory response induced by *M.tb* attracts more macrophages that engulf apoptotic debris containing bacilli, and further intracellular bacterial replication ensues. Furthermore, some of the infected macrophages migrate to new sites where they attract additional uninfected macrophages and serve as niduses for new granuloma formation [81]. *M.tb* can also induce macrophage death by necrosis in some settings, for example through the induction of lipoxins and anti-inflammatory eicosanoids that suppress TNF production [21, 83]. One consequence of necrotic cell death is the release of extracellular mycobacteria, which are capable of even more exuberant growth in the extracellular milieu than they are within macrophages [20, 21]. Therefore, while apoptosis facilitates bacterial dissemination and necrosis promotes unchecked extracellular mycobacterial growth, optimal control of *M.tb* probably involves curbing mycobacterial replication within macrophages in a manner that does not induce cell death.

*2.4.2. Status in Infants.* Infants do not usually form lung cavities with an abundance of extracellular bacteria as observed in adolescent and adult patients [11, 13]. Interestingly, lung cavities are occasionally seen in the absence of effective immune containment, in contrast to adolescents and adults where destructive immune responses contribute to cavity formation [84]. Future studies are needed to elucidate the

mechanistic underpinnings of the scarcity of cavities in young children, and the possibility that macrophage cell death pathways are altered during infancy seems ripe for exploration. For example, diminished TNF-driven necrosis as a result of low TNF production by both innate and adaptive immune cells may provide a partial explanation. The fact that HIV-infected adults exhibit a similar phenotype [85–87] suggests that diminished T cell function (as discussed below) could be a contributing factor. Another intriguing hypothesis is that infant macrophages have a higher intrinsic predilection for apoptosis, a possibility that requires further investigation.

### 2.5. Pattern-Recognition Receptor Signaling

*2.5.1. Role in TB.* *M.tb* components are recognized through multiple pattern–recognition receptors that trigger an inflammatory response. Important receptors include TLRs, cytosolic NOD like receptors (NLRs), C-type lectin receptors, and DC-SIGN [88–90]. Among the TLR family, TLR2, TLR4, and TLR9 play the most prominent roles in innate immunity to TB [90, 91]. TLR2 forms heterodimers with TLR1 or TLR6 and has been implicated in recognition of multiple mycobacterial cell wall glycolipids. TLR4 is activated by mycobacterial heat shock protein 60/65, whereas TLR9 recognizes unmethylated CpG motifs in bacterial DNA. Critical downstream cytokines induced by TLR- and NLR-mediated signals include the cytokines TNF, IL-1, and IL-12. Some pattern-recognition receptors also elicit an anti-inflammatory response in response to *M.tb*. The mannose receptor is a C-type lectin receptor expressed at high levels on alveolar macrophages. Man-LAM and other major components of the mycobacterial cell wall are natural ligands for the mannose receptor and their recognition suppresses IL-12 production [92–94]. DC-SIGN is expressed primarily on DCs. Engagement of DC-SIGN by mycobacterial components (including Man-LAM) in the presence of simultaneous TLR stimulation promotes an anti-inflammatory response, including IL-10 production [95].

*2.5.2. Status in Infants.* Compared to adult myeloid cells, neonatal macrophages and DCs exhibit an altered pattern of TLR-mediated cytokine production, with decreased amounts of proinflammatory cytokines including TNF, IL-1, IL-6, and IL-12 and increased amounts of the anti-inflammatory cytokine IL-10 [96, 97]. Rather than simply reflecting an immature immune system, this probably represents a coping strategy to deal with the massive bacterial colonization that takes place during infancy, offering a means to avert a cytokine storm that might otherwise pose a serious inflammatory threat. The molecular mechanisms underlying this altered signaling are poorly understood, and in most cases, it is not clear if the altered responses are intrinsic to the signaling pathways or a result of suppression by extrinsic factors. Immune cells in human cord blood seem to express the same quantity of TLRs and downstream signaling molecules as those found in the peripheral blood of adults [98–100]. Reduced production of IL-12 in neonates has been correlated with the instability of a transcriptional complex that restricts induction of the IL-12p35 subunit [101]. On the other hand, extrinsic suppressive factors may play a role; for example, newborn plasma contains high concentrations of adenosine, which in turn causes high intracellular cAMP levels, that may enforce a bias against proinflammatory cytokine responses [96]. The distinct roles in the immune response to TB played by TNF, IL-1, IL-12, and IL-10, each differentially regulated in infants, will be discussed below. It is important to note that IL-6 also contributes to TB protection and exhibits altered expression levels during infancy [96, 97, 102]. Thus, although IL-6 may also play a role in the heightened vulnerability of infants to TB, it will not be discussed further in this review because little is currently known about its mechanistic mode of action during TB.

### 2.6. Tumor Necrosis Factor

*2.6.1. Role in TB.* Central in the innate immune response by macrophages and DCs is their production of the inflammatory cytokine TNF, which plays multiple roles in immunity against TB [20–22]. Although the critical role of TNF was discovered in the mouse model of TB [103], its importance in human TB was subsequently verified by the finding that TNF blockade causes TB reactivation in latently infected individuals [104]. In addition to its role in activating macrophages to control intracellular *M.tb*, TNF is also important for chemokine production and recruitment of immune cells to the granuloma [105, 106]. Interestingly, TB disease in adolescents and adults is promoted in individuals genetically predisposed to produce either low or high amounts of TNF, whereas immune control is promoted by the production of intermediate TNF levels [107]. This confounding observation has been at least partially explained by the finding that both low and high TNF states lead to necrotic cell death of infected macrophages and enhanced extracellular bacillary growth, whereas intermediate TNF levels can curb intracellular bacterial growth without triggering macrophage necrosis [108, 109]. Importantly, corticosteroids that are routinely given in conjunction with antibiotics to all patients with TB meningitis, in an attempt to reduce inflammation-related sequelae, have recently been shown to benefit only those TB patients predisposed to overproduce TNF. In TB meningitis patients who produce low amounts of TNF, corticosteroids are not helpful and may even be detrimental [108].

*2.6.2. Status in Infants.* TNF production by human neonatal macrophages and DCs is greatly diminished. Although the capacity to produce TNF gradually improves with age, adult levels of TNF production are not achieved until after one year of age [68, 96, 110]. While adolescents and adults may develop TB disease associated with exacerbated TNF production [107], it seems likely that most TB disease in infants is associated with a failure to produce enough TNF. This raises the question as to whether adjunctive steroid therapy should be given to infants with TB meningitis, because steroid therapy in adolescents and adults only benefits those with a propensity to produce high amounts of TNF [108]. Although several studies have demonstrated a benefit for steroids in TB

meningitis in older children, adolescents, and adults [111], its utility in infants with TB meningitis has not been specifically addressed in a prospective trial. There is an urgent need to perform such a study because steroids are now routinely administered for all cases of TB meningitis, regardless of age. Given the inherent deficiency in TNF production in infants, it is plausible that infants might receive no benefit from this therapy, and may even be harmed.

### 2.7. Role of Other Cytokines Altered during Infancy

*2.7.1. IL-1.* The finding that mice lacking MyD88 (the signaling adaptor molecule utilized by most membrane-bound TLR) are extremely susceptible to TB was originally interpreted to reflect the critical role of TLR for innate recognition of *M.tb* [112–114]. Mice lacking individual or combinations of specific TLR, however, exhibited much more subtle susceptibility phenotypes [115–117], probably reflecting the redundancy of pattern-recognition receptors that operate in TB. A role for IL-1 was then considered, because MyD88 also serves as the adapter protein for IL-1 receptor signaling, and TB susceptibility of mice lacking the IL-1 receptor is essentially identical to that of mice lacking MyD88 [118, 119]. Thus, IL-1 receptor signaling is sufficient to explain the requirement of MyD88 for TB resistance. Subsequent studies have implicated both IL-1 receptor-binding members of the IL-1 family (IL-1$\alpha$ and IL-1$\beta$) in contributing to immune resistance to TB [119, 120]. In mice, IL-1$\alpha$ and IL-1$\beta$ play a dramatic role in immune protection, at least as great as any of the factors that are better appreciated to mediate TB resistance, including TNF, IL-12, and IFN-$\gamma$. A role for IL-1 in human immunity against TB is supported by several studies showing an association between polymorphisms in the IL-1 or IL-1 receptor genes and host resistance [121–124]. The mechanisms by which IL-1 mediates protection against TB are largely unknown. Although IL-1 can serve as a costimulatory molecule for T cells, particularly for the production of IL-17 [125], mice lacking IL-1 receptor signaling have profound susceptibility even before T cell responses are initiated [73, 74]. Thus, IL-1 plays an important innate role in TB, and the appearance of severe necrotic lung lesions in mice lacking IL-1-transmitted signals suggests that IL-1 may regulate cell death pathways crucial to TB pathogenesis [118, 119]. Recently, IL-1$\beta$ production during later stages of mouse infection has been shown to be tightly regulated by IFN-$\gamma$-induced nitric oxide [126].

*2.7.2. IL-12.* IL-12 is a heterodimeric molecule (IL-12p70) composed of the IL-12p40 and IL-12p35 subunits. It is produced by macrophages, and even more so by DCs, and its primary role in TB is to promote the expansion and maintenance of IFN-$\gamma$-producing T cells, which in turn activate macrophages to kill *M.tb*, or at least to curb its replication [127]. Mice or humans deficient in either IL-12 or the receptor through which it signals are extremely susceptible to TB disease [127–130]. IL-12 also has the potential to promote IFN-$\gamma$ by innate cells, including $\gamma\delta$ T cells, Natural killer (NK) cells, Natural killer T cells, CD1 group 1-restricted T cells- and the recently characterized mucosal-associated invariant T cells [131, 132], but further studies are needed to define the roles of these innate cell types in TB immunity.

*2.7.3. IL-10.* IL-10 is an anti-inflammatory cytokine with pleiotropic immunoregulatory effects. Among these effects is its ability to restrict the differentiation of IFN-$\gamma$-producing T cells and to modulate IFN-$\gamma$-mediated signal transduction [133–135]. In mice, strong induction of IL-10 restricts protective immunity to TB, and in humans, a genetic polymorphism that results in enhanced innate production of IL-10 increases TB susceptibility [136, 137]. Despite its potential to negatively impact TB immunity, IL-10 probably also serves a host-protective role by limiting deleterious inflammatory damage to host tissues [138]. Thus, an optimal immune response to *M.tb* probably involves tightly regulated production of IL-10.

*2.7.4. Status of IL-1, IL-12, and IL-10 in Infants.* IL-1 and IL-12 production is diminished in human neonates, whereas IL-10 production is greatly increased. Although production of proinflammatory cytokines gradually increases, adult capacity is not achieved until after one year of age for IL-1, and after two years of age for IL-12 [22, 80, 139–141]. Conversely, the production of IL-10 gradually decreases, but amounts comparable to those produced in adults are not seen until after one year of age [96, 110, 140].

### 2.8. Antimicrobial Peptides

*2.8.1. Role in TB.* Antimicrobial peptides are produced and utilized by phagocytes and lung epithelial cells. Production of LL-37, one of the few bactericidal peptides that effectively kill *M.tb*,is upregulated in response to vitamin D and microbial interaction [142–144]. During *M.tb* infection, human beta defensin-2 (HBD2) is produced in the human lung by epithelial cells in response to IL-1 and TNF stimulation [145]. In addition to endogenous antimicrobials, human alveolar macrophages can utilize antimicrobial components obtained from cytotoxic T cells [146, 147] and apoptotic neutrophils alike [148]. Neutrophils alone carry a vast arsenal of antimicrobial peptides such as $\alpha$-defensins, lactoferrin, cathelicidin, and lysozyme prepacked in granules [149]. These potent granular contents are utilized upon granule fusion with phagosomes [150] but may also be deployed directly to the outside of the phagocyte to fight extracellular pathogen or be delivered to macrophages. In addition to the direct mycobactericidal effects of antimicrobial peptides, some can also influence the chemotaxis of immature DCs and memory lymphocytes [151] and serve as a link between innate and acquired immune responses. Overall, these innate immune pathways restrict the unchecked replication of *M.tb* during the first few weeks of infection and also set the stage for an appropriate adaptive response, which is ultimately required for successful immune control of *M.tb*.

*2.8.2. Status in Infants.* HBD2 and LL-37 are detectable in lung aspirates from healthy neonates and in increased amounts during pulmonary or systemic infections [152, 153].

However, the level of expression is low and *in vitro* stimulation of human fetal lung tissue with IL-1$\beta$ and IFN-$\gamma$ only induces a 2-fold increase in expression, in contrast to a 10-fold induction in adult tissue [152]. It is likely that the overall lack of IL-1$\beta$, IFN-$\gamma$, and TNF and general phagocyte inactivity combine to minimize the production of antibactericidal peptides, providing the newborn with reduced protection against pulmonary TB.

## 3. Adaptive Immunity

### 3.1. CD4 T Cells

*3.1.1. Role in TB.* CD4 T cells are key antimycobacterial components of the adaptive immune response [20, 154–157], and TB is a leading cause of death in CD4 T cell lymphopenic HIV patient [7]. Key cytokines elaborated by protective Th1 effector cells include IFN-$\gamma$ and TNF [20, 154–156] although CD4 T cells can also restrict *M.tb* replication by an IFN$\gamma$- and TNF-independent mechanism in mice [158]. Cytokines generated by Th2 cells, including IL-4 and IL-13, are detrimental to the protective response [159], partly by inhibiting autophagy-dependent killing of intracellular *M.tb* [160].

Interestingly, the contribution of CD4 T cells in controlling TB may impact CD8 T cell function. Previous work has shown that CD4 T cell help is necessary for the induction of optimal CD8 T cell responses, particularly in the face of chronic infection, and CD8 T cells without CD4 T cell help lose effector function over time [161, 162]. This important role for CD4 T cells was originally discovered in mice [132, 161–164], but subsequently verified in nonhuman primates [165], and probably explains why HIV-infected humans with reduced numbers of CD4 T cells eventually lose CD8 T cell function [161, 162]. Such CD8 T cell exhaustion occurs gradually, with IL-2 production, cytolytic function, proliferative capacity lost at early stages, TNF production lost somewhat later, and IFN-$\gamma$ production lost only very late [161–164]. The precise nature of CD4 T cell help for CD8 T cell responses is unclear, but possibilities include production of cytokines, including IL-2, licensing of antigen presenting cells, delivering survival signals, and controlling lymph node cellular input [166–168]. The relevance of these findings for TB has been demonstrated more recently. Maintenance of functional CD8 T cells in chronic mycobacterial infections in mice, including TB, requires help from CD4 T cells [169–171].

*3.1.2. Status in Infants.* Altered infant CD4 T cell function has been widely described in both mice and humans. CD4 T cells from infants are characterized by poor proliferative capacity and diminished production of Th1 cytokines, including IFN-$\gamma$ [172–175], as well as an increased propensity for Treg induction in the periphery [176, 177]. *In vitro* analyses of infant T cell responses have revealed clear differences in the intrinsic function of infant and adult T cells [178]. In fact, the Th2 bias of infant and neonatal CD4 T cells is reflected at the level of chromatin structure, as the Th2 cytokine loci in young mouse and human T cells is hypomethylated and poised for rapid transcription [179, 180]. An important factor explaining these intrinsic differences is that most T cells in infants are recent thymic emigrants (RTEs) [181, 182], those T cells that have recently completed thymic maturation and egress, whereas RTEs comprise only a small percentage of adult T cells [183]. CD4 RTEs are impaired in IL-2 and IFN-$\gamma$ secretion and skewed to Th2 responses both *in vivo* and *in vitro* [181, 184–186]. Because of the important role of CD4 T cells in providing help to CD8 T cells, the diminished capacity of infant CD4 T cells likely restricts the function of CD8 T cells as well.

Despite these well-established biases of infant T cell responses, it is important to acknowledge that these tendencies are not absolute. Both BCG-immunized and *M.tb*-infected infants clearly mount a readily detectable population of IFN-$\gamma$-producing T cells [187, 188]. Future studies are needed to determine whether more subtle differences in their protective properties such as the kinetics of their expansion, tissue homing, longevity, or polyfunctionality may help to explain the immune vulnerability of infants to TB.

### 3.2. CD8 T Cells

*3.2.1. Role in TB.* Appreciation of CD8 T cells as active components of the antimycobacterial adaptive immune response has come only recently [155]. CD8 T cells may be activated through cross presentation of mycobacterial antigens by DCs that have taken up apoptotic infected cells [189], but can also directly recognize *M.tb*-infected cells [190]. CD8 T cells help control *M.tb* replication by producing IFN-$\gamma$, as well as by perforin-mediated cytolysis of infected macrophages and direct killing of *M.tb* (reviewed in [155, 191]).

*3.2.2. Status in Infants.* Mouse studies have shown that CD8 RTEs have a reduced capacity to mediate cytolysis and to produce IFN-$\gamma$ [184, 192]. Consistent with these findings, CD8 T cells from human infants exhibit a reduced capacity to produce IFN-$\gamma$ and granzyme B in the absence of exogenous IL-12 [193]. *In vivo* activated RTE-derived CD8 T cells are skewed to short-lived effector T cells and away from the memory precursor compartment [194]. Importantly, antigen-specific CD8 RTEs generate impaired memory T cells, demonstrating that if T cells first see antigen as RTEs, cell fate decisions are impacted long after [192].

### 3.3. Delayed Adaptive Immune Response

*3.3.1. Role in TB.* Despite the essential role of IFN-$\gamma$ producing T cells in immune protection against *M.tb*, the correlation between the magnitude of the IFN-$\gamma$-producing T cell response and the degree of protection is actually very poor. A large study of BCG-immunized infants recently revealed no correlation between protection and the frequency or cytokine expression profile of mycobacteria-specific T cells present in the blood [187]. Mouse studies have similarly shown no correlation between protection and IFN-$\gamma$ producing T cell frequencies [195]; however, the rapidity by which *M.tb*-specific, IFN-$\gamma$-producing T cells reach the site of infection

in the lung has been shown to be a critical predictor of protection [20, 196]. Unfortunately, the adaptive immune response to *M.tb* is significantly delayed compared to that observed in response to other pathogens [196]. Humans recently exposed to *M.tb* do not become tuberculin skin test positive until ~6 weeks after exposure [197, 198], whereas the T cell response to most pathogens peaks 1-2 weeks after exposure. Similarly, mice experimentally infected with *M.tb* via aerosol also exhibit a delayed T cell response that does not peak until several weeks after infection [20, 196], and the tractable nature of the mouse model has recently been utilized to dissect out many aspects of this delay. *M.tb*-specific T cell responses cannot be initiated until *M.tb* is ferried to the lung-draining lymph node by migratory DCs [39, 75]. This process does not occur until the second week of infection because *M.tb* replicates "under cover" in alveolar macrophages for a prolonged period [20, 196, 199]. *M.tb*-induced lipoxins contribute to this pathogen-induced loitering by suppressing apoptosis [200]. After their eventual release from alveolar macrophages, *M.tb* bacilli are phagocytized by and released from neutrophils before a significant number of bacteria are available for acquisition by DCs [201]. After *M.tb* is finally ferried to the lung draining lymph node by DCs, the effector T cell response is delayed even further by the expansion of pathogen-specific Treg cells at this site [202, 203]. *M.tb*-specific effector T cells do not reach the lung in sufficient numbers to begin to curb bacterial replication until three weeks after infection and take yet another week to peak in numbers [20, 196]. In the meantime, the delay enables *M.tb* to replicate exuberantly and establish a niche in the lung that facilitates high-burden chronic infection.

*3.3.2. Status in Infants.* Whether adaptive immunity takes even longer to develop in infants than in older children and adults has not been formally studied. Clinical experience, however, suggests that this may be the case. Infants exposed to family members with active TB often take up to three months for detection of *M.tb*-specific, IFN-$\gamma$-producing T cells by a positive tuberculin skin test or IFN-$\gamma$ release assay. The infant immune system has deficits in multiple factors that shape the rapidity of the adaptive immune response to TB. As previously discussed, infant macrophages, neutrophils, and DCs are present in low numbers and also have chemotactic deficits that impair their recruitment to sites of inflammation. These alterations have the potential to significantly delay the trafficking of *M.tb*-infected DCs to the lung-draining lymph node that is a prerequisite for initiating the *M.tb*-specific T cell response. Even after *M.tb* is delivered to the lymph node and T cell priming is initiated, effector T cell expansion may be slowed further by the increased propensity of infants to induce Treg responses [176, 177]. Taken together, it seems likely that infants have an even greater delay in the initiation of their adaptive immune response to TB than do older children and adults. Thus, infant T cells may not only be biased against making IFN-$\gamma$, but also against arriving in the lung in a timely manner.

Recent evidence shows that the T cell response of newborns immunized with BCG is quite delayed, peaking at 10 weeks after immunization [204]. This finding has important implications for the timing of an infant booster vaccine, which probably should not be administered until several weeks after this peak response [205]. Whether immunized adults would respond with different kinetics to BCG immunization is not known.

## 4. Practical Considerations

The vulnerability of infants to TB is profound and perhaps intractable. Nevertheless, there are several practical considerations. For perinatal TB, prevention is better than a cure (at least it is more easily achieved), and there are several opportunities to prevent neonatal *M.tb* infection and disease that are currently underutilized. Despite the fact that most neonatal TB is acquired from mothers with active disease, TB screening of women during the antenatal and postnatal period is often not adequately implemented in high-burden settings [206]. Such screening, however, could be readily integrated into primary antenatal care or (preventing mother-to-child transmission of HIV) PMTCT programs. Exposed newborns without signs of diseases should be offered TB prophylaxis. Even though TB diagnosis is difficult in infants, treatment should be started as early as possible to prevent complicated disease.

Infants in high TB endemic countries should be vaccinated with BCG, which is effective in preventing severe forms of disease. Given the enhanced susceptibility of neonates to mycobacterial infection, it seems a paradox that mycobacterium BCG is most effective when given to neonates. One possible explanation may be that BCG reaches a higher bacterial load for a prolonged period of time in infants, and this ultimately drives an enhanced antimycobacterial T cell memory response, despite the deficiencies in infant immunity outlined here. Another possibility is that infant effector T cells have an increased propensity to migrate to the uninfected lung [181, 184–186], which might play a dominant role in preventing progression to active TB. In any case, unraveling this conundrum will undoubtedly provide key insights into how long-lived immunity against TB can best be achieved and help inform the design of a desperately needed new and improved TB vaccine. Of interest, observational studies have suggested that BCG may also have a nonspecific beneficial effect on survival [207–209].

As highlighted in this review, TB is a fundamentally different disease in infants than in older children and adults. In older individuals, TB disease can be caused by an inappropriate and excessive inflammatory response to infection [107], whereas infant TB is a disease in which the inflammatory response is inadequate. Because the immune pathogenesis of infant TB is unique, TB immunotherapies that are beneficial for older individuals may be inappropriate for infants. As a possible case in point, adjunctive corticosteroid therapy for TB meningitis is currently routine for all ages, including infants. This practice stems from studies that have clearly shown modestly improved outcomes for TB meningitis in older children and adults receiving steroid adjunctive therapy [111]. The efficacy of steroids for infant TB meningitis, however, has not been specifically examined. Recently, the benefit of steroids for TB meningitis in adolescents and adults

was found to be limited exclusively to a subset of individuals with a genetic propensity to produce high levels of TNF [108]. Steroids were not beneficial, and possibly detrimental, in individuals with lower capacities for TNF production. Because infants have an inherently restricted capacity to produce TNF, infants are unlikely to have TB meningitis that is caused by a hyperinflammatory state. Spurred by the growing problem of drug-resistant *M.tb*, potential immune-based TB therapies are gaining increased attention and research funding. Decisions to employ any TB immunotherapy in infants should not be made by extrapolating findings from studies of older individuals.

## 5. Summary

Inflammatory responses are dampened in infants, at least in part, to accommodate colonization with beneficial commensal bacteria. Unfortunately, key factors required for immune protection against TB are also down regulated. Macrophages, neutrophils, and DCs are present in low numbers and have impaired function. Their capacity to produce proinflammatory cytokines, including TNF, IL-1, and IL-12, is reduced, while their capacity to produce anti-inflammatory cytokines, including IL-10, is increased. Furthermore, infant T cells have an inherent bias against IFN-$\gamma$ production, and favor production of cytokines such as IL-4 and IL-13 that are counterproductive during TB. Because of these immune alterations, infants are 5–10 times more likely to progress to active TB after infection and are also more likely to manifest severe, disseminated forms of disease. It is interesting to consider this immune dampening during infancy in light of the emerging idea that individuals susceptible to TB may represent both ends of the inflammatory spectrum; those that have a genetic predisposition to mount an inflammatory response to TB that is either too low or excessive may be susceptible, whereas those with a propensity for an intermediate response may be protected [108, 109]. If this principle proves to be generalizable for human TB, the reduced inflammatory response during infancy may shift some individuals that might otherwise be susceptible to hyperinflammatory TB into the protected zone, whereas many others that might otherwise be protected may become vulnerable due to their diminished capacity to mount an appropriate inflammatory response. The vulnerability of infants to TB and the unique nature of their immune response have practical implications for public health interventions, vaccine design, and immunotherapy.

## Conflict of Interests

The authors declare that they have no conflict of interests.

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*Review Article*

# Neonatal Immune Adaptation of the Gut and Its Role during Infections

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The intestinal tract is engaged in a relationship with a dense and complex microbial ecosystem, the microbiota. The establishment of this symbiosis is essential for host physiology, metabolism, and immune homeostasis. Because newborns are essentially sterile, the first exposure to microorganisms and environmental endotoxins during the neonatal period is followed by a crucial sequence of active events leading to immune tolerance and homeostasis. Contact with potent immunostimulatory molecules starts immediately at birth, and the discrimination between commensal bacteria and invading pathogens is essential to avoid an inappropriate immune stimulation and/or host infection. The dysregulation of these tight interactions between host and microbiota can be responsible for important health disorders, including inflammation and sepsis. This review summarizes the molecular events leading to the establishment of postnatal immune tolerance and how pathogens can avoid host immunity and induce neonatal infections and sepsis.

## 1. Introduction

Harboring trillions of microbes, the intestinal mucosa represents a complex ecosystem playing a dual role in host defense. Permanently exposed to enteric microbes, the mucosa has to provide an efficient protection against pathogenic microbes, and, on the other side, has to maintain tolerance towards commensal flora. The innate immune system has evolved to provide mutual profit to both the host and microbiota. Commensal bacteria, expressing unique enzymes, contribute to the digestion of dietary substances as well as the synthesis of food supplements [1]. They also confer protection against pathogenic bacteria though competition for space and nutriments [2, 3]. Commensal flora induces innate immune signaling which favors the differentiation and maturation of the immune system, the maintenance of the barrier integrity, and restricts commensal flora to the lumen [4–8]. On the other hand, the innate immune system has to be controlled and the intestinal mucosa develops mechanisms of tolerance that enable microflora to thrive and mechanisms of defense to provide an efficient response in case of invasion by pathogens. This subtle balance of the innate immune signaling is tightly controlled, and disturbance of this host-commensal relationship may cause inappropriate response of the innate immune system leading to inflammation, organ dysfunction, infections, sepsis, or cancer [9–11].

The intestinal surface is covered by a monolayer of polarized epithelial cells which, from birth to death, represents the only border separating the microbes of the intestinal lumen from the host. The challenge is particularly complex since the essential function of gut is to exchange nutriments with the content of the lumen, representing the major part of body nutrition. Conversely, the direct contact between the intestinal bacteria and the epithelial cell surface has to be minimized and controlled to avoid an inappropriate activation of the immune system. During ontogeny, the formation of the primitive gut starts early and is initiated from cells of the endoderm [12–14]. In mice at E6, definitive endodermal cells are specified during gastrulation. At E8.5, the endodermal tube is initiated by the fold of the endodermal lining at the anterior and posterior ends, creating anterior and caudal intestinal portals. After gut tube formation at E9–9.5, the simple epithelium turns into a pseudostratified epithelium. Specific intestinal markers, such as villin, first

appear in the hindgut at E9 [15]. Between E9.5–14.5, while the gut length and circumference increase, the primitive gut tube is patterned along the anterior-posterior axis. A transitional period in the course of which the epithelium turn stratified was thought to occur, but a recent study shows that this event may not take place [16]. Around E14.5–15, gut epithelium begins a remodeling process with the emergence of finger-like protrusions called villi on the previously flat luminal surface, providing efficient nutrient absorption. Of note, unlike the small intestine, villi are lost during fetal development of the colon mucosa. Cell proliferation, firstly homogenous along the epithelium, becomes limited to the intervillus regions where gland-like invaginations (named crypts) secondarily start to form, creating a protected stem cell niche. These groups of stem cells migrate in a crypt-villus axis and are behind the different cell phenotypes of the intestinal epithelium. The level of maturity of neonatal gut at birth differs between species and depends on the length of the gestation period (Figure 1). Whereas human and guinea pig small intestine presents mature crypt-villus architecture at birth, crypts emerge 12–15 days after birth in mice during the weaning period [17]. In humans, the fetal gut is structurally mature from week 19 of gestation, and all the cellular components of the gastrointestinal immune system are already present during the fetal life. For example, T cells are identified around 12 weeks of gestation [18]. Nevertheless, the gastrointestinal immune system remains immature at birth, since antigenic stimulation of the colonizing microflora is required for its full maturation.

Cytodifferentiation goes along the villus/crypt axis formation. The immature primitive stem cells localized in the crypts lead to the formation of distinct lineages of intestinal epithelial cells based on their functions: enterocytes, goblet cells, enteroendocrine cells, and Paneth cells [19]. Notch-mediated signaling pathway triggers epithelial cell differentiation and is essential for gut homeostasis [20]. Enterocytes are absorptive cells which represent 90% of intestinal epithelial cells. The apical surface is lined by a microvilli-covered brush border where essential enzymes and transporters for nutrition are expressed. Secretory cells are divided in three types: goblet cells, enteroendocrine cells, and Paneth cells. Goblet cells are the most abundant secretory lineage in the gut epithelia and are involved in the production of highly glycosylated mucins generating a mucus matrix acting as a protective barrier by covering the gut mucosa [21]. It has been suggested that they could participate in the delivery of luminal antigens to subepithelial antigen-presenting cells [22]. They are located throughout the epithelial surface and their number increases from the proximal small intestine to the colon. Enteroendocrine cells are divided in more than 16 subtypes identified in mouse intestine depending on hormones or other signal mediators secreted. Their role as immune sensors is still unclear, even though it has been shown that they express a variety of innate immune receptors and respond to microbial stimulation [23]. Paneth cells, located at the bottom of the crypt of the small intestine, produce and secrete antimicrobial peptides and soluble mediators in the lumen, creating a niche for stem cells and reinforcing the mucus layer. Of note, they are lacking in the colon, as well as in the intestine of some species such as *Xenodon merremii* [24]. Stem cells allow a constant renewal of gut epithelium which must be maintained throughout the course of life. Transit-amplifying cells, after about two days in the crypt, divide 4-5 times before being terminally differentiated into one of the specialized intestinal epithelial cell types. In adult mice, around three days after the end of their differentiation, the cells reach the top of the villus, enter in apoptosis, and are exfoliated to the gut lumen [25]. The different cell types of the epithelium appear at different times during gut formation. In mice, Paneth cells appear after birth during the emergence of crypts in the small intestine whereas enteroendocrine cells are already present around E10. After birth, cell proliferation is low in the intestine of neonate mice until weaning in correlation with suckling diet and increases around 10–12 days after adaptation of the gut epithelium to solid nutrient components [26, 27]. Importantly, transcriptional repressor B lymphocyte-induced maturation protein 1 (Blimp1) is highly expressed in the developing and postnatal intestinal epithelium until the suckling to weaning transition. It has been shown that this factor is accountable for the developmental switch responsible for postnatal intestinal maturation and governs the suckling to weaning transition of the epithelium [28, 29]. The generation of new mouse models, such as the multicolor Cre-reporter R26R-Confetti mice, will probably bring new insights in the development and maturation of the intestine [30].

This review focuses on the major mechanisms and factors that are crucial for the establishment of the immune intestinal tolerance in the first weeks after birth, as well as the maintenance of a life-time homeostasis. Finally, the postnatal dysregulations of these processes possibly leading to infant inflammatory diseases such as neonatal infections and sepsis will be addressed.

## 2. Postnatal Colonization of the Gut

In normal conditions, fetal gastrointestinal tract is thought to be sterile. However, studies have suggested that fetal gut can be exposed to microorganisms invading the amniotic fluid, which can be associated with preterm delivery [31, 32]. It is also well known that prenatal exposure of the mother to bacterial components can influence intestinal epithelial development and function in newborn, as well as sensitivity to inflammatory diseases such as necrotizing enterocolitis [33–35]. Thus, prenatal exposure of the gut to bacteria may modulate immediate postnatal adaptations inducing tolerance toward colonizing bacteria. During birth, the intestinal mucosa undergoes a dramatic transition from a protected site to a densely colonized environment [36, 37]. Delivery allows the first contact between gut epithelium and microorganisms. Newborns are mainly exposed to microorganisms from the maternal mucosa and endotoxins of the environment. In mice and humans, after birth, facultative anaerobic or microaerophilic bacteria such as *Lactobacilli* and *Streptococci* are dominant. Few days later, *Enterococci* and Enterobacteriaceae appear and generate a decrease of local oxygen concentration by their metabolic activities, favoring the colonization by *Bifidobacteria*, *Bacteroides* spp. and

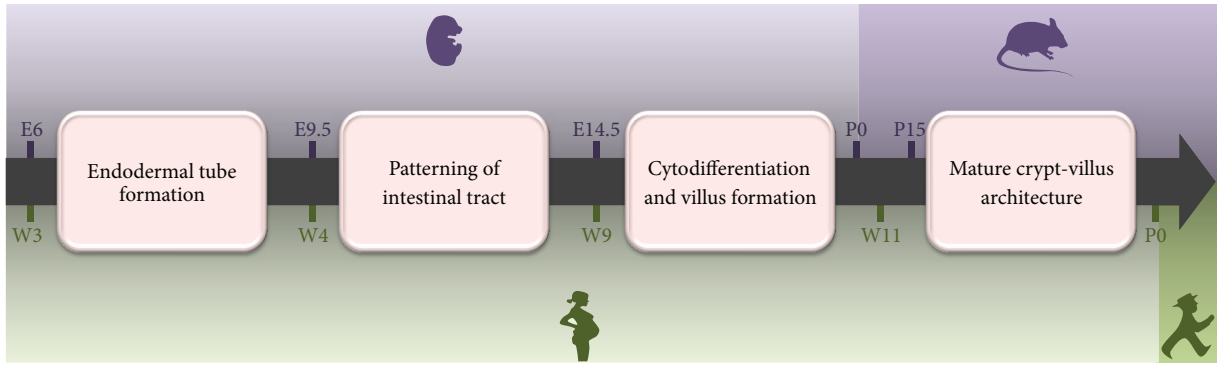


Figure 1: Time line of intestinal development in human and mice. Definitive endodermal cells are specified during gastrulation (mouse: embryonic day 6 E6, human: week 3 W3) and initiate the formation of the primitive gut tube, fully formed at E9.5 in mice and W4 in humans. At later stages, the tube is patterned along the anterior-posterior axis. Cytodifferentiation and villus formation take place from E14.5 in mouse and from W9 in human. In mice, crypt formation starts around day 15 after birth (postnatal day 15, P15), whereas in humans, mature crypt-villus architecture is already defined during fetal period from W11.

*Clostridium* spp. [38–40]. Less is known about the longitudinal pattern of colonization after birth and the differences along the length of the gastrointestinal tract. Interestingly, transcriptome analyses show significant spatial differences in the response to colonizing microflora in the jejunum and colon compared to the ileal segment [41].

The colonization of the gut is influenced by several factors. The first microbial exposure of the newborn, as well as the delivery mode (i.e., vaginal delivery versus caesarian-section) has been shown to influence the postnatal gut microbiota composition [42]. Associations between some specific constituents of microbiota in human newborn day 4 after birth and the concentration of specific microbial groups at day 120 have been demonstrated, suggesting that early gut microbiota may influence later microbiota [43]. Colonization of the gut of caesarian-section-born infants appears to be delayed compared to vaginal delivery born infants. Moreover, caesarian-section born human babies have a different colonization pattern compared to vaginal delivery born ones [44]. However, vaginal microbes of the mother seem to not settle in neonate gut and change rapidly with suckling [45]. Although neonatal rodents are exposed to greater numbers of environmental microbes than humans, similar findings have been shown in culture-based studies demonstrating that the initial flora of the neonates is mainly composed of the vaginal and fecal microorganisms of maternal origin [46]. Diet (i.e., breast milk versus formula) is also a factor influencing the composition of the microflora. *Bifidobacteria* is dominant in the microflora of breast milk fed neonates, whereas formula-fed infants microflora harbor a majority of *Bifidobacteria*, *Bacteroides* spp., and *Clostridium* spp. [47]. Under the influence of the diet (i.e., milk to solid food) and especially after weaning, the composition of the microbiota changes rapidly. Gut microflora has fully matured in children around the age of 4 and after 3 to 5 weeks in mice [37, 48] and remains relatively stable throughout life [49, 50]. Other factors, such as hygiene, environment, and lifestyle, also influence the initial composition of the microflora, but at a lower extend once the microflora is stabilized. Indeed, the microflora of marital partners does not have a significantly greater similarity in their composition than unrelated individuals, even if these partners live in the same environment and have similar dietary habits [51]. Besides, the microbiota of monozygotic twins living separately is notably more similar than the microbiota of unrelated individuals [52].

The mature microbiota contains a complex and dynamic population of more than 1000 different microbial species in the human gastrointestinal tract, reaching $10^{12}$ cfu/g of gut contents in the large bowel [53]. The collective genome of the whole microbes, the microbiome, may contain more than 100 times the number of genes of the mammalian genome [54]. Gut microbiota is increasingly considered as a "metabolic organ" inside the gut intestinal tract, which acts in physiology to develop functions that humans have not evolved for their own. The impact of the microbiota on gut physiology, metabolism, and health has been shown to be largely influenced by microbial activities, like fermentation of food components not digested by the upper gastrointestinal tract such as nondigestive carbonate [55]. Interaction between host and bacteria in the gut mucosa is, of course, essential for host digestive efficiency and intestinal physiology, and plays a major role in the establishment of immune postnatal tolerance after birth, as well as in the maturation of the gut-associated lymphoid tissue [27, 56, 57]. Doubtlessly, the microbiota and the immune system of the host interact in a two-way street: while bacteria induce immune maturation, the host immune system regulates the number and the composition of the bacteria.

## 3. Adaptation of the Gut Mucosa to Bacterial Colonization

*3.1. Crosstalk between Bacteria and Host Cells.* The first exposure of the gut to bacterial ligands occurs during the passage though the birth canal or shortly thereafter. This interaction between colonizing bacteria and host cells has been shown to be crucial for the establishment of intestinal tolerance (Figure 1). Bacterial ligands are recognized by innate immune receptors, such as Toll-like receptors (TLRs), which

are expressed by intestinal epithelial cells throughout fetal, neonatal, and adult life. Both TLR2 and TLR4 are expressed in human fetal tissue from 18 weeks of gestation [58]. TLR1-5/9 and TLR1-9 have been detected in human small intestinal tissue and colon, respectively and TLR1-9 and TLR1-4/9 in murine small intestinal tissue and colon respectively [59]. Intestinal epithelial cells also express the cytosolic helicases retinoic acid-inducible gene I Rig-I and melanoma differentiation-associated gene 5 Mda5, sensing the presence of RNA and Nod-like receptors such as Nod1 and Nod2, sensing the DAP-type tripeptide motif or the muramyl-di-peptide motif of peptidoglycan, respectively. Epithelial Nod1 and Nod2 seem to synergistically protect the colon during *Salmonella enterica* subsp. *enterica* sv. Typhimurium (abbreviated *Salmonella* Typhimurium) infections [60].

Interestingly, we showed that the postnatal exposure of epithelial cells to lipopolysaccharide (LPS), the endotoxin found in the outer membrane of Gram-negative bacteria which activates TLR4, drives the neonatal innate immune tolerance in epithelial cells [27, 38]. This state renders epithelial cells hyporesponsive to TLR stimuli and protects the gut during the maturation of the mechanisms involved in adult intestine homeostasis. At birth, the first contact between the LPS of the bacteria and the epithelial TLR4 induces the production of microRNA (miR)-146a, a small noncoding RNA. miR-146a specifically inhibits the translation of the interleukin-1 receptor-associated kinase (IRAK) 1, an essential kinase of the TLR4 pathway. In other words, the activation of TLR4 during birth induces the activation of an inhibitory loop through the expression of miR-146a, shutting down the ability of the cell to respond to TLR agonist. This $IRAK1^{low}$ state protects the gut during the first contact with bacteria, since the administration of an anti-miR-146a to neonates reestablishes the expression of IRAK1 in epithelial cells as well as TLR susceptibility and induces inflammation, apoptosis, and damages in the gut mucosa during bacterial colonization. On the contrary, the administration of a miR-146a mimic to caesarian-section born mice, which normally fail to decrease the epithelial IRAK1 level due to the lack of TLR4 activation in LPS-free conditions after birth and develop intestinal damages during colonization, rescues the intestinal phenotype. Moreover, the $IRAK1^{low}$ state sustains the expression of specific genes that are important for cell maturation, survival and nutrient absorption. The switch to an $IRAK1^{high}$ state progressively occurs after day 14, when cell proliferation increases, crypt-villus architecture appears and immune tolerance is acquired. The $IRAK1^{high}$ epithelial cells are able to respond to LPS stimulation and are fully functional to activate an inflammatory response against pathogens. Strikingly, the $IRAK1^{low}$ state in epithelial cells during the postnatal period seems to be maintained by an active constitutive signal mediated by internalized endotoxin. The loss of endotoxin is correlated to the increase of proliferation and allows the change from an $IRAK1^{low}$ to an $IRAK1^{high}$ state. Even though TLR signaling sensitivity is decreased in the neonatal intestinal epithelium, a number of other innate immune receptor signaling remain fully functional during this period. For example, the helicases Rig-I and Mda5 mediate the antiviral defense against rotavirus infection by promoting interferon $\lambda$ production [61, 62].

Epithelial crosstalk with colonizing bacteria seems to be essential for immune tolerance since a specific epithelial lack of the proinflammatory transforming growth factor (TGF) $\beta$-activated kinase 1 (TAK1) leads to early inflammation, Tnf-dependent induction of apoptosis, tissue damages, and postnatal mortality [63]. Similarly, spontaneous mucosal damages have been observed during early postnatal development of mice specifically deleted in epithelial p65/RelA NF-$\kappa$B subunit. In inhibitor $\kappa$B kinase (Ikk) 1/2 (also known as Ikk-$\alpha$ and $\beta$) knockout and myeloid differentiation primary response gene 88 (Myd88) dominant-negative mice, translocation of commensal bacteria is increased and inflammation occurs due to a lack of homeostatic signaling. Accordingly, epithelial-specific deficiency of Nemo (also known as Ikk-$\gamma$) and Ikk1/2 in a Myd88 knockout background does not cause inflammation [64]. Clearly, an active dialogue between colonizing bacteria and epithelial cells exists and is essential for the maintenance of homeostasis in the gut. With the use of germ-free mice and the development of high-throughput sequencing, this concept has been extensively studied, highlighting that this interaction drives several aspects of innate and acquired immune response of the host [65, 66]. In germ-free mice, the absence of microbiota impairs the immune development of the intestine, and mice exhibit underdeveloped gut-associated lymphoid tissue including rudimentary Peyer's patches and intestinal follicles, reduced number of $CD4^+$ T cells and immune effector molecules as wells as intraepithelial lymphocytes (IELs), decreased MCH class II expression on antigen-presenting cells, and decreased immunoglobulin A (IgA) production [67–70]. Reconstitution of the microbiota or even inoculation of specific bacterial components such as polysaccharide A (PSA) largely corrects these deficiencies and reconstructs aspects of the mucosal immune system [71]. Thus, the presence of the microbiota in the gut allows the development of an extensive and activated intestinal immune system. Recent findings have given some keys about the way the microflora influences the development of the immune system. After detection of the microorganisms by innate immune receptors, epithelial cells produce cytokines such as IL-10 and TGF-$\beta$ which can regulate professional immune cells of the lamina propria. The balance between Th1, Th2, and Th17 responses has to be fine-tuned to maintain homeostasis. Particularly, $CD4^+CD25^+$ regulatory T ($T_{reg}$) cells contribute to the maintenance of self-tolerance, suppress inflammatory response by secreting anti-inflammatory cytokines, and can inhibit the function of antigen-presenting cells. The activation of intestinal epithelial cells by some commensals such as *Clostridium* spp. induces the production of TGF-$\beta$ which in turn allow the accumulation of IL-10-producing $Foxp3^+$ $T_{reg}$ cells [8]. Also, migratory $CD103^+$ $CCR7^+$ dendritic cells are conditioned by microbial and epithelial-derived factors and promote the differentiation of $Foxp3^+$ $T_{reg}$ cells as well as IgA production by B cells [72]. Epithelial cells also react to the alterations of the immune system, and the gene expression profile can switch from metabolic activities to epithelial host

defense in the absence of IgA, for example [73]. Meanwhile, segmented filamentous bacteria (SFB) mediate Th17-cell as well as Th1 and $T_{reg}$ population initiation, raising protection against bacterial infections [5]. Also, acetate from *Bifidobacterium longum* enhances intestinal barrier integrity through induction of antiinflammatory and antiapoptotic genes and protects mice from lethal infection with *Escherichia coli* O157:H7 [74]. The different mechanisms by which pathogens and commensals differentially activate the immune system of the host in the intestine remain partially understood and are extensively studied currently. It has been proposed that the immunologic distinction between pathogens and the microbiota is mediated by host mechanisms, and also *via* the recognition of specialized molecules evolved by symbiotic bacteria to enable commensal colonization, such as PSA from *Bacteroides fragilis* which activates TLR2 pathway on $Foxp3^+$ $T_{reg}$ to engender mucosal tolerance [75].

*3.2. The Intestinal Mucus Layer as a Protective Barrier for the Host against Bacteria.* The mucus barrier covering the intestinal mucosa forms a protective physical shield against bacteria and limits the microbial contacts with the host cells. The small intestine and the colon harbor different type of mucus layers. The small intestine is covered by a single layer of mucus mainly composed of the protein mucin 2 MUC2, released at the crypt openings [76]. Probably covering the villi, the mucus is unattached to the epithelium and is permeable to bacteria which can be trapped in the pores of the MUC2 network, bind the hydrophobic CysD mucin domains conferring stickiness to mucus, or bind the polymorphic and variable mucin glycans though their adhesins [76, 77]. By contrast to the small intestine for which a nonpermeable mucus layer would be detrimental for nutrition function, the colonic mucosa is covered by a dense and thick double layer of mucus [78]. The inner layer is attached to the epithelium and forms a compact bacteria-free coat, of around 50 $\mu$m thickness in mouse and up to several hundred $\mu$m in human. Secretions of goblet cells allow the inner mucus layer to be renewed and the upper part is converted to the loose permeable outer mucus layer which expands until 5 fold in volume due to endogenous protease activities acting on the MUC2 mucin, creating a habitat for the intestinal microbiota [79]. Bacteria utilize the released mucin monosaccharides as an energy source in addition to undigested carbohydrates from the food, thus producing short fatty acids able to diffuse through the inner mucus layer for the host.

The mucus layer clearly plays a major role in host/microbiota homeostasis. In mice deficient in MUC2, bacterial contact with the host mucosa is increased, leading to the development of spontaneous colitis, and later on, colorectal cancer [80, 81]. As described before, commensal bacteria generally use the mucus and adhere to the mucus matrix. Some of them, such as SFB, penetrate the mucus and directly interact with epithelial cells for the maturation of mucosal specialized immune cells [5, 6]. Moreover, some pathogenic bacteria such as *Listeria monocytogenes* and *Salmonella* Typhimurium developed the ability to penetrate the mucus matrix to invade the epithelium by targeting goblet cells specifically [82, 83].

At birth, the production of mucin is low in the gut, especially in species exhibiting a nonfully mature intestine at birth. In mice, proliferation of the epithelium is extremely low until the second week after birth and is correlated with the number of goblet cells and mucus production [27, 84]. During the first 2 weeks, the intestinal epithelium developed specific strategies to tolerate the colonizing bacteria, such as secretion of neonate-specific antimicrobial peptides and constitutive active downregulation of the innate immune TLR4 pathway. By using mice with enterocyte-specific deletion of TLR4, Sodhi et al. demonstrated that TLR4 signaling prevents goblet cells differentiation by inhibiting Notch signaling, independently of the microbiota [85]. These findings might explain the low number of goblet and the low production of mucus during the 2 first weeks after birth, since the TLR4 signaling pathway is downregulated during this period [27]. Moreover, soluble factors contained in maternal milk also contribute to the protection of the neonates during breast feeding time and will be discussed later on. Of note, human milk contains mucin 1 and 4 which can bind pathogenic bacteria such as *Salmonella* Typhimurium and by competition with the host immune receptors, inhibit the invasion of epithelial cells [86]. Also, human milk oligosaccharides favor the selection of specific bacteria such as *Bifidobacterium infantis* which are able to consume them *via* mucus-utilization pathways, facilitating milk and solid food digestion [87].

Soluble factors are also released by mucosal cells into the forming mucus layer and reinforce the protection provided since they are jammed in a gradient manner in the mucus matrix. Apart from antimicrobial peptides, some secreted enzymes modify microbial ligands and thus prevent innate immune recognition and activation by the host. Among them, the intestinal alkaline phosphatase (IAP), contained in enriched vesicles released by epithelial cells, impairs LPS recognition by dephosphorylating LPS molecules and limits bacterial growth [88]. Likewise, the amidase peptidoglycan recognition protein-2 (Pglyrp-2) secreted by intraepithelial lymphocytes cleaves the muramyl dipeptide of the peptidoglycan, which impairs the recognition by the immune intracellular receptor Nod2 [89].

*3.3. Antimicrobial Peptides Regulate Commensal Flora and Protect against Pathogens.* Among the secreted molecules involved in the establishment of tolerance and in homeostasis, antimicrobial peptides play an important role. In addition to their bactericidal effects, antimicrobial peptides exert immunomodulatory functions, such as proinflammatory and chemoattractive activities, wound healing activation, and dendritic cell responses modulation [90]. In mammals, these ancient gene-encoded antibiotics are divided in two families: Defensins and Cathelicidins. $\alpha$- and $\beta$-defensins consist of about 30 amino acids forming a triple-strand $\beta$-sheet structure with three intramolecular disulfide bonds. Defensins exhibit a broad range of bactericidal activity against Gram-positive and Gram-negative bacteria and also against fungi, viruses, and protozoa. Since they are highly cationic, they interact with the negatively charged

phospholipids of the outer membrane of bacteria to disrupt the membrane integrity [91]. In the gut, Paneth cells, located in the crypt of the small intestine, produce constitutively high amount of $\alpha$-defensins, human $\alpha$-defensin (HD) 5, and HD6 in humans and more than 20 (also named cryptdins) in mice [92]. In mice, Paneth cells also produce another related family of antimicrobial peptides, the cryptdin-related sequence (CRS) peptides [93]. $\alpha$-defensins secretion counts for 70% of the secreted bactericidal activity in Paneth cells. On the other hand, $\beta$-defensin proteins are expressed in the colon, although mRNAs have been detected in the small intestine. $\beta$-Defensins are regulated on the transcriptional level after innate immune activation. Conversely, $\alpha$-defensins are posttranscriptionally regulated by proteolytic cleavage by the matrix metalloproteinase 7 (MMP7 or matrilysin) in mice and by the endoprotease trypsin after secretion in humans. Notably, MMP7-deficient mice exhibit alterations in the composition of the microbiota [94]. They also have been shown to be more susceptible to *Salmonella* Typhimurium infections, oppositely to humanized mice expressing HD5 which are more resistant [95, 96]. Studies of the expression of defensins during development show some discrepancies, probably due to the use of different experimental approaches and techniques (mRNA versus protein levels, use of oligonucleotide probes detecting several members of this conserved family, etc.). Nevertheless, it seems that expression of $\alpha$-defensins 4 and 5 in mice is microbiota dependent, since germ-free mice exhibit a reduced level [97]. During postnatal development, it has been also noticed that $\alpha$-defensins 1,3, and 6 exhibit a gradual increase whereas $\alpha$-defensins 2 and 5 exhibit a rapid increase correlated with the appearance of crypts and Paneth cells after 2 weeks.

Cathelicidins are secreted in the gut by a variety of cell types, including neutrophils, mast cells, and epithelial cells. Mature cathelicidins result from the proteolysis of the C terminus of cathelin-domain-containing protein precursors, hCAP18 in human and CRAMP in mice. In cattle and pig, the diversity of this family of peptide is much more diverse. As defensins, the antimicrobial activity of cathelicidins is related to their cationic amphipathic properties, but they differ in their structure since they form $\alpha$-helical or $\beta$-hairpin structures. Strikingly, cathelicidins have been shown to play a major role in the establishment of tolerance in neonates. Especially in mice, CRAMP is highly expressed at birth and during the first 2 weeks of life, independently of the enteric microbiota. The expression gradually disappears with the formation of the crypts and appearance of Paneth cells secreting $\alpha$-defensins, which then seem to take over the major antimicrobial activity in the small intestine [98]. Of note, similar changes in the composition of antimicrobial peptides and in bactericidal activity during the postnatal period in humans have been detected [99]. It also has been shown that CRAMP-deficient neonates are more susceptible to *Listeria monocytogenes* infections [98]. The eventual role of CRAMP in the establishment and selection of the enteric microflora still remains to be investigated.

At a lower extent, Paneth cells also secrete antimicrobial proteins, such as lysozyme P, secretory phospholipase A2, and the recently discovered C-type lectins Reg3$\beta$ and Reg3$\gamma$ [92]. Interestingly, it has been recently shown that, during the initial colonization of the gut, Reg3$\beta$ and Reg3$\gamma$ production known to be secreted is not only restricted to Paneth cells and absorptive enterocytes, since mRNAs were detected in goblet cells of small intestine and proximal colon between day 14 and 28 [100]. Recently, the active role of enterocytes in the control of bacterial load at the mucosal surface has been demonstrated by the fact that they produce Reg3$\gamma$ through a pathway involving the interleukin (IL)-1R and TLR adaptor molecule Myd88 [101]. This production is at least in part supported by an intrinsic regulatory loop mediated by interleukin-22 (IL-22)-producing ROR$\gamma$t$^+$ NKp46$^+$ lymphocytes [102]. Reg3$\gamma$ is essential to keep a 50 $\mu$m bacteria-free zone above the small intestine epithelial surface and the antimicrobial effect is related to the capacity of specifically targeting native peptidoglycans on bacterial surfaces [103]. Specific deletion of Myd88 in intestinal epithelial cells results in increased number of mucus-associated bacteria, translocation of bacteria, decrease of the expression of Reg3$\gamma$ as well as MUC2, and differences in the composition of microbiota [104]. Myd88-dependent expression of Reg3$\gamma$ is also particularly important against Gram-positive bacterial infections, such as *Listeria monocytogenes* [105].

*3.4. Maternal and Soluble Factors Participating to the Establishment of Tolerance in the Gut.* Immunological priming can start prenatally, and maternal immune-active components derived from the placenta can influence the development of the gut immune system [34]. Secretory antibodies, such as IgG, are mainly transferred *via* the placenta in human and mice during the prenatal period [106]. At birth, the immaturity of the gut renders the neonate particularly exposed to microbes, and additionally to all the mechanisms previously cited in this review, maternal factors transmitted to the neonate through breast milk bring supplementary immunoprotection and help for the development of the immune system. Of note, amniotic fluid contains similar components compared to colostrum and has been shown to favor immune tolerance towards colonizing microflora, and administration to preterm pigs delivered by caesarian-section is protective against necrotizing enterocolitis [107]. Human milk is composed of 40 g/L lipids, 8 g/L proteins, 70 g/L lactose, and 5–15 g/L oligosaccharides. Colostrum and early breast milk contain large amount of IgA, immune cells such as neutrophils, macrophages, colostral corpuscules and lymphocytes, as well as soluble mediators such as cytokines (interleukins, INF-$\gamma$, TGF-$\beta$, etc.), hormones and growth factors (insulin, EGF, VEGF, CSF, etc.), nonspecific immune factors (oligosaccharides, lactoferrin, lysozyme, etc.) and even certain microRNAs [108–111]. In humans, a breast-fed infant consumes around $10^8$ immune cells per day, consisting of 55–60% macrophages, 30–40% neutrophils, and 5–10% lymphocytes. Maternal macrophages persist in the lumen of the neonate's gut during the first postnatal week and have even been found in the systemic circulation [112]. Also, maternal milk participates to the maturation of adaptive immune system since microRNAs associated with T-cell and B-cell differentiation have been detected [111].

As pointed before, analyses of the intestinal microbes of breast-fed human infants revealed that maternal milk also plays a role in shaping the microbiota. The breast-fed neonate is provided with 0.25–0.5 grams per day of secretory IgA antibodies *via* absorption of maternal milk. Maternal IgA restrict immune activation and microbial attachment by binding nutritional and microbial antigens. The appearance of IgA secreted by the neonate is correlated with weaning and plasma cells maturation. Of note, the specific deletion of Myd88 in intestinal epithelial cells induces a downregulation of polymeric immunoglobulin receptor, the epithelial IgA transporter, underlying the importance of Myd88 signaling in gut homeostasis [104]. Lactoferrin limits the pool of free iron and suppresses bacterial growth. Interestingly, miR-584 has been shown to induce the expression of the lactoferrin receptor in epithelial cells during the neonatal period [113]. The presence of oligosaccharides that can be utilized by specific bacteria such as *Bifidobacterium longum* spp. *infantis* also influences the composition of the neonatal microflora [114]. Interestingly, functional similarities between mammalian milk and crop "milk" produced by pigeons, flamingos, and emperor penguins to feed their young have been shown not only at the nutrition level, but also for the establishment of the immune system of the neonate and the maturation of the microbiota [115], which is particularly compelling from an evolutionary point of view. Thus, maternal milk contains factors to help the neonate to establish the microflora and to fight against pathogens. On the other hand, it also has been shown that it contains living bacteria (<3 log cfu/mL) and a range of bacterial components such as bacterial DNA [116]. Indeed, bacterial translocation from the mouse gut is increased during pregnancy and lactation, and bacterially loaded dendritic cells in the milk are thought to contribute to neonatal immune imprinting [117].

## 4. Neonatal Innate Immune Response, Infections, and Sepsis

*4.1. Immune Stimulation, Epithelial Barrier Disruption, and Alteration of Microbiota.* Proper development of immune tolerance is necessary for the maintenance of gut homeostasis and an efficient response against pathogens. Dysregulations of the mechanisms involved cannot only lead to inappropriate intestinal inflammation against microbiota such as inflammatory bowel diseases in some cases, but can also increase the susceptibility to bacterial infections and lead to neonatal sepsis [118]. In both human and mice, the immune response towards infections in the neonate is generally reduced compared to the adult response. Defects in mucosal immunity or even a response to infectious challenge can result in a dysbiosis, characterized by an altered commensal colonization of the gut. The disruption is usually a transient phenomenon, which is solved with the resolution of the infection and characterized by the return of the changed microbiota to baseline. However, some pathogens cleverly exploit host immunity to favor their invasion, such as *Salmonella* Typhimurium, *Citrobacter rodentium,* or *Campylobacter jejuni* [119]. The host response drives the disruption of the microbiota which enhances pathogen colonization and persistence, suggesting that host innate responses select for a characteristic microbiota composition.

Pathogenic bacteria also developed species-specific mechanisms to cross the epithelial barrier though their interaction with host cell receptors. For example, *Salmonella* Typhimurium utilizes the receptor for epidermal growth factor (EGF) of epithelial cells, and the entry of the bacteria is coincident with tyrosine phosphorylation of the receptor. Also, both *Salmonella* Typhimurium and EGF induce patterns of host tyrosine phosphorylations that are remarkably similar [120]. *Salmonella* Typhimurium has also developed innate immune evasion mechanism such as O-antigen expression during apical intestinal epithelial invasion which delays the recognition of LPS by TLR4 [121]. In contrast, *Listeria monocytogenes* enters the enterocyte by using a zipper mechanism [122]. The mechanisms used by invasive *Escherichia coli* are different, and their capacity to inhibit NF-$\kappa$B activity allows them to damp the inflammatory response of the host [123]. Inflammation leads to production of nitric oxide, which is known to alter expression and localization of the tight protein zonulin ZO-1, ZO-2, ZO-3, and occludin, and increases epithelium permeability which favors bacterial translocation [124]. As pointed before, bacterial translocation is also observed under physiological conditions, since systemic bacterial DNA has been detected in healthy volunteers, suggesting a role for bacterial translocation in the development of the immune system [125, 126].

The immaturity of the neonatal immune system explains the age-dependent differences of the immune responses against pathogens as well as the susceptibility to different type of infections. For example, newborns are highly susceptible to *Shigella flexneri*, the causative agent of human bacillary dysentery, due to the lack of Paneth cells during early postnatal development. Also, MMP7-deficient mice show an increased inflammation and higher bacterial load after oral infection compared to wild type [127]. Similarly, the susceptibility to rotavirus is restricted to children under the age of 6 in human and is highest in between day 3 and 11 in mice. Interestingly, an upregulation during infancy of TLR3 expression on intestinal epithelial cells, which are the prime target of rotavirus, has been observed. This increase might contribute to the age-dependent susceptibility to rotavirus infection [62, 128]. Also, neonates have been shown to be more prone to *Salmonella* Typhimurium infections, correlating with an age-dependent increase of INF-$\gamma$, important for epithelial defense against intracellular pathogens [129].

*4.2. Microbial Pathogenesis of Neonatal Sepsis.* Neonatal sepsis (NS) is a major cause of morbidity and mortality among newborn infants, occurring in 1 to 10 per 1000 newborns, with a mortality rate of 15 to 50% [130, 131]. More than 10% of the neonates develop an infection during the first month of their life [132]. Despite the fact that symptoms are the same as an adult sepsis, the immune response of the neonate during NS is different. Due to the fact that the adaptive immune system of the neonate is not mature, the response induced is controlled by the innate immune system [133, 134]. Besides, no association was found between the TNF-$\alpha$-308 G/A

polymorphism blood culture-proven sepsis in very low birth weight infants, whereas the TNF-$\alpha$-308 A allele is associated with higher sepsis in adult [135]. NS is clinically associated with a systemic infection during the 4 first weeks after birth, which can lead to pneumonia or meningitis. According to the time of symptoms appearance, NS is considered as early-onset neonatal sepsis (EONS) during the first 72 hours after birth, or late-onset neonatal sepsis (LONS) afterwards. This distinction largely contributed to improve the diagnostics and the treatment of this pathology, particularly because of the identification of the causative microorganism which varies depending on the age of the infant and also because of the origin of the infection depending on the time of appearance.

A number of pathogens have been associated with NS and the predominant agents are bacterial, but viruses including herpes simplex and enteroviruses have also been associated with fulminant neonatal sepsis with high mortality [136, 137]. Causative agents for EONS are mostly microorganisms colonizing the maternal genital tract [138]. The most frequent microorganisms involved in EONS are Coagulase-negative *staphylococci* (CoNS), Group B *streptococci* (GBS), *Escherichia coli*, *Listeria monocytogenes*, and *Haemophilus influenzae* [138–140]. LONS are mostly caused by microorganisms from the external environment, often carried by care staff or by horizontal transmission [141]. The most frequent microorganisms involved in LONS are GBS, CoNS, and *Enterobacteriaceae*, including *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* [138, 142].

CoNS are responsible for 50% of LONS and more than half of very low birth weight infants are infected with CoNS, unlike full-term infants [143]. The recognition of CoNS by the innate immune system may have serious implications for preterm infants, since, for example, the expression of TLR4, although similar on term neonatal and adult monocytes [144], is significantly reduced in preterm infants [145]. The formation of biofilms by CoNS is essential for the pathogenicity of the bacteria by protecting themselves against host defense [146]. The polysaccharide intercellular adhesin (restricted to a subpopulation of *Staphylococci epidermidis*) and the poly-g-DL-glutamic acid (ubiquitous among *Staphylococci epidermidis* strains) protect the bacteria against cathelicidin and human $\beta$-defensin 3 [147, 148]. Moreover, the immaturity of the neonatal complement system impairs the capacity of neonates to fight against biofilm-associated *Staphylococci epidermidis* infections [149]. Interestingly, a study, dealing with the effects of lactoferrin with antibiotics commonly used in neonatal practice against CoNS, shows a synergic action, demonstrating that lactoferrin may be a promising agent to improve treatments of NS caused by CoNS [150].

If GBS is a component of the normal mucosal flora, in contrast, invasive GBS disease constitutes a rare event for which neonates present an increased risk. Neonates respond with a powerful inflammatory cytokine response to GBS and exhibit at the same time several deficiencies of components of the clearance mechanisms [151, 152]. Host factors underlying postnatal maturation and directly influencing elimination of GBS are likely to contribute to GBS sepsis in newborns. Extracellular GBS lipoproteins are known to interact with TLR2/6 and thereby contribute to GBS sepsis pathogenesis, and Myd88-dependent signaling is essential in innate immune response against GBS. Also, type I interferons (IFNs) and IFN-$\gamma$, IL-6, IL-12, and IL-18 contribute significantly to the course of GBS neonatal sepsis [153, 154]. The recognition of GBS and other Gram-positive bacteria by macrophages and monocytes relies on bacterial single-stranded RNA and phagocytosis induced NO in a Myd88 and UNC-93B-dependent manner but independently of known nucleotide-sensing TLRs [155, 156]. Recently, the activation of NLRP3 inflammasome, leading to production of IL-1$\beta$ and IL-18, was also shown to be involved in host defenses against this pathogen [157].

*Listeria monocytogenes* represents an opportunistic pathogen which mainly infects immunocompromised patients, pregnant woman, elderly persons, and neonates [158]. In neonatal infections, *Listeria monocytogenes* can be transmitted from mother to child *in utero* or during vaginal delivery. In a neonatal mouse model, neonatal mice overproduce IL-10 during infection, which is known to trigger a detrimental effect [159]. Indeed, IL-10 blockade in neonates is protective during both early and late infection, whereas this effect is only observed at early stage in adult mice [160]. As pointed before, the cathelicidin CRAMP is highly expressed during the neonatal period and plays a prominent role in the protection of the newborn against pathogenic enteric bacteria, and particularly against *Listeria monocytogenes* [98]. Activation of the PI3 kinase and Rac1 *via* a TLR2-MyD88-dependent pathway facilitates the phagocytosis of *Listeria monocytogenes* by murine macrophages. In intestinal epithelial cells, *Listeria monocytogenes* is recognized by immune receptors such as Nod2 and Ipaf, and the NADPH oxidase (Nox) 4-dependent production of reactive oxygen species (ROS) allows horizontal intercellular communication. This mechanism favors the amplification of the immune response against bacteria [161].

Some recent studies have highlighted a significant reduction in GBS EONS with the increased use of prophylactic antibiotics, leading to an increase of rates of non-GBS infection and particularly an increase in EONS caused by *Escherichia coli* [140]. Enteropathogenic *Escherichia coli* (EPEC) destroys intestinal microvilli and suppresses phagocytosis to facilitate efficient infection [162]. The bacterial protein Hek, which promotes adherence to and invasion into cultured epithelial cells, has a key role in the transcytosis of *Escherichia coli* across the intestinal epithelial barrier [163]. Moreover, gut barrier dysfunction was recently shown to be mediated by an increase of HMGB1 following LPS administration, supporting the deleterious effect of *Escherichia coli* on bacterial translocation and potentially sepsis [164]. In a murine model of enterotoxigenic *Escherichia coli* (ETEC) infection, pretreatment with lactoferrin led to nearly full protection of gut-associated tissue, intact microvilli and decreases of activated cells [165]. Lactoferrin has also an inhibitory effect on the adherence of ETEC to epithelial cells [166].

*4.3. Factors Involved in the Pathogenesis of Neonatal Sepsis.* Commonly, associated risk factors to NS are maternal and environmental exposure, immune status, as well as the weight

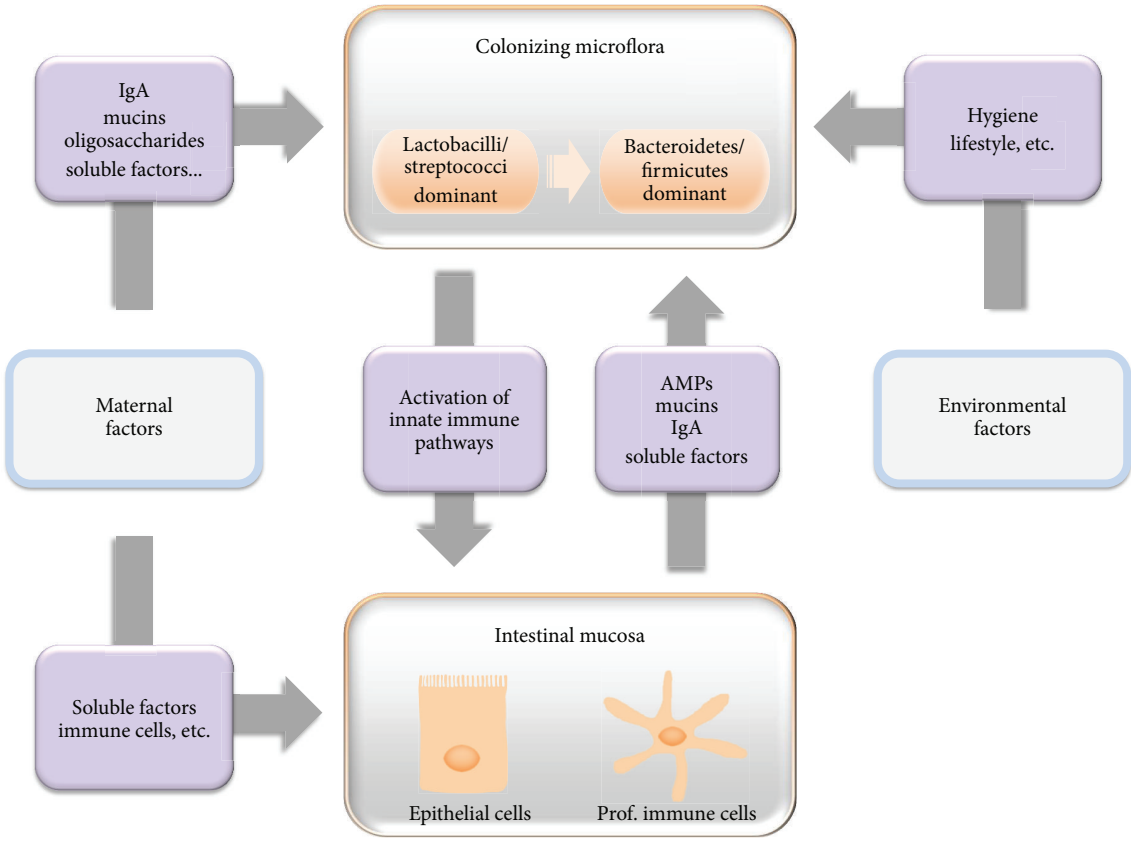


Figure 2: Factors involved in postnatal intestinal innate immune adaptation. The colonizing microflora and the intestinal mucosa interact in a two-way street to establish life-time tolerance and mutualism between each other. While bacteria activate innate immune pathways in host epithelial and immune cells inducing immune maturation and tolerance, mucosal cells produce factors (antimicrobial peptides AMPs, mucins, immunoglobulin A IgA, etc.) to control the number and the composition of bacteria. Microflora is also influenced by environmental factors (hygiene, lifestyle, etc.). Maternal factors, such as IgA, mucins, oligosaccharides, or other soluble factors, can modulate the microflora, and contribute to improve host immune defense and maturation (maternal immune cells, soluble factors, etc.).

of the neonate at birth, and the time of the gestation period, making preterm and very low birth weight infants (<1500 g at birth) particularly susceptible [140]. The integrity of the intestinal barrier is a must to prevent the dissemination of microorganisms in the systemic compartment. Moreover, the level of permeability of the gut plays a key role in the pathogenesis of inflammatory pathologies such as ischemia-reperfusion or necrotizing enterocolitis, another inflammatory disorder leading to necrosis of the gut in neonates and particularly preterm infants [38, 167, 168]. Of note, an association between *Pseudomonas aeruginosa* sepsis and necrotizing enterocolitis has been shown [169]. Disruption of the neonatal barrier can be due to antibiotic treatment, hypoxia, or remote infection [170, 171].

Dysbiosis of the intestinal microbiota also predisposes the intestine of neonates to inflammation. Indeed, many operational taxonomic units, which are frequently detected in healthy controls, were not detected in LONS cases [172]. This data suggests that a lack of colonization by various normal or nonpathogenic bacteria, rather than the presence of a pathogen, might increase the risk of LONS. Based on this finding, it has been proposed that a delay in colonization by proteobacteria, which is normal and immunologically well tolerated during the initial weeks of microbiota development, might result in an excessive immune response that compromises the integrity of the mucosal barrier, thereby allowing translocation of bacteria into the circulation resulting in LONS and extensive inflammation. Moreover, neonates developing sepsis present a low microbial diversity compared to healthy infants [173]. The specific mechanisms linking the intestinal microbial changes to sepsis remain unclear, but recent studies favor the idea that disruption of the normal intestinal microbiota and induction of the inflammasome are potential mechanisms [174, 175]. Moreover, the number of *Bifidobacteria*,known to colonize the healthy newborn intestine soon after birth and likely contribute to normal intestinal development, is lower in LONS infants compared to healthy controls [172, 176].

A protective effect of human breast milk against infection and sepsis/meningitis in very low birth weight as well as in full-term infants has been described [177]. The large amount of glycans in the milk seems to protect the neonate from many bacterial, viral, fungal, and other pathogens [178, 179]. Secretory IgA of the milk is known to inhibit the association of bacteria with the gut mucosa and reduce bacterial penetration in the gut. In neonates, IgA supplementation is known

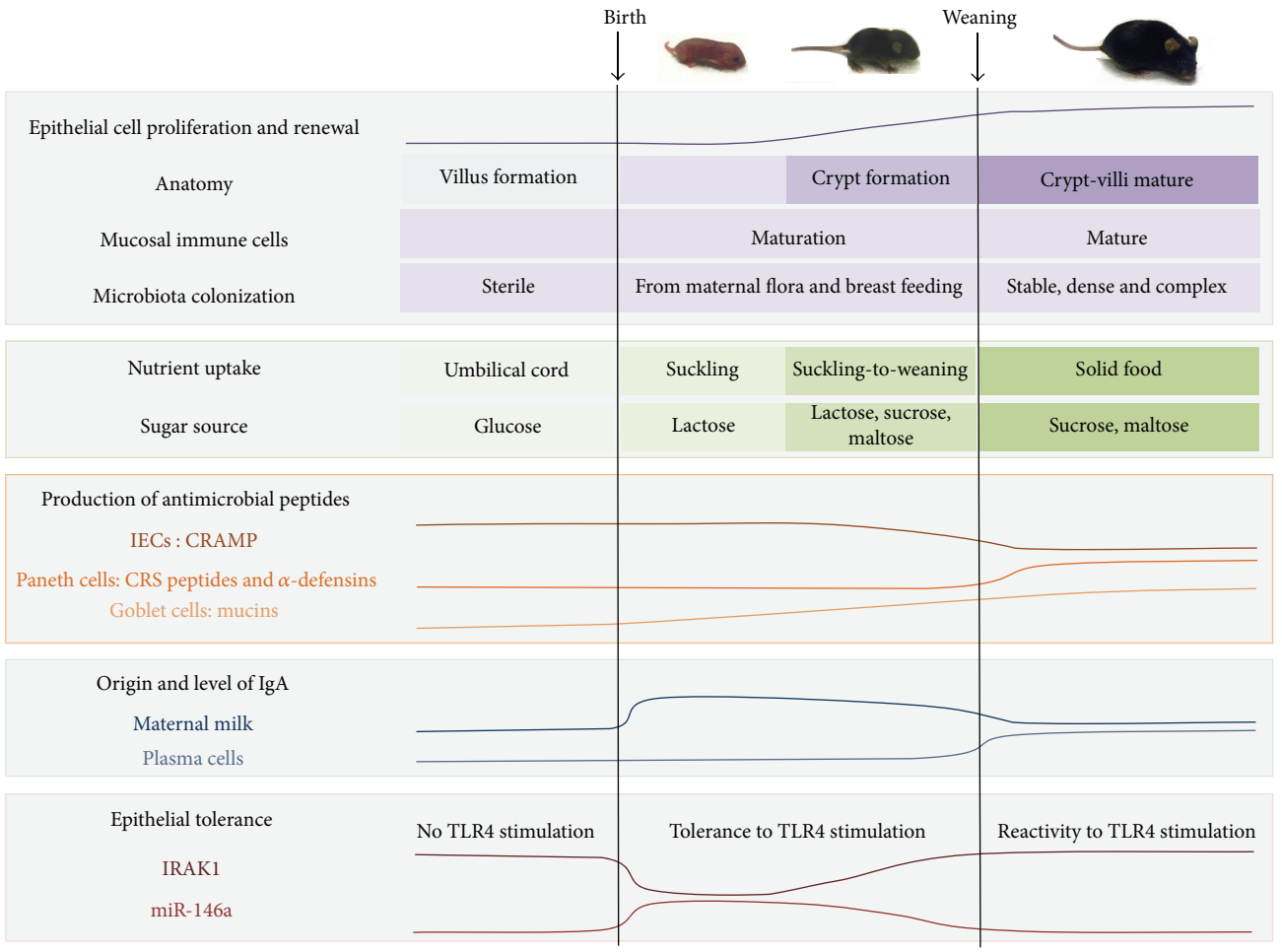


Figure 3: Summary of changes taking place in the intestine during the fetal period, neonatal period, and adult period. Please refer to the text for details. IECs: intestinal epithelial cells: CRAMP: cathelin-related antimicrobial peptide; CRS: cryptdin-related sequence; IgA: immunoglobulin A; TLR: toll-like receptor; IRAK1: interleukin-1 receptor-associated kinase 1; miR-146a: microRNA 146a.

to avoid bacterial translocation by enhancing gut mucosal barrier function and therefore neonatal gut-origin sepsis [180, 181]. As pointed before, lactoferrin, which is present in human milk, is a component of innate immunity and has antimicrobial activity. In several *in vitro* and *in vivo* models, lactoferrin shows potent protective effect on infections with enteric microorganisms, such as *Staphylococci epidermidis*, *Escherichia coli*, and rotavirus [182–184]. Moreover, a recent study has shown the beneficial effects of oral lactoferrin prophylaxis for the prevention of sepsis and necrotizing enterocolitis in preterm infants [185].

The pathology of sepsis involves highly complex interactions between pathogens, immune response of the host, and multiple downstream events leading to organ dysfunction and death. Studies in twins suggest that genetic factors are also involved and contribute to variations in susceptibility to infections. Candidate genes have been suggested to play a role in the pathogenesis of sepsis [186, 187]. Several polymorphisms associated with neonatal sepsis have been identified in genes playing a role in host innate immunity: the phospholipase A2, the pattern recognition receptors TLR2 and TLR5, the anti-inflammatory cytokine IL-10, and the serum mannose-binding lectin (MBL) [188, 189]. Moreover, mutations of genes also involved in the innate immune system have been associated with sepsis in very low birth weight infants, such as CD14, TLR4, NOD2, IL-6, and MBL [190]. Identification of these genetic variations may allow the development of new diagnostic tools and more accurate predictors, as wells as the improvement of the classification of sepsis.

## 5. Conclusion

Outside the uterus, the neonate, which is a unique host immunologically, is exposed to environmental microbes and endotoxins. Immune adaptation of the gut to extrauterine life is extremely important and complex (Figures 2 and 3). External factors such as breast feeding, environment, or delivery mode, as well as genetic factors influence this process which is largely microbiota dependent. The microbiota is an essential complex and multifunction ecosystem which functions as an extra organ, shaping the immune system of the host, and is by itself sculpted by the host immunity. Indeed, interactions between microbiota/microbes components and

intestinal epithelial cells largely drive the establishment of homeostasis during the neonatal period and also allow its maintenance during adult life. Pattern recognition receptors, seen initially as the first sentinel in the fight against microbes, play also obviously a major role in the tolerogenic response, and their signaling needs to be tightly fine-tuned spatially and temporally. Since the same receptors can have both beneficial and deleterious effects, the understanding of these aspects requires sustained efforts and probably more work on the animal models used currently, as well as on the tools to study the microbiota. Breaking the balance between all the players of the immune tolerance can induce inflammatory diseases and an increased susceptibility to infections. In neonates, the failure to establish immune tolerance leads to important mortality and morbidity and is particularly crucial for the survival of premature infants that are even more susceptible to infections and sepsis. Despite major advances in neonatal intensive care, infections and sepsis continue to be an important cause of death. A better understanding of the key mechanisms involved to establish and keep the balance between all the players of the immune tolerance will allow the discovery of efficient therapeutic and prophylactic tools to improve the medical care of those infants.

## Conflict of Interests

The authors declare that they have no conflict of interests.

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*Review Article*

# Role of Pore-Forming Toxins in Neonatal Sepsis

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Protein toxins are important virulence factors contributing to neonatal sepsis. The major pathogens of neonatal sepsis, group B Streptococci, *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus*, secrete toxins of different molecular nature, which are key for defining the disease. Amongst these toxins are pore-forming exotoxins that are expressed as soluble monomers prior to engagement of the target cell membrane with subsequent formation of an aqueous membrane pore. Membrane pore formation is not only a means for immediate lysis of the targeted cell but also a general mechanism that contributes to penetration of epithelial barriers and evasion of the immune system, thus creating survival niches for the pathogens. Pore-forming toxins, however, can also contribute to the induction of inflammation and hence to the manifestation of sepsis. Clearly, pore-forming toxins are not the sole factors that drive sepsis progression, but they often act in concert with other bacterial effectors, especially in the initial stages of neonatal sepsis manifestation.

## 1. Introduction

The birth canal, that is, the area between the fully dilated uterus and the outside of the vagina, harbours a polymicrobial community that may fulfil the definition of a biofilm [1]. Next to apathogenic species, such as *Lactobacillus* spp., potentially pathogenic bacteria including group B streptococci (GBS), *Escherichia coli* (*E. coli*), *Listeria monocytogenes* (*L. monocytogenes*), and *Staphylococcus aureus* (*S. aureus*) are found in the vagina of up to 20% of women. During birth, the fetus needs to pass from the sterile uterus through these bacteria. Accordingly, aspiration of bacteria during birth is regarded as a major cause of neonatal sepsis in the first three to seven days of life (early-onset sepsis). In line with this model, early-onset sepsis is predominantly caused by GBS, *E. coli*, *L. monocytogenes*, and *S. aureus*. Yet most infants successfully control the bacteria at the mucocutaneous surfaces.

Subsequent to aspiration, bacteria like *GBS* can proliferate to striking densities in the neonatal lung, as shown in newborn primates with neonatal GBS pneumonia ($10^9$–$10^{11}$ colony-forming units (CFUs)/g lung tissue, [2]). The antimicrobial quality of the local pulmonary environment, for example, the concentration of surfactant, may be important for the metabolic activity in the bacterial community and therefore for the expression of bacterial virulence factors such as bacterial toxins [3].

Sepsis imposes a major threat to newborn infants. It is estimated that sepsis causes over half a million neonatal deaths annually, thereby accounting for about 15% of all neonatal deaths worldwide [4]. Whereas sepsis causes approximately 2.5% of infant deaths in developed countries, it is responsible for up to 50% of neonatal deaths in developing countries [5, 6]. Moreover, neonatal sepsis often occurs as meningoencephalitis, which leaves almost 50% of affected patients with lifelong disabilities [7]. On the other hand, GBS, *E. coli*, and *S. aureus* are normal components of the mucocutaneous microbiome, and it is impossible to predict the risk to an individual baby.

## 2. Bacterial Membrane-Damaging Toxins

The first membrane-damaging bacterial toxin was described by Paul Ehrlich in 1898 [8], who found that *Clostridium tetani* extracts lyse erythrocytes. Today, three different mechanisms of membrane damage by proteinaceous agents can be delineated. First, toxins can solubilise target membranes

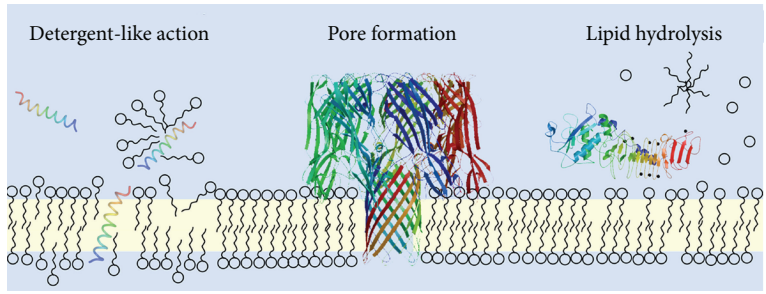


Figure 1: Ways to damage a lipid membrane. There are various mechanisms of membrane damage by protein toxins. Amphiphilic toxins can integrate into the membrane and essentially solubilise the lipid membrane like a detergent (structure: *S. aureus* $\delta$-toxin, PDB ID 2KAM). Similarly, the membrane lipids can be hydrolysed by phospholipases also resulting in the destruction of the membrane (structure: *Clostridium, perfringens* $\alpha$-toxin, PDB ID 1KHO, [165]). By far the largest class of membrane damaging toxins is that of the pore-forming toxins (structure: $\alpha$-toxin from *S. aureus*, PDB ID 7AHL, [100]). These toxins integrate as stable channels into the lipid bilayer, thus creating an aqueous connection between the cytosol and the extracellular space of the target cell.

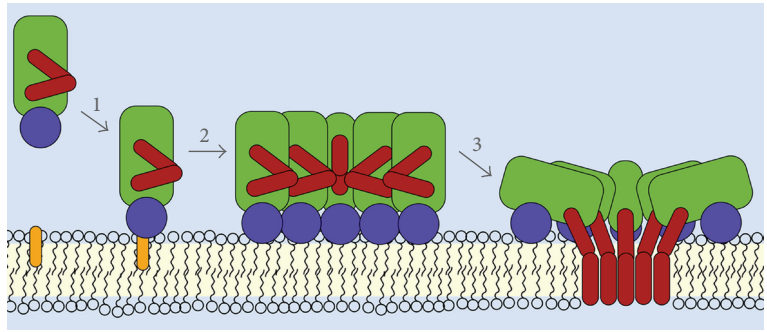


Figure 2: Pore formation is a dynamic process with structurally and functionally distinct states. Distinct molecular states exist on the path to membrane pore formation by PFTs. The toxin is secreted by the bacterial pathogen into the extracellular medium in a water-soluble form, usually as a monomer. Upon engagement of the membrane via binding to a receptor (step 1), for example, a membrane lipid or protein, the monomers assemble to a prepore oligomer (step 2). The membrane beneath the prepore oligomer remains intact and is only punctured once the prepore refolds to the membrane-inserted pore oligomer (step 3). This step usually goes along with considerable structural rearrangements.

acting essentially as amphiphilic surfactants. $\delta$-Toxins from various staphylococcal species [9, 10] and the cyclolipopeptides from *Bacillus subtilis* are prominent examples [11] (see Figure 1). Second, toxins can act as phospholipases and damage membranes by enzymatic hydrolysis of phospholipid ester bonds. $\beta$-Hemolysin from *S. aureus*, for instance, is a sphingomyelin-specific phospholipase, which cleaves sphingomyelin to ceramide and phosphorylcholine. However, the large majority of membrane damaging proteins belong to the class of pore-forming proteins/toxins (PFTs). PFTs, which make up approximately 30% of all protein toxins in pathogenic bacteria [12], have evolved in all domains of life. They are secreted as water-soluble proteins and subsequently integrate into foreign membranes.

## 3. Mechanism of Membrane Pore Formation by Pore-Forming Toxins

Common structural themes of protein/membrane association are insertion of transmembrane $\alpha$-helices or $\beta$-sheet barrel arrangements, anchoring by prosthetic glycolipids, or direct linkage to hydrophobic lipid tails, such as myristic or palmitic acid. Most pores or channels allowing for communication across biological membranes are formed by integral membrane proteins spanning the lipid bilayer. However, PFTs form pores by acting initially extraneously of the lipid bilayer. They start out as soluble molecules and then turn themselves into integral membrane proteins, with a membrane-spanning region that defines the pore. Pore formation is a dynamic process with structurally and functionally distinct states (see Figure 2). Initial binding to the membrane, for example, to a membrane lipid or protein receptor, is followed by homotypic oligomerisation to a prepore state on the membrane surface (Figure 2, arrows 1 and 2). In this state, the protomer configuration resembles that of the soluble monomer, and the whole oligomer still stands prone to the membrane with an intact lipid bilayer beneath the assembled ring. In Figure 3(a1), this is depicted for pneumolysin from *Streptococcus pneumoniae*, a close homologue of listeriolysin from *L. monocytogenes* and, interestingly, also of perforin secreted by cytotoxic T cells [13], and of the complement membrane attack complex [14, 15], which indicates that bacterial attack and immune defence employ the same mechanisms. This prepore state

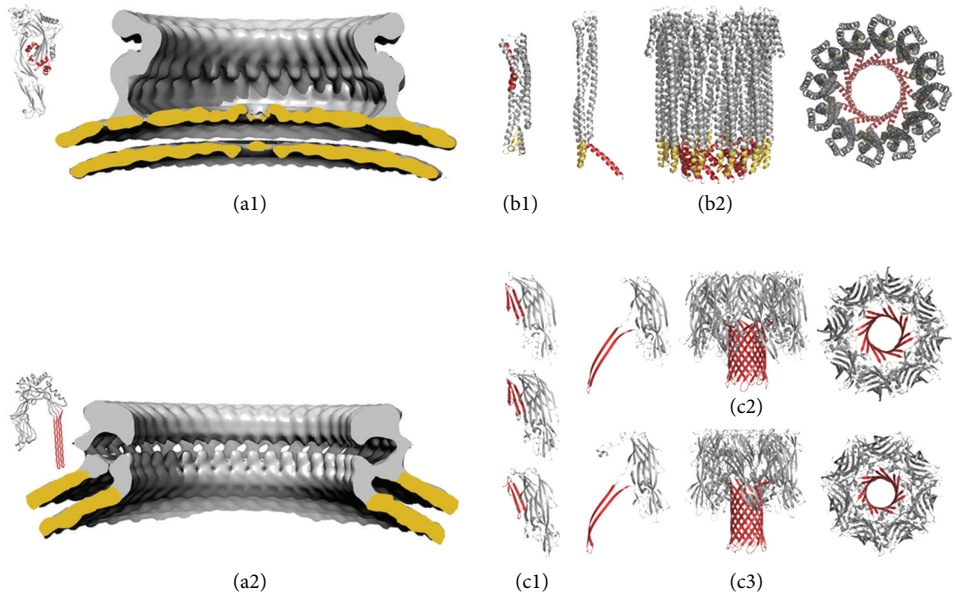


Figure 3: Structures of PFTs that are important for neonatal sepsis. Panel (a) shows available structures of cholesterol-dependent cytolysins to illustrate listeriolysins' mechanism of pore formation. (a1) displays the crystal structure of the soluble, monomeric form of perfringolysin from *Clostridium perfringens* (left, PDB ID 1PFO, [57]). The cryo-electron microscopy (cryo-EM) reconstruction of the prepore (EM databank: 1106) of the listeriolysin homologue pneumolysin from *Streptococcus pneumoniae* displayed on the right revealed that the protomer configuration in the prepore resembles that of the soluble monomer [16]. Lipid membrane is coloured yellow. Molecular modeling of the protomer fitted into the cryo-EM pore structure below (EM databank: 1107) revealed the considerable structural rearrangements that accompany membrane pore formation. The $\alpha$-helices that refold into $\beta$-sheets are coloured in red. Panel (b) shows the different structures available for ClyA from *E. coli*, (b1) the soluble state (PDDid 1QOY, [148]) monomer and (b2) a protomer from the dodecameric pore state, which is shown as side and top view on the right (PDB ID 2WCD, [149]). Pore-lining $\alpha$-helices are in red and the $\beta$-tongue in yellow. Panel (c) shows the PFTs from *S. aureus*. (c1) shows from top to bottom LukF (PDB ID 1LKF, [113]), LukF-PV (PDB ID 1PVL, [114]), and LukS-PV (PDB ID 1T5R, [115]). (c2) shows the octameric pore structure of $\gamma$-hemolysin (PDB ID 3B07, [116]), protomer on the left, side and top views on the right. (c3) displays the heptameric pore structure of the AFT pore (PDB ID 7AHL, [100]), individual protomer, side and tops views. The $\beta$-stem that unfolds into the membrane lining, extended $\beta$-hairpin is shown in red.

then undergoes drastic conformational rearrangements to be inserted as a stable pore into the membrane (see Figure 2, arrow 3). This rearrangement can even involve the refolding of $\alpha$-helices in the soluble state to $\beta$-sheets in the membrane-inserted form [16]. While this general mechanism of pore formation can be proposed for nearly all PFTs, the structural changes, which the individual proteins undergo, remain largely elusive. Detailed mechanistic models on how hydrophilic proteins can suddenly change their solubility and integrate into biological membranes are available only for a few PFTs involved in neonatal sepsis (see Figure 3). Not surprising when considering that one not only needs to be able to study the solution state in sufficient structural detail, for example, via X-ray crystallography, but the structure of the membrane state also needs to be resolved. PFTs are classically divided into two main groups based on the structural motifs that form the pore [17–19]. Pores can be formed by $\alpha$-helices, $\alpha$-PFTs or by $\beta$-sheets, $\beta$-PFTs. For certain pore-forming proteins, it was recently proposed that lipids might play a direct role in pore formation, but our understanding of how this can be achieved is limited by the available structural data to date [20–22]. The structures, where known, of the soluble and membrane states of the PFTs discussed in this review are displayed in Figure 3. However, not all membrane pores are equal. The pore diameter, for instance, of the $\alpha$-hemolysin membrane heptamer is considerably smaller than that of listeriolysin with up to 50 protomers (cf. Figures 3(a) and 3(c)). Clearly, the size of the membrane pore has important consequences for the targeted cell, as a large pore diameter is not selective for what it can conduct across a membrane, potentially mediating diffusion of larger molecules, such as ATP or even small proteins.

Interestingly, clearing of toxin pores from host membranes, a mechanism that is of marked importance when considering the amounts of toxin that are produced during sepsis, seems also to be size dependent. Cholesterol-dependent cytolysins, such as listeriolysin, can induce $Ca^{2+}$-dependent resealing of membrane pores by induction of endocytosis [23–25] but $\alpha$-hemolysin from *S. aureus* cannot [26]. This is counterintuitive, as van der Goot and coworkers nicely state [27] that small pores are harder to repair than larger ones.

## 4. Bacterial Pore-Forming Toxins in Pathogens Causing Neonatal Sepsis

In the major pathogens isolated from newborn infants with sepsis, PFTs are key virulence factors. They initiate a multitude of events ranging from direct necrotic cell deaths

to the induction of signalling cascades, for instance, $Ca^{2+}$-mediated signalling [27]. Prominent PFTs in the context of neonatal sepsis are listeriolysin O from *L. monocytogenes*, $\beta$-hemolysin/cytolysin from GBS (*Streptococcus agalactiae*), $\alpha$-hemolysin and cytolysin A from *E. coli*, and $\alpha$-hemolysin, $\gamma$-hemolysin, and the leukocidins from *S. aureus*. It has long been appreciated that PFTs are especially important during initiation of bacterial infections through induction of necrosis and apoptosis of host epithelial and endothelial cells, which promotes microbial invasion and subverts defence mechanisms. However, PFTs may also contribute to sepsis by receptor mediated or membrane damaging mechanisms in immune cells, which respond with the formation of inflammatory mediators, as was shown for $\beta$-hemolysin/cytolysin from GBS [28] and listeriolysin O [29, 30] and $\alpha$-hemolysin and Panton-Valentine leukocidins from *S. aureus* [31, 32].

## 5. $\beta$-Hemolysin/Cytolysin from Group B Streptococci

*Streptococcus agalactiae* (group B streptococci, GBS) are the major cause of sepsis and meningitis in newborn infants without underlying disease in the western world. In addition, they are a significant cause of invasive infections in pregnant woman and immuneocompromised patients [33, 34]. The pore-forming toxin $\beta$-hemolysin/cytolysin is one of the main virulence factors of GBS. It has been implicated in the pathogenesis of early- [35] and late-onset neonatal sepsis, although its role in both cases remains controversial. Rabbits infected with wild-type GBS had significantly higher bacterial blood counts than those infected with GBS mutants lacking the $\beta$-hemolysin/cytolysin [35]; mortality also increased dramatically. Similarly, in a neonatal rat model of meningitis wild-type GBS induced more neuronal damage in the cortex and the hippocampus than cytolysin-deficient mutants [36]. The clinical outcome score, assessed in this study by weighted changes and motor activity, decreased profoundly upon presence of the cytolysin. The $\beta$-hemolysin/cytolysin lytic protein agent is thought to be encoded by the cylE gene of the cyl operon [37], since expression of cylE induces $\beta$-hemolytic activity in nonhemolytic *E. coli*. However, the exact molecular nature of the protein component responsible for hemolysis and pore formation remains obscure, as it has evaded purification to homogeneity as of yet. Interestingly, $\beta$-hemolysin/cytolysin is also necessary for the synthesis of an orange carotenoid pigment [38], with which it also associates [39]. Hemolytic activity of partially purified $\beta$-hemolytic activity containing the carotenoid pigment could be inhibited by addition of the lipid dipalmitoylphosphatidylcholine (DPPC), the major component of surfactant in the lung. Accordingly, surfactant deficiency may explain in part the particular susceptibility of preterm infants to GBS sepsis and meningitis [40, 41]. As outlined above, breaching of epithelial barriers by GBS is the first step in sepsis pathogenesis. Accordingly, it appears to be important that $\beta$-hemolysin/cytolysin mediates not only injury of lung epithelial [40] and of lung microvascular endothelial cells [42] and invasion of brain endothelial cells [43] but also injury of professional phagocytes [39] and neurons [36]. Moreover, GBS mutants lacking clyE were more readily cleared from mouse and human blood [39]. Interestingly, Rubens and colleagues initially proposed that $\beta$-hemolysin/cytolysin was no longer needed for systemic disease manifestation once the epithelial barriers have been breached [44]. However, pro- and anti-inflammatory activity of $\beta$-hemolysin/cytolysin in macrophages was demonstrated, indicating more profound immunomodulatory functions of the cytolysin [45, 46]. One direct or indirect molecular $\beta$-hemolysin/cytolysin target with important implications for mononuclear phagocyte activation is the NLPRP3 inflammasome. Activation of the NLRP3 inflammasome requires GBS expressing $\beta$-hemolysin/cytolysin. This pathway is essential in a mouse model of GBS sepsis, where deficiency in NLRP3 or its signalling partners apoptosis-associated speck-like protein and caspase-1 increases lethality and bacterial dissemination [28]. Yet direct evidence for binding, engagement, and activation of TLRs by the $\beta$-hemolysin/cytolysin is not available.

A second pore-forming toxin of GBS is the CAMP factor that has long been used for microbiological identification of GBS, since it characteristically synergizes with secreted sphingomyelinase of *S. aureus* to lyse erythrocytes on blood agar plates [47–49]. However, its role in neonatal sepsis is not clear, as it was not required for systemic infection in a mouse model of GBS infection [50].

## 6. Listeriolysin O from *Listeria monocytogenes*

*Listeria monocytogenes* (*L. monocytogenes*) is a Gram-positive bacterium that causes early- and late-onset neonatal sepsis and meningitis. *L. monocytogenes* has the capacity to breach the intestinal barrier, thereby causing food-borne listeriosis, the blood-brain barrier, causing meningitis, and the maternal-placental barrier, causing early-onset listeriosis. Listeriolysin O (LLO), a member of the PFT class of cholesterol-dependent cytolysins (CDCs), is a major virulence factor of *L. monocytogenes* with multivalent functions [51]. In the late 1980s, Kathariou et al. and Portnoy et al. reported that *L. monocytogenes* mutants lacking functional LLO were avirulent in mice [52, 53]. Furthermore, LLO mutants did not induce secretion of cytokines such as TNF-$\alpha$, IL-1$\beta$, or IFN-$\gamma$, when injected intravenously into C57BL/6 mice [54]. Recently, a single-gene signature-tag-based approach was used to assess the contribution of individual amino acids of LLOs to its virulence in mice [55].

Based on structural homology with other toxins such as pneumolysin from *Streptococcus pneumoniae* [56] and perfringolysin from *Clostridium perfringens* [57], common pore-forming properties can be proposed [58] (see Figure 3(a)). LLO engages cholesterol as a native membrane receptor in dependence on the two amino acids threonine 515 and leucine 516, oligomerises to a prepore complex of up to 50 monomers, and forms a membrane pore in a concerted refolding step with each protomer contributing two beta-hairpins to the membrane-spanning $\beta$-barrel, which originates from five $\alpha$-helices in the soluble state [16, 59–61]. The

allosteric monomer assembly and prepore refolding process were recently shown to rely on an undecapeptide sequence (483-ECTGLAWEWWR-493), which was originally thought to be soley responsible for cholesterol binding [62]. The exact nature of the membrane pore remains controversial, as arciform pores, that is, membrane pores with a seemingly incomplete protein ring lining the aqueous membrane hole, are often observed in electron microscopic and atomic force microscopic imaging of various CDCs [22, 63–65]. Moreover, LLO does not lose its membrane targeting properties after incubation with cholesterol [66].

*Listeria* are classical intracellular pathogens [67] and LLO pore formation was traditionally thought to only mediate escape of *Listeria* from the phagolysosome [68]. This concept was based on the finding that LLO was active only at acidic and not at neutral pH, which is found in the maturing phagolysosome [58]. However, host factors also play an important part in regulating the activity of LLO in the phagolysosome. LLO hijacks the reductive capacity of the $\gamma$-interferon-inducible lysosomal thiol reductase GILT to maintain cysteine 484 of LLO in its reduced thiol state [69], thus greatly increasing bacterial escape from the phagolysosome. Intriguingly, CDCs were originally, that is, before the identification of cholesterol as a membrane receptor, termed sulfhydryl-activated [70] or thiol-activated, oxygen-labile cytolysins [71], as chemical reduction activated the toxins towards hemolysis of red blood cells. Additionally, the cystic fibrosis transmembrane conductance regulator (CFTR), which transports chloride not only across the apical plasma membrane of epithelial cells in the lung but also into the phagolysosomes of macrophages, potentiates LLO oligomerisation on the phagosomal membrane and its lytic activity and phagolysosomal escape of *L. monocytogenes* [72]. However, the role of LLO extends beyond mediating phagosomal escape. LLO reduces formation of reactive oxygen species (ROS) by inhibiting the NADPH oxidase NOX2 in RAW 264.7 macrophages [73]. This activity seems to rely on pore formation in the phagosomal membrane and prevents degradation of bacteria inside the phagosome. Pore formation at the plasma membrane of target cells induces the dynamin-/F-actin-dependent but clathrin-independent uptake of *L. monocytogenes* into HepG2 cells [74]. This finding questions the traditional model of LLO pore-forming activity being strictly dependent on the low phagosomal pH [58], whereby premature lysis of target cells by (secreted) LLO is prevented [75]. Residual lytic activity and structural integrity of LLO at neutral pH [74, 76, 77] are in line with LLO-mediated calcium influx into epithelial Hep-2 [78] and HEK 293 cells [79] along with concomitant *L. monocytogenes* uptake. Indeed LLO seems to form pores at neutral pH in the plasma membrane, which do not result in lysis of the target cell but rather in uptake of the pathogen [74]. As pneumolysin from *S. pneumoniae* can replace LLO in the uptake of *L. monocytogenes* into HepG2 cells, CDCs from other bacterial pathogens may similarly induce cellular uptake. The contribution of TLR signalling in response to CDC has been subject of several studies. As examples, the LLO homologues anthrolysin (*Bacillus anthracis*) and pneumolysin can signal via TLR4 [80, 81]. On the other hand, LLO induces an inflammatory cellular response in a TLR-independent fashion [82]. Moreover, LLO activity at the plasma membrane induces clustering of lipid rafts [83], suppression of antigen-induced T-cell activation [84], inflammasome activation, and histone H3 dephosphorylation [30], all of which might contribute to sepsis progression either at the stage of heightened inflammation or at later stages of immune suppression.

## 7. $\alpha$-Hemolysin, $\gamma$-Hemolysin, and Leukocidins from *Staphylococcus aureus*

*Staphylococcus aureus* (*S. aureus*) is well recognised as a significant cause of neonatal sepsis [85]. Around ten bacteria are sufficient to colonise the umbilical cord. After birth, *S. aureus* can colonise the upper respiratory tract in up to 40% of infants [86]. *S. aureus* produces a number of PFTs with distinct specificity for target cell membranes. Although most clinical isolates produce the PFTs $\alpha$-hemolysin, bicomponent $\gamma$-hemolysins, and bicomponent leukocidins, none of these toxins was found to be a necessary and sufficient virulence determinant of neonatal sepsis. In contrast, other factors such as the antigenic, peptidoglycan-associated protein A [87], superantigens [88], and sphingomyelinase C ($\beta$-hemolysin, [89]) contribute to host invasion, subversion of the immune system, and sepsis manifestation. However, there is evidence that pore formation by $\alpha$-hemolysin ($\alpha$-toxin, AFT) contributes to the pathogenesis of sepsis [90]. As an example, erythrocyte lysis by AFT could be directly imaged [91]. Downregulation of AFT expression *in vivo* clearly reduces virulence of *S. aureus* [92, 93]. In a model using C57BL/6J mice, AFT activates the NLRP3 inflammasome, thereby promoting necrotising pneumonia [94]. Moreover, monoclonal antibodies to AFT are protective against staphylococcal pneumonia [95]. Indeed, a nonhemolytic variant of AFT was used to vaccinate rabbits, and antisera could be used to passively immunize mice against an otherwise lethal challenge with wild-type *S. aureus* [96]. In a mouse model of mastitis, coagulase and AFT proved to be the primary virulence determinants [97]. Heat-inactivated *S. aureus* and an AFT mutant greatly reduced the bacterial burdens in a mouse brain abscess model and attenuated the expression of inflammatory mediators [98]. In an *in vivo* model of corneal virulence, AFT also proved to be a decisive virulence factor [99].

AFT is one of the best-studied PFTs to date. It was the first toxin of which the membrane structure was solved by X-ray crystallography (see Figure 3(c3), [100]). AFT consists of 293 amino acids and oligomerises on the plasma membrane of target cells to a heptamer (potentially a hexamer) prior to membrane insertion and pore formation. It was shown to have an important role in bacterial pathogenesis, especially by its ability to induce necrotic cell death [90], by which it can cause vascular leakage when perfused into the lung [101]. Recently the metalloprotease ADAM10 was identified as the membrane receptor of AFT [102]. At low toxin concentrations, ADAM10 is required to mediate the cytotoxic effects of AFT. Interestingly, binding of AFT to ADAM10 resulted in the upregulation of ADAM10 in alveolar epithelial cells and concomitant cleavage of E-cadherin. This

leads to epithelial barrier disruption thereby aggravating staphylococcal pneumonia in mice [103]. Moreover, activation of the NLRP3-inflammasome by AFT might contribute to later stages of sepsis, although the molecular mechanism underlying inflammasome activation remains elusive at this stage [31]. In this respect, it is interesting to note that, whereas direct TLR activation has not been demonstrated for AFT, NOD2-dependent sensing of *S. aureus* was dependent on AFT [104].

Next to AFT, *S. aureus* expresses the bicomponent cytotoxins leukocidins (Luk) and the $\gamma$-hemolysins (Hlg). Bicomponent implies that class S toxins (LukS-PV, LukE, HlgA, HlgC, and LukS) have to associate with class F toxins (LukF, LukD, LukF-PV, and HlgB) in a 1 : 1 stoichiometric ratio to form a functional oligomer before insertion into the membrane (see Figure 3(c)). Pathogenic *S. aureus* can produce several different bicomponent toxin pores, among the most prominent are LukE/LukD [105], Panton-Valentine leukocidin LukS-PV/LukF-PV, $\gamma$-hemolysins LukF/HlgA [106], HlgA/HlgB, HlgB/HlgC, and the M/F-PV-like leukocidins, all of which might be expressed at different stages during sepsis. The Panton-Valentine leukocidin (PVL), which was first isolated from furuncles in 1936 [107], is probably the most widely studied member. In a mouse model, secreted PVL promotes tissue invasion and causes necrotizing pneumonia, via mechanisms including upregulation of protein A and other adhesins [108]. PVL is an important factor in the early stages of skin infection, as shown in a rabbit skin infection model [109]. Association of *pvl* and *spa* (protein A) genes seems to be an important virulence determinant in methicillin-resistant *S. aureus* (MRSA). Moreover, PVL was reported to directly bind to TLR2 and induce inflammation in the mouse lung [110]. LukE/LukD promotes systemic bacterial growth *in vivo* by specifically targeting neutrophils [111]. Interestingly, via engagement of their native receptor CCR5 (CC-motif-chemokine-receptor type 5), LukE/LukD toxin pores clear antigen-presenting cells (macrophages and dendritic cells) and *S. aureus*-specific CCR5-positive Th1/Th17 cells [112], thus greatly contributing to the spread of *S. aureus* in the host.

AFT, Hlg, and the leukocidins belong to the class of $\beta$-pore-forming toxins [17]. Despite their moderate sequence identity (around 30% for the pairwise alignment with AFT), they share a common structural fold (see Figure 3(c)). The structures of the soluble, monomeric LukF (Figure 3(c1), top, [113]), LukF-PV (Figure 3(c1), middle, [114]), LukS-PV (Figure 3(c1), bottom, [115]), and the octameric, membrane complex of Hlg (LukF/HlgA, Figure 3(c2), [116]) are available. A common molecular mechanism could be proposed in which a prestem, triple stranded $\beta$-sheet in the soluble monomer, refolds to a double, stranded, membrane-inserted $\beta$-sheet (Figures 3(c2) and 3(c3), left), resulting in a hexadeca- or tetradecastranded $\beta$-barrel that penetrates the membrane.

The target membrane specificities of *S. aureus* PFTs hint towards their role in neonatal sepsis. Whereas AFT has a broad specificity and might thus be involved at the initial stages of sepsis manifestation, where the lung epithelium needs to be breached, bicomponent leukocidins and Hlgs mainly attack polymorphonuclear neutrophils, macrophages and lymphocytes [117, 118]. Bearing in mind that PVL does not attack lymphocytes and Hlg can be hemolytic, at least *in vitro*, their restricted but highly directed mode of attack predisposes the bicomponent leukocidins to be important factors for the subversion of the immune system once the initial barrier has been breached.

## 8. $\alpha$-Hemolysin and Cytolysin A from *Escherichia coli*

Pathogenic *Escherichia coli* (*E. coli*) cause around 25% of invasive neonatal sepsis [119], and antibiotic resistance is an emerging threat in this context [120]. Generally, *E. coli* can persist in the intestine as a normal constituent of the intestinal microbiota. However, extraintestinal pathogenic *E. coli* (ExPECs) are the most common gram-negative bacterial species isolated from neonates with bacterial infections, and neonatal mortality from gram-negative sepsis remains high [121]. Urinary tract infections of pregnant women can lead to aspiration of ExPECs during partition with subsequent uncontrolled growth in the lung of the newborn and potential progression to a systemic infection. Pathogenic *E. coli* often secrete the pore-forming toxin $\alpha$-hemolysin (HlyA, CylA), a 107 kDa member of the RTX class of toxins [122], which is usually associated with strains causing uropathogenic infections [123]. Deletion of HlyA in *E. coli* greatly reduced mortality and cytokine production as compared to the isogenic wild type bacteria in an intravenous infection model [124]. HlyA furthermore induced hemorrhagic bleeding of bladder tissue and exfoliation of urothelium when pathogenic bacteria were administered into the urethra [125]. HlyA is encoded by at least 50% of all ExPEC clinical isolates [126]. Around 80% of meningitis- and sepsis-associated *E. coli* belong to the K1 serotype [127, 128]. The *E. coli* K1 strain RS218 expressed HlyA in a zebrafish model of systemic infection [126] and the hlyA gene was present in more than 40% of *E. coli* from the genital tracts of pregnant women [129]. As a member of the repeats in toxin (RTX) family of hemolysins, the hlyA gene is part of the chromosomal hlyCABD operon, which also encodes a type 1 ABC transporter for secretion of the PFT. The amino acid toxin repeats, which are located in the C-terminal portion of the protein, are composed of the sequence GGXGCDXUX (with U being a large hydrophobic residue). These repeats are responsible for $Ca^{2+}$ binding, which is a prerequisite for membrane association and pore formation by the N-terminal, hydrophobic, and acylated domain of HlyA [130]. Interestingly, other pore-forming proteins, such as the human cytotoxic lymphocyte encoded perforin, also require binding of $Ca^{2+}$ for membrane association and pore formation [131]. HlyA oligomerises on the plasma membrane of target cells, where it accumulates in cholesterol-rich microdomains [132, 133]. However, rather than cholesterol being a direct, lipid membrane receptor as in the case of the CDCs listeriolysin or pneumolysin, cholesterol seems to contribute to the physicochemical environment necessary for HlyA membrane engagement. The exact nature of the pore formation mechanism is under considerable debate. It seems now accepted that HlyA forms membrane pores as an oligomer, at least in artificial membrane mimics [134–136], albeit possibly heterogeneous in size [137]. In this respect,

it is intriguing that the P2X7 receptor and pannexin 1 were found to mediate HlyA-dependent pore formation [138, 139]. Sublytic doses of HlyA initiate degradation of paxillin and proteolytic cascades inside epithelial cells and macrophages, thus attenuating the inflammatory host response and promoting epithelial exfoliation [140]. Similarly, HlyA inhibits epithelial cytokine production potentially promoting epithelial invasion of *E. coli* [141].

Another PFT of *E. coli* is ClyA (also termed hemolysin-E or SheA), which is expressed by various pathogenic and nonpathogenic *E. coli* including K12 strains, that are also found in clinical isolates of neonatal meningitis [142], bacteremia [143, 144], and neonatal sepsis [145]. However, three neonatal meningitis K1 strains isolated by Ludwig and colleagues harboured deletion mutations at the clyA gene locus [146]. Nevertheless, a synergistic enhancement of extraintestinal infection was recently reported between nonpathogenic *E. coli* K12 and pathogenic ExPEC strains in a mouse model of septicaemia [147], which hints towards a potential involvement of ClyA. ClyA is a 34 kDa protein belonging to the class of $\alpha$-PFTs. The mechanism of pore formation is well understood, as crystal structures for the soluble [148] and membrane state [149] are available (see Figure 3(b)). Upon association with the membrane, insertion of the so-called $\beta$-tongue induces a series of substantial structural rearrangements in the now membrane-anchored monomer, resulting in a perpendicular position to membrane with the amphiphatic helix a1 now lying along its surface. After oligomerisation to a dodecamer, helices a1 become inserted into the membrane forming a 130 Å hollow cylinder with a 30 Å aperture protruding through the membrane.

## 9. Synopsis and Medical Outlook

Neonatal sepsis is a syndrome caused by systemic inflammation and defined by clinical criteria such as tachycardia, respiratory distress, temperature instability, and unusually high amount of immature immune cells in the blood. Pathogenic bacteria that are aspirated by the fetus in the birth canal during parturition can cause neonatal sepsis if their infection is not controlled locally by the innate defence mechanisms of the respiratory and alveolar epithelia. Toxins, either proteinaceous or of other molecular nature, are important factors contributing to neonatal sepsis. While endotoxins such as the lipopolysaccharide (LPS) from Gram-negative bacteria contribute to the sepsis phenotype by activating monocytes and macrophages via Toll-like receptor 4 binding, pore-forming proteinaceous exotoxins act by permeabilising target membranes of host cells. Whereas the membrane targeting effects of PFTs, that is, engagement of membrane, oligomerisation, and pore formation, are well defined, their secondary downstream effects are manifold, owing to the fact that defined ionic and molecular gradients across cellular membranes modulate a diverse set of signalling cascades. Unregulated cell death and its consequences are important in neonatal sepsis [150]. The PFTs described above can cause direct necrotic cell death, which in the case of *E. coli*, GBS, and *S. aureus* contributes to overcoming the epithelial and endothelial barriers in the lung. LLO of *L. monocytogenes* is critical for cell invasion and cell-to-cell spread of this intracellular pathogen and thus also contributes to breaching the epithelial barriers of the host. Necrotic cell death can release proinflammatory cytokines from leukocytes thus contributing to the inflammatory storm during sepsis. Interestingly, several PFTs discussed above can induce the inflammasome: AFT [31], $\beta$-hemolysin/cytolysin [28], LLO [29, 151], HlyA [140], and leukocidins [32, 152]. Inflammasome activation ultimately may contribute to hyperinflammation in newborn infants similar to what has been shown in GBS and *E. coli* sepsis in mice [28, 153]. PFTs can also elicit and alter apoptosis of host immune cells, that is, AFT via caspase-2 [154, 155], leukocidins via activation of caspases 3 and 9 [156], LLO via release of cytochrome C from mitochondria [157], $\beta$-hemolysin/cytolysin independently of caspase activation [36, 39], HlyA [158], and ClyA [159], thus potentially contributing to the *apparent immunodeficiency* of the patient that is characteristic during stages of sepsis [160]. Despite the formation of hydrophilic channels by all PFTs, the ways in which they induce the inflammasome are varied, hinting towards the fact that functions of PFTs at cellular membranes are more subtle than might be expected from their general mode of action. In this respect, effects of sublytic concentrations of PFTs have recently been explored. For instance, sublytic doses of LLO induce mitochondrial network disorganisation with transient alteration of the metabolic state of the target cell, thus weakening the cell for *L. monocytogenes* entry without destroying it [161]. Moreover, targeting of organs by PFTs might also contribute to the septic phenotype. GBS $\beta$-hemolysin/cytolysin, for example, had marked effects on cardiomyocyte contractility and viability [162].

Due to their role in neonatal sepsis and bacterial infection in general, PFTs present attractive therapeutic targets. In cases where membrane receptors have been defined, specific inhibitors, akin to viral entry inhibitors, might prevent membrane binding and pore formation. Monoclonal antibodies against PFTs that prevent membrane binding and/or refolding to the pore state could be a way of neutralising the toxin, at least in the blood stream. Moreover, vaccines based on PFTs may be used for immunizing women and thereby protecting newborn infants through placental transfer of specific immunoglobulins, given the fact that pneumolysin from *S. pneumoniae* is considered a vaccine candidate [163]. Indeed, novel vaccines based on AFT are currently developed [164]. As PFTs elicit specific cellular responses, it might, however, also be promising to designing therapeutics based on the pathways that the toxins induce in the target cell, as has been proposed for p38 MAPK and $\beta$-hemolysin/cytolysin from GBS [45]. In any case, it is exactly these cellular responses towards PFTs that need to be investigated in the context of neonatal sepsis in the future to improve therapeutic strategies.

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*Review Article*

# Age-Dependent Differences in Systemic and Cell-Autonomous Immunity to *L. monocytogenes*

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Host defense against infection can broadly be categorized into systemic immunity and cell-autonomous immunity. Systemic immunity is crucial for all multicellular organisms, increasing in importance with increasing cellular complexity of the host. The systemic immune response to *Listeria monocytogenes* has been studied extensively in murine models; however, the clinical applicability of these findings to the human newborn remains incompletely understood. Furthermore, the ability to control infection at the level of an individual cell, known as "cell-autonomous immunity," appears most relevant following infection with *L. monocytogenes*; as the main target, the monocyte is centrally important to innate as well as adaptive systemic immunity to listeriosis. We thus suggest that the overall increased risk to suffer and die from *L. monocytogenes* infection in the newborn period is a direct consequence of age-dependent differences in cell-autonomous immunity of the monocyte to *L. monocytogenes*. We here review what is known about age-dependent differences in systemic innate and adaptive as well as cell-autonomous immunity to infection with *Listeria monocytogenes*.

## 1. Introduction

*L. monocytogenes* is an opportunistic pathogen that mainly affects very young, old, or immune compromised individuals [1]. Epidemics of listeriosis are associated with high mortality rates and continue to cause widespread concern [2–9]. The fact that the newborn in particular suffers a much higher risk of severe outcome suggests that deficiencies exist in the host defense of the newborn versus the young adult against *L. monocytogenes* [10].

Host defense against infection can broadly be categorized into systemic immunity and cell-autonomous immunity [11, 12]. Systemic immunity is crucial for all multicellular organisms, increasing in importance with increasing cellular complexity of the host. Differences in innate as well as adaptive systemic immunity between the neonatal versus adult host in response to *L. monocytogenes* infection undoubtedly contribute to their difference in clinical response, and they are summarized here [10, 13–17]. However, from plants to humans, the ability to control infection at the level of an individual cell equates firmly with survival of the host [18]. This capacity for cell intrinsic self-defence is called cell-autonomous immunity [12]. Cell-autonomous immunity is operationally defined by a minimal set of genetically encoded antimicrobial defense factors that enables an infected host cell to resist a pathogen [18]. In higher organisms, cell-autonomous immunity following microbial exposure is characterized by the rapid induction of a transcriptional program [19]. Successful execution of this defense program is necessary for the survival of not only the single cell but also the host [18]. We postulate that much of the increased risk to suffer and die from *L. monocytogenes* infection in the newborn period is a consequence of age-dependent differences in cell-autonomous immunity to *L. monocytogenes*.

## 2. Systemic Immunity to Listeriosis

The increased susceptibility of neonates to suffer from severe listeriosis is a well-documented clinical phenomenon. However, the mechanisms leading to this susceptibility are only

incompletely understood. *Listeria monocytogenes* has been used extensively in mouse infection models to elucidate the inner workings of the immune system in response to pathogenic challenge. While mice mimic certain aspects of human immunity and pathogen susceptibility, the model has certain limitations, and it is unknown how closely it parallels clinical susceptibility to *L. monocytogenes.* Our knowledge about the human response to *Listeria* infection is confined primarily to results obtained from *in vitro* experiments. Elucidation of the ontogeny of host innate and adaptive immune development [20, 21] has also added to our conceptual understanding of age-dependent differences in immunity; however, their relevance to infection with *Listeria* is not clear. In this section, we will detail the key contributing effectors of the host systemic innate and adaptive immune response to *Listeria,* weaving together information from mechanistic studies in animal infection models and human studies in primary cells.

### 2.1. Innate Immune Response

*2.1.1. Innate Immune Response in the Mouse.* The first line of defense against *Listeria* is the gastrointestinal barrier. Within intestinal crypts, Paneth cells produce antimicrobial effectors including lysozyme, phospholipase A2, and alpha defensins. *L. monocytogenes* infects intestinal epithelial cells and is also taken up from the intestine through Peyer's patches and macrophages of the lamina propria. From there, bacteria disseminate to the liver, spleen, and mesenteric lymph node through the blood and lymph [22], often carried within host monocytes [23].

Within these tissues, bacteria are initially taken up by resident macrophages, which produce chemokines to promote recruitment of monocytes and neutrophils to the site of infection. Recruitment of monocytes to sites of infection is central to the early control of murine *L. monocytogenes* infection, as shown by the increased susceptibility of mice lacking CCR2 or CCL2, the receptor and ligand for monocyte recruitment [24, 25]. Following migration, monocytes differentiate locally into macrophages and a subset of TNF/iNOS producing dendritic cells (TipDCs) [26]. Infected macrophages secrete TNF-$\alpha$, IL-12p70, and IL-18, cytokines that activate NK cells and CD8+ "bystander" T cells to produce IFN-$\gamma$ [27–30]. IFN-$\gamma$ production at early time points is required to activate macrophages in order to kill intracellular bacteria. NK cells have typically been regarded as the primary early producers of IFN-$\gamma$ in the mouse, but this assumption has been called into question by evidence that "bystander" CD8+ T cells can produce IFN-$\gamma$ at early time points in an antigen-independent manner. In fact, based on transfer of NK or CD8+ T cells into IFN-$\gamma$-deficient recipient mice, CD8+ T cells provide more effective "bystander" protection than NK cells [30, 31]. IFN-$\gamma$ is also required for the differentiation of murine monocytes into TipDCs, though NK cells appear to be the primary source of IFN-$\gamma$ for this differentiation process [32]. During *L. monocytogenes* infection of mice, $CD11b^{+}$ $CD11c^{int}$ myeloid lineage cells are the main source of TNF-$\alpha$ and iNOS, which are both crucial mediators of the murine anti-*Listeria* response [26, 33, 34]. Cells of the myeloid lineage, such as TipDCs, are also primary producers of IFN-$\beta$ following *L. monocytogenes* infection in mice [26, 35, 36]. As will be discussed in a later section, high levels of type 1 IFNs (IFN-$\alpha$ and IFN-$\beta$) have been implicated in promoting apoptosis of several cell types, and mice deficient for the type 1 IFN receptor are more resistant to *L. monocytogenes* [37, 38].

In mice, the immediate wave of neutrophil migration, which occurs between 30 minutes and 4 hours after infection, is driven by the production of formyl peptides [39]. Following migration into the tissues, neutrophils kill extracellular bacteria through secretion of bactericidal granules and neutrophil extracellular traps (NETs); this appears to be of greater importance in the mouse liver than the spleen [40]. However, the role of neutrophils in defense against *L. monocytogenes* remains somewhat controversial as initial neutrophil depletion studies suggested essentiality of these cells in early infection, but the antibody used has since been found to bind inflammatory monocytes as well as neutrophils [40]. More recent studies utilizing the murine neutrophil-specific Ly6G-specific 1A8 antibody indicate that depletion of neutrophils prior to infection causes 10–1000-fold higher *Listeria* burdens within the first 3 days of infection, while initiation of neutrophil depletion alongside infection has no effect [41, 42]. These data suggest that neutrophils primarily contribute to controlling *L. monocytogenes* early during infection.

Dendritic cells (DCs) are key for antigen presentation to T cells, priming of T cells, and cytokine production in the response to *L. monocytogenes*. In mice, conventional DCs (cDCs) undergo maturation following phagocytosis of *L. monocytogenes*. Within the cDC subset, CD8$\alpha$+ DCs contain the highest bacterial burden, generate high levels of IL-12, and are particularly potent at priming T cell responses [43–45]. CD8$\alpha$+ DCs are proficient at cross-presentation of antigens from phagocytosed material including dead or dying cells, via the MHC-I pathway [46], while CD8$\alpha$− DCs are central to presentation through MHC-II class molecules [47]. Additionally, CD8$\alpha$+ DCs have also been implicated in providing intracellular transport of bacteria from the marginal zone to the periarteriolar lymphoid sheath (PALS), where *L. monocytogenes* grows profusely and causes lymphocyte apoptosis [48]. This was further demonstrated by marked resistance to *Listeria* in mice deficient for the transcription factor Batf3, which specifically lack CD8$\alpha$+ DCs. Thus, DCs are crucial in activating *Listeria*-specific T cells but possibly also contribute to early containment of bacterial replication.

*2.1.2. Innate Immune Response in the Human.* Very little is known about the human systemic innate immune response in listeriosis. Following ingestion of *L. monocytogenes* in contaminated food, bacteria are known to mediate uptake into human epithelial cells through interaction of the protein internalin A with the host protein E-cadherin [49, 50]. This mechanism of oral infection is not conserved in mice due to a single polymorphism in E-cadherin, which renders mice highly resistant to oral listeriosis [51]. Experiments in other models including the guinea pig have begun to reveal fundamentals of bacterial uptake and dissemination following oral *L. monocytogenes* infection, but the availability of tools for

these models remains limited [22]. Much remains to be done in order to elucidate *L. monocytogenes* pathogenesis immediately after oral ingestion, utilizing models that utilize either humanized mice or murinized *L. monocytogenes* to allow dissection of mechanisms relevant for bacterial uptake from the gastrointestinal tract [52–54]. *In vitro* models of infected human primary cells and cell lines have indicated the likely response of some key cell types to *L. monocytogenes;* however, these experiments give no indication of the relative importance or specific role played by host cells *in vivo* in human listeriosis. Clinical susceptibility of individuals with genetic-, infection-, or medication-induced immunodeficiencies has provided some insights. For example, an increased risk for severe listeriosis is noted among individuals receiving immunosuppressive medications that interfere with cell-mediated immunity and production of TNF-$\alpha$ [28, 55, 56].

*2.1.3. Innate Immune Response in the Neonatal Mouse Model.* Our knowledge about neonatal listeriosis is severely limited, despite the fact that this age group suffers so severely from this infection. A much lower dose of *L. monocytogenes* is required to result in systemic infection in newborn rather than in adult mice; however within the first two weeks of life, newborn mice gradually develop adult-level resistance to *L. monocytogenes* [57]. Heightened susceptibility of neonatal mice is also noted if they are infected systemically [58]; therefore, age-dependent differences within the gastrointestinal tract are unlikely to be the sole cause for the increase in neonatal susceptibility to severe listeriosis. In mice, neonatal susceptibility correlates with delayed systemic production of innate cytokines and activation of NK cells [57, 58]. At birth, mice have dramatically fewer CD8$\alpha$+ DCs and much lower IL-12 production in response to antigen. These levels gradually increase, reaching adult levels sometime after day 10 of life [59]. In a murine neonatal listeriosis model, splenocytes from infected neonates showed reduced transcription of T-helper-type-I (Th1-) supporting cytokines (IL-12p70 and IFN-$\gamma$) following restimulation, as compared to infected adults [60]. Neonatal mice also produced elevated levels of the cytokine IL-10 compared to adults upon infection with *L. monocytogenes* [61], and the survival-increasing and CFU-reducing benefits of IL-10 blockade were of substantially longer duration and of enhanced effect in neonates. Interestingly, it was shown that activation of phagocytes with IFN-$\gamma$ prior to infection substantially increased resistance of newborn mice to *L. monocytogenes* [58, 62]. Monocyte chemotaxis to the site of infection is also delayed in neonatal mice [63]. These findings cumulatively suggest that neonates generate an altered innate cytokine response to *L. monocytogenes* infection in comparison with adults. While these differences likely contribute to neonate susceptibility, the mechanisms responsible and their applicability to human infection are not yet clear.

*2.1.4. Innate Immune Response in Human Neonates.* For the human neonate we can only extrapolate from general concepts of innate immune ontogeny to possible mechanisms leading to age-dependent differences in susceptibility to *Listeria* infection. For example, adhesion and chemotaxis (directed migration) of human neonatal neutrophils and monocytes are markedly deficient in comparison to adult cells [64, 65]. Furthermore, innate cytokine responses of neonates markedly differ from those of adults. For example, TLR-induced generation of proinflammatory cytokines such as TNF-$\alpha$ and IL-1$\beta$ differ in the neonate depending on the stimulant, reaching adult-level production between 1-2 years of age. During this time period, production of IL-10, IL-6, and IL-23 undergoes a slow decline from a perinatally higher than adult level [20, 21]. And while significantly reduced at birth, the ability of TLR agonists to induce type I IFN production reaches adult-like levels within only a few weeks of life. The last group of TLR-induced cytokines to reach adult-level production is the Th1-supporting innate cytokines IFN-$\gamma$ and IL-12p70 [20, 21, 66–69].

These patterns are noteworthy because IFN-$\gamma$, IL-12p70, and TNF-$\alpha$ have key protective roles in the murine innate immune defense against *Listeria,* while IL-10, which neonates make more of, has been shown to increase susceptibility to *Listeria* infection in mice [70, 71]. The low production of type I IFNs in neonates versus adults is notable as well; however, the age-dependent difference here is opposite of what might have been expected based upon the available data. In animal models, type I IFN appears to be detrimental, and *in vitro* studies of human primary cells indicate that high levels of type I IFN promote cell death in several cell types central to *Listeria* defense, as will be discussed in a later section. Thus, the precise impact of low type I IFN production in human neonates is not yet known.

*2.2. Adaptive Immune Response.* Effectors of the innate immune system are capable of controlling infection only over the short term in mice; in fact, SCID mice (deficient for B and T cells) are capable of restraining infection [72] but cannot achieve sterilizing immunity. Thus, the innate immune system must also activate the adaptive immune system for final and complete clearance of *Listeria*. The murine adaptive immune response peaks about 1 week after infection with *L. monocytogenes.* It has been demonstrated in mouse infection models that T cell responses are central to clearance of *L. monocytogenes* infection, with humoral responses playing only a minimal role [29, 73]. As described above, antigen presentation through both the MHC-I and MHC-II pathways is primarily mediated by DCs, activating CD8+ and CD4+ T cells specific for *Listeria* antigens, respectively [44]. Of the two, CD8+ cytotoxic T cells play a more important role in control of listeriosis than CD4+ cells [74], though the relative importance of several known potential mechanisms of protection is still a matter of debate. The innate cytokine IL-12p70 is important for the expansion phase of the CD8+ T cell response [75]; IL-12p70 appears to activate T cells into full effector cells necessary for control of *L. monocytogenes* infection. The role of CD4+ T cells requires IFN-$\gamma$ production by these cells and likely involves the reciprocal activation of macrophages [76]. CD4+ cells appear to be important for the initial stage of CD8+ T cell priming and for memory longevity [29, 77, 78]. Murine $\gamma\delta$ T cells are also known to play a role in IFN-$\gamma$ production during infection [79]. While it is not

known how closely the mouse model mimics the adaptive immune response to clinical listeriosis in the human, the susceptibility of individuals with AIDS or those undergoing treatment to suppress cell mediated immunity indicates that T cells likely perform a central role in human defense against listeriosis as well [28].

Some crucial mediators of adaptive immune defense against *Listeria* appear to differ qualitatively or quantitatively in neonates. At birth, neonatal CD4+ T cells in mice appear to be Th2 biased [80]. In addition, neonatal CD4+ Th1 cells have been shown to undergo apoptosis when reexposed to antigen, whereas Th2 cells do not [81]. Another potential difficulty of the neonatal response to infection stems from the fact that murine lymphoid cells are limited in number early in life; therefore, a suitable expansion of cells could be difficult to attain [82]. Finally, the reduced production of innate IL-12p70 and increased production of IL-10 by neonatal innate cells upon stimulation would be expected to lead to suboptimal activation of CD8+ T cells and thus increased susceptibility to listeriosis [10, 20, 21, 83, 84]. The human adaptive response to neonatal listeriosis has not been adequately examined.

In summary, differences in innate immunity between neonate and adult have been defined [10]; however, few of these differences correlate well with the high-risk period for human neonatal listeriosis typically restricted to the first 6–8 weeks of life [85]. It thus appears likely that factors other than age-dependent differences in innate immune function must also play a role in the increased susceptibility of the human newborn to severe infection with *L. monocytogenes*. While differences for the human newborn versus adult adaptive immune response have been defined [17], the human is already capable of initiating and sustaining strong, protective Th1-type responses prior to birth [86]. Thus again, age-dependent differences in adaptive immunity alone cannot explain the overall increased risk for severe outcome of infection with *L. monocytogenes* early in life. Containment of infection ultimately depends on the interaction between the intracellular *L. monocytogenes* and the infected host cell. The next section will cover this primary battleground.

## 3. Cell-Autonomous Immunity: The Cell as a Battleground

Cell-autonomous immunity is defined as the ability of a single cell to resist infection, while systemic immunity is expressed as resistance of the entire host to infection, that is at the organismal level. For infection with *L. monocytogenes* the differentiation between systemic immunity and cell-autonomous immunity is not as clear, as one of the main target cells infected by *L. monocytogenes* is the monocyte, which is an integral part of the innate immune system, and also the effector arm of the adaptive immune system. For example, as outlined above, T cell interactions with monocytes are critical for survival of the host following *L. monocytogenes* infection. However, T cells do not kill *Listeria*; rather, T cells only lyse infected cells [14], in the process releasing viable bacteria [87]. The main function of the T cell in defense against *L. monocytogenes* instead is to support the monocyte response. Elegant experiments conducted in mice decades ago already clearly identified that age-dependent susceptibility to primary infection with *L. monocytogenes* correlates best with age-dependent differences in monocyte function [57, 58]. Since then, we have learned that for the host not to succumb to *L. monocytogenes*, phagocytes such as monocytes/macrophages have to rapidly trap and kill the ingested bacteria [57, 87–89]. We now also know that, from the moment *L. monocytogenes* binds the monocyte, a response is set into motion that aims to destroy the bacteria [90]. In adult mice, this cell autonomous immune response of the monocyte has been found to be essential for protection from severe listeriosis [32, 87, 91, 92]. This strongly suggests that age-dependent differences in systemic immunity are the result of age-dependent differences in cell autonomous immunity of human monocytes to *L. monocytogenes*. Given the importance of cell autonomous immunity for neonatal infectious disease, it is remarkable how often this form of somatic self-defence is either overlooked or underappreciated [18]. This is particularly true for listeriosis. In this section, we review what is known about age-dependent differences in the cell autonomous immune response of the monocyte to *L. monocytogenes*.

*3.1. Monocyte Recognition of Listeria. L. monocytogenes* is recognized by monocytes via several distinct pathways, each setting in motion a host cellular response that involves hundreds of genes [93–95].

(i) The extracellular and phagosomal Toll-like receptor (TLR)/MyD88-dependent recognition pathway induces expression of inflammatory cytokines such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$) as well as reactive oxygen (ROS) and nitrogen (RNS) species in order to kill ingested *L. monocytogenes* [96–101]. Multiple *L. monocytogenes* ligands that are recognized at both the host cell surface and within a vacuole contribute to the MyD88-dependent response to *L. monocytogenes* [13]. This pathway is clearly important for host resistance as we and others have shown that MyD88-deficient mice are extremely vulnerable to *L. monocytogenes* infection [15, 102]. While TLR/MyD88 sensor function appears well developed early in life [103], downstream effector responses are strikingly different in the human newborn as compared to the young adult [10]. As discussed in the previous section, TLR-induced cytokine generation differs between neonates and adults. Additionally, MyD88-induced production of ROS or RNS is also strikingly reduced inearly as compared to adult life [104–107]. This suggests that the activity of multiple MyD88-dependent effector mechanisms essential for protection from severe infection with *L. monocytogenes* is functionally altered early in life. The period between birth and 6 weeks of age represents the highest risk period for severe infection with *L. monocytogenes* in the human newborn. This period best correlates with

the period of low type I IFN production following TLR/MyD88-dependent stimulation, suggesting a possible functional connection [10]. However, the TLR/MyD88 dependent response of human neonatal monocytes to *L. monocytogenes* has not yet been investigated.

(ii) The cytosolic STING/IRF3-dependent pathway in mice leads to the robust expression of interferon-$\beta$ (IFN-$\beta$) and other interferon stimulated genes (ISG) controlled by the transcription factor IRF3 [108]. Induction of IFN-$\beta$ by cyclic dinucleotides secreted by cytosolic *L. monocytogenes* is entirely STING dependent *in vitro* and *in vivo* [109, 110], as STING functions as the direct host receptor for cyclic dinucleotides [111]. To our knowledge, the developmental pattern of the cytosolic pathway has not been examined in any detail in human monocytes. In mice, IFN-$\beta$-mediated signals can be harmful or protective for the *L. monocytogenes*-infected mouse, depending on the relative activity of concomitant TLR/MyD88 signalling [87]. In mice, production of IFN-$\beta$ during *L. monocytogenes* infection appears restricted to monocytes and macrophages, with no induction of expression in lymphocytes, neutrophils, or dendritic cells [35]. Cell-type specific differences in IFN-$\beta$ production in response to *L. monocytogenes* infection have not been examined in humans. It is however important to note that while IRF3-dependent production of type-1 IFN in human newborns is reduced as compared to adults [10], production of IFN-$\beta$ in humans in response to *L. monocytogenes* is not dependent on IRF3 (as it is in the mouse) but appears p38 MAPK-dependent [112, 113]. Thus, the role of this pathway for human neonatal listeriosis is not clear.

(iii) Activation of the inflammasome pathway by *L. monocytogenes* leads to proteolytic release of IL-1$\beta$ and possibly to inflammatory cell death called pyroptosis [114, 115]. In mice, *L. monocytogenes* can activate the inflammasome via three different cytosolic sensors: NLRP3, NLRC4, and/or AIM2 [115–124]. Murine IFN-induced GTP-binding protein 5 (GBP5) binds NLRP3 subunits and assembles them into a functional complex during *L. monocytogenes* infection of IFN-$\gamma$-activated murine macrophages (reviewed by [18]). However, inflammasome activation in response to *L. monocytogenes* has also been described as NLRP3 independent, partially NLRC4 dependent, and fully AIM2 dependent [115]. Alum, the most common vaccine adjuvant, exerts part of its function via activation of the inflammasome [125]. Alum-induced responses significantly decline over the first 2 years of life [126], suggesting age-dependent differences in at least some inflammasome activities. However, the developmental pattern of the various inflammasome pathways in humans in response to *L. monocytogenes* has not been elucidated. The importance of the inflammasome pathway for age-dependent susceptibility to *L. monocytogenes* thus is not known.

*3.2. Fate of Listeria inside the Monocyte.* Entry of *L. monocytogenes* into monocytes/macrophages occurs via phagocytosis [43, 127]. This process is initiated after *Listeria* is bound by complement that together with the listerial protein internalin B functions as ligands for complement receptors on phagocytes. In addition, scavenger receptors recognize lipoteichoic acid, a component of the listerial cell wall [128]. Once bound by either scavenger or complement receptors, the bacteria are internalized into a phagosome. The phagosome then undergoes a series of transformations via sequential interaction with subcompartments of the endocytic pathway, eventually maturing into a phagolysosome. During this process, engulfed bacteria are exposed to a range of pH-dependent host microbicidal effectors that include ROS and RNS, iron scavengers and exporters, lactoferrin and natural resistance-associated macrophage protein 1 (NRAMP1), antimicrobial peptides and proteins (e.g., defensins, cathelicidins, lysozyme as well as other carbohydrate hydrolases, phospholipases, and various proteases and peptidases) that permeabilize and degrade the ingested bacteria. Production of several of these key molecules has been found reduced in early life [129]; however, precise roles have not been ascribed to any with respect to human or murine neonatal infection with *L. monocytogenes*.

The ability to escape from the phagosome enables *L. monocytogenes* to avoid certain destruction and to instead replicate in the cytosol [130]. This phagosomal escape can occur as rapidly as 30 min after bacterial cell entry [130–132]. The escape of *L. monocytogenes* from the single-layer membrane vacuoles is assisted by virulence-associated bacterial molecules (listeriolysin O (LLO) and phosphatidylinositol-phospholipases (e.g., PC-PLC and PI-PLC)), as well as several host derived factors, such as the $\gamma$-interferon-inducible lysosomal thiol reductase (GILT) [133, 134]. While LLO is absolutely required for phagosome vacuolar escape in mice, it is dispensable in human cells, where the phospholipases are critical instead [135].

The intracellular fate of phagocytosed *L. monocytogenes* depends on the speed of phagosome maturation versus listerial escape. This dynamic host-pathogen interactive process [130] has not been examined at all in human neonates. From studies in the murine host we know that IFN-inducible GTPases are centrally involved in restricting listerial escape from the phagosome [11]. At least two families of IFN-inducible GTPases—the 21–47 kDa immunity-related GTPases (IRGs) and the 65–73 kDa GBPs—regulate intracellular traffic of phagosomes containing bacteria. Over 20 IRGs have been identified in mice, while the human genome only contains two (reviewed by [18]). Murine Irgm1 is known to target the early *L. monocytogenes* phagosome, where it directs trafficking of bacteria-containing phagosomes and endosomes along microtubules towards maturing phagolysosomes. And the IFN-$\gamma$-induced guanylate-binding protein 7 (Gbp7) is known to direct the assembly and activation of ROS producing NOX2 holoenzymes specifically on phagosomes containing *L. monocytogenes* [11].

At least four other murine Gbps—Gbp1, Gbp6, Gbp7, and Gbp10—confer cell-autonomous immunity to listerial infection [136]. Mice deficient in Gbp1 display significantly

increased susceptibility to *L. monocytogenes* [136]; this systemic *in vivo* phenotype is directly attributable to a role for Gbp1 in cell-autonomous immunity of the macrophage, resulting in delayed and reduced transport of antimicrobial peptides, autophagic machinery, and components of the NADPH oxidase to the phagosomal compartments that contain *L. monocytogenes* (reviewed by [18]). Identification of interacting partners for Gbps has begun to reveal some of the specific molecular mechanisms involved in Gbp-mediated listerial killing (reviewed by [11, 18]). Gbp1 interacts with the ubiquitin-binding proteins, delivering ubiquitinated *L. monocytogenes* to autolysosomes. Gbp7 recruits the autophagy protein ATG4B, which drives the extension of autophagic membranes around bacteria within damaged bacterial compartments and assembles NOX2 on these compartments. And as mentioned above, Gbp5 binds NLRP3 to promote specific inflammasome responses during the infection of IFN-$\gamma$-activated murine macrophages by *L. monocytogenes*. Gbps thus seem essential for cell-autonomous immunity of the murine monocyte/macrophage to *L. monocytogenes* [137]. Unfortunately, nothing at all is known about either expression or function of GBPs in human neonatal monocytes.

Autophagy is a process by whichcytoplasmic materials, including bacteria, are targeted to lysosomes for degradation (reviewed in [19, 138, 139]). Autophagy has been shown to target *L. monocytogenes* within intact phagosomes, damaged phagosomes, and those found in the cytosol [140]. Therefore, *L. monocytogenes* must successfully evade killing by the autophagy system at all stages of its residence within host cells. *L. monocytogenes* has developed strategies to prevent being taken up by the autophagosome. For example, ActA recruits host proteins to disguise *L. monocytogenes* from ubiquitination and thus prevent autophagic recognition [141, 142]. InlK is another surface protein that contributes to listerial escape from autophagy [143] via recruiting the major vault protein (MVP) to evade ubiquitination and autophagic recognition [138, 144]. In murine cells, expression of LLO is necessary for the induction of the autophagic response, specifically at the early time points after infection; this suggests a role for permeabilization of the vacuole in the induction of the autophagic pathway. However, it is the expression of the phospholipases that allows *L. monocytogenes* to escape from autophagosomes [145, 146]. The importance of autophagy in limiting *L. monocytogenes* replication has been demonstrated *in vivo*, as mice deficient in autophagy exhibit increased bacterial load and decreased survival following infection [147]. The above-mentioned family of GTP-binding proteins again features prominently in autophagy as well: Gbp1 directs ubiquitin-associated *L. monocytogenes* to the autophagy machinery via binding to autophagy receptors [148–150]. To our knowledge, autophagy itself has never been examined as a function of age, not in humans or in mice; thus nothing is known about the role of autophagy in human neonatal listeriosis.

*3.3. Fate of the Listeria-Infected Monocyte.* *L. monocytogenes* induces cell death in multiple immune and nonimmune cell types (reviewed in [89]). Of all the cell death pathways induced by *L. monocytogenes*, T lymphocyte apoptosis is the best understood. *In vivo*, *L. monocytogenes* infection of mice is followed by rapid, synchronous, and extensive depletion of lymphocytes surrounding the periarteriolar lymphoid sheaths (PALS) in the spleen [27, 151]. The death of T lymphocytes in the PALS induced by *L. monocytogenes* is apoptotic in nature and precedes activation of T cells [152]. Importantly, the dying lymphocytes are not themselves infected with *L. monocytogenes*, indicating that apoptosis is caused by a factor extrinsic to the dying cell [88, 153, 154]. Dendritic cells can also respond with apoptosis to infection with *Listeria* (reviewed in [89, 155]). Most of the known pathways for the induction of apoptosis (Fas/FasL signaling, TNF-RI signaling, and perforin) were however shown not to be relevant in the development of the apoptotic lesions following infection of mice with *L. monocytogenes*. Only TNF-related apoptosis-inducing ligand (TRAIL) deficiency/soluble DR5 (TRAIL antagonist), type I interferon receptor deficiency (IFNABR−/−), and granzyme deficiency [37, 38, 156–158] reduced T cell apoptosis *in vivo* following infection, suggesting they are involved. Treatment with type I interferon primes resting lymphocytes to undergo apoptosis induced by LLO [37]. Murine DCs and macrophages infected with *L. monocytogenes* produce massive amounts of type I interferon [43, 94, 159]. And IFN-abR −/− mice are more resistant to *L. monocytogenes* infection and display reduced apoptosis of splenic lymphocytes [37, 38]. The direct positive correlation between the strength of type I interferon induction, apoptosis, and virulence of particular strains of *L. monocytogenes* in mice further supports the importance of type I IFN for *Listeria*-induced apoptosis [160]. The proapoptotic effect of type I interferon on lymphocytes negatively influences the murine host systemic immune response to *L. monocytogenes* following infection, likely via induction of IL-10 [37, 161].

Data regarding the mechanisms by which *L. monocytogenes* induces cell death of monocytes and macrophages are inconsistent and somewhat contradictory, with evidence for apoptosis as well as pyroptosis, and necrosis [89]. Importantly, when *L. monocytogenes* kills the infected monocytes by necrosis, it is rendered less virulent [114]. Caspase-1-dependent cell death (pyroptosis) also reduces bacterial survival [115, 162, 163]. Thus, to promote its pathogenesis, *L. monocytogenes* must avoid killing infected monocytes via either necrosis or pyroptosis [109] and instead promote apoptosis [89]. Neonatal monocytes respond to innate stimulation with apoptosis at higher frequency [164], but this difference was detected following LPS stimulation. Nothing at all is known about the type of cell death induced in human neonatal monocytes infected with (or exposed to) *L. monocytogenes*.

*3.4. Regulation of Cell-Autonomous Immunity in the Monocyte.* Recent evidence suggests that epigenetics may play a role in regulating cell autonomous immunity. The transcriptional status of a gene is tightly linked to the structure of chromatin; transcriptional regulation of gene expression can be achieved via epigenetic regulatory mechanisms [138]. *L. monocytogenes* is known to reprogram host chromatin structure during infection to benefit its own survival (reviewed in

Table 1: Age-dependent differences in systemic immunity to *L. monocytogenes*.

| Effector | Role in listeriosis | Neonatal mouse | Neonatal human |
|---|---|---|---|
| Neutrophils | Chemotaxis | ? | Decreased |
| | Extracellular bacteria killing | ? | ? |
| Resident tissue macrophages | Production of chemokines | ? | ? |
| | Production of TNF$\alpha$, IL-12p70, IL-18 | Reduced IL-12p70 | Reduced IL-12p70 |
| Monocytes | Chemotaxis to infection site | Reduced | Reduced |
| | Differentiation to TipDCs and macrophages | ? | ? |
| Dendritic cells (DCs) | Antigen presentation | Reduced | ? |
| | Production of IL-12p70 | Reduced | Reduced |
| −CD8$\alpha$+ DCs | Bacterial transport to PALS | ? | ? |
| −TNF$\alpha$+ iNOS + DC (TipDC) | Production of TNF$\alpha$, iNOS | ? | ? |
| NK cells | Production of IFN$\gamma$ | ? | ? |
| CD4+ T cells | CD8 + Priming | ? | ? |
| | Cytokine production | Reduced | Reduced |
| CD8+ T cells | Bystander production of IFN$\gamma$ | ? | ? |

[135, 138]). For example, *L. monocytogenes* induces acetylation of histone H4 as well as phosphorylation and acetylation of histone H3 specifically at the IL-8 promoter, leading to its downregulation in a p38 MAPK- and MEK1-dependent manner [165]. However, modulation of the monocyte epigenome can also work to the benefit of the host following for example BCG vaccination [166]. Neonatal mice are in fact completely protected from an otherwise lethal dose of *L. monocytogenes* if given BCG prior to infection with *L. monocytogenes* [57, 58]. As neonatal immunization of human newborns with BCG reduces neonatal mortality unrelated to tuberculosis, that is, nonspecifically [167], it may well be that regulation of cell autonomous immunity to *L. monocytogenes* is mediated via changes in epigenetics. While it is known that epigenetic modifications of immune-related genes vary with age [168], the role of epigenetics in cell autonomous immunity to *L. monocytogenes* remains hidden for now.

Table 2: Age-dependent differences in cell autonomous immunity to *L. monocytogenes*.

| Effector | Role in listeriosis | Neonatal mouse | Neonatal human |
|---|---|---|---|
| Recognition of *L. monocytogenes* | (i) TLR/Myd88 | ? | ? |
| | (ii) Cytosolic surveillance | ? | ? |
| | (iii) Inflammasome | ? | ? |
| Intracellular fate of *L. monocytogenes* | (i) Phagocytosis | ? | ? |
| | (ii) Autophagy | ? | ? |
| | (iii) IFN-inducible GTPases | ? | ? |
| Fate of *L. monocytogenes*-infected monocyte | (i) Apoptosis | ? | ? |
| | (ii) Necrosis | ? | ? |
| | (iii) Pyroptosis | ? | ? |

## 4. Conclusion and Outlook

Age-dependent differences in systemic innate and adaptive immunity to infection with *L. monocytogenes* very likely play a key role in the increased morbidity and mortality of the newborn. Several possibly relevant innate and adaptive immune response differences between newborn and adult have already been delineated; however few of these have been assigned clear functional roles in the host defence against *L. monocytogenes* (Table 1). Cell autonomous immunity seems particularly relevant following infection with *L. monocytogenes*; as the main target, the monocyte, is also centrally important to innate as well as adaptive systemic immunity to listeriosis. Thus, the outcome of infection of the monocyte is likely of paramount significance to systemic immunity of the host. However, currently nothing at all is known about age-dependent differences in cell autonomous immunity of the monocyte to infection with *L. monocytogenes* (Table 2). Given the many differences between murine and human listeriosis, studies aimed at identifying the molecular mechanisms relevant to age-dependent differences in cell autonomous immunity to infection with *L. monocytogenes* cannot indiscriminately be extrapolated from mouse to humans but will need to be conducted or at least confirmed in primary human monocytes. Identifying these aspects is likely to produce insights into not only pathogenesis but also interventions. Furthermore, the same age-defined high-risk period of severe listeriosis in the human (0–6 weeks) also represents high-risk periods for other relevant pathogens such as herpes simplex virus and group B streptococcus [169–176]. Thus, delineating the underlying mechanisms responsible for age-dependent risk for severe listeriosis potentially has broader implications.

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*Review Article*

# The Immunologic Basis for Severe Neonatal Herpes Disease and Potential Strategies for Therapeutic Intervention

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Herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2) infect a large proportion of the world's population. Infection is life-long and can cause periodic mucocutaneous symptoms, but it only rarely causes life-threatening disease among immunocompetent children and adults. However, when HSV infection occurs during the neonatal period, viral replication is poorly controlled and a large proportion of infants die or develop disability even with optimal antiviral therapy. Increasingly, specific differences are being elucidated between the immune system of newborns and those of older children and adults, which predispose to severe infections and reflect the transition from fetal to postnatal life. Studies in healthy individuals of different ages, individuals with primary or acquired immunodeficiencies, and animal models have contributed to our understanding of the mechanisms that control HSV infection and how these may be impaired during the neonatal period. This paper outlines our current understanding of innate and adaptive immunity to HSV infection, immunologic differences in early infancy that may account for the manifestations of neonatal HSV infection, and the potential of interventions to augment neonatal immune protection against HSV disease.

## 1. Introduction

Young infants are highly vulnerable to infections due to changes that occur in the immune system during the transition from fetal to postnatal life [1–3]. Herpes simplex virus (HSV) infection exemplifies this paradigm [4]. When HSV infection occurs within the first several weeks of life, the majority of infants will die without treatment. However, acquisition of HSV after this period is typically mild or even asymptomatic. In this paper, we explore what is known about mechanisms by which immunologic control of HSV infection may be impaired in early infancy. Furthermore, we discuss the implications of these findings for developing interventions to better prevent and treat neonatal HSV infection and suggest directions for future research.

## 2. Herpes Simplex Virology and Pathogenesis

The herpes simplex viruses (HSV-1 and HSV-2) are members of the neurotropic $\alpha$-herpesvirus subfamily of the *Herpesviridae* family of viruses (reviewed in [5, 6]). This family includes a variety of enveloped, icosahedral capsid-containing, linear double-stranded DNA viruses with relatively large genomes, many of which cause diverse diseases in humans. All members of the family share the capacity to remain latent in the infected host and are capable of periodic reactivation and spread to new hosts.

Outside the newborn period, primary infection in immunocompetent individuals may cause gingivostomatitis, pharyngitis, or ulcerative genital lesions [7], but infection is frequently subclinical [8, 9]. In most chronically

infected individuals, reactivation of virus is either asymptomatic or at most leads to bothersome mucosal lesions [10].

Spread of HSV within the population generally results from reactivation of virus from latently infected neurons within sensory ganglia and anterograde axonal transport to the innervated mucosa, with subsequent viral replication in the epithelium and shedding [11]. The grouped vesicular and/or ulcerative lesions typical of HSV may or may not occur during these episodes, and subclinical genital shedding of HSV-2 is as common in those without any history of genital lesions as it is in those with such a history [12]. Episodes of asymptomatic genital shedding appear to decrease over time, with reactivation occurring more than twice as often in the first three months after primary first-episode HSV-2 genital infections than in subsequent three-month periods [13]. However, short bursts of asymptomatic viral reactivation occur surprisingly frequently for both oral and genital HSV, with about half of genital mucosal reactivations lasting less than 12 hours and more than 70% of these episodes occurring without symptoms [14].

## 3. Epidemiology and Clinical Manifestations of Neonatal HSV Infection

HSV infections are common, with seroprevalences in American adults of about 60% for HSV-1 and 17% for HSV-2 [15]. HSV generally initiates infection at mucosal surfaces and spreads along sensory neurons to establish latency within ganglia (typically the trigeminal or sacral ganglia) [16]. Neonatal HSV infection (defined as occurring before 28 days after birth) occurs in between 1 in 12,500 and 1 in 1,700 live births in the United States, which in combination with its high morbidity makes it a major public health concern (reviewed in [4]). Less than half of neonatal HSV infections occur in the setting of long-standing maternal infection, with a risk of transmission of <1% even when virus is detectable in the maternal genital tract at the time of delivery. In contrast, when a woman acquires HSV late in pregnancy, the risk of neonatal HSV is 25%–50%; this scenario accounts for about 50%–80% of all cases of neonatal HSV [4]. The difference in transmission risk between women with established and recent HSV infections suggests the importance of transplacental maternal antibody [17–21], as discussed in greater detail later, as well as the higher viral titers present during primary maternal infection.

HSV-1 is commonly associated with oral mucosal infection and HSV-2 with genital infection. However, genital infection with HSV-1 is increasing in prevalence, with recent studies suggesting it surpasses HSV-2 as a cause of genital infection in several different populations [22–29]. It has been speculated that this observation is related to a recent trend of acquiring oral HSV-1 infection later in life along with an increase in oral sex in young adults; this results in a population susceptible to genital HSV-1 infection at initiation of sexual activity [30]. Importantly, several studies suggest that both symptomatic and asymptomatic genital shedding of HSV-1 is less frequent than that of HSV-2 [13, 31–33], which may have implications for neonatal infection.

*3.1. In Utero Infection.* Intrauterine HSV infection is associated with hydrops fetalis and fetal death. Surviving infants of *in utero* HSV infection have symptoms at birth similar to other congenital infections, including microcephaly, hydranencephaly, chorioretinitis, and rash, although the presentation is highly variable [18, 79]. Although it is highly morbid, *in utero* HSV infection accounts for <5% of all neonatal cases, or approximately 1 per 250,000 deliveries [80, 81]. It is unclear why fetal infection occurs so infrequently, for example, compared to cytomegalovirus, but multiple factors may be involved [82]. First, detection of HSV DNA in peripheral blood, though relatively common during primary infection, is rare during established disease, even with clinical reactivations [83]. Second, the maternal-placental interface appears to have fairly effective mechanisms to block the spread of HSV. Interestingly, even in cases with severe or disseminated maternal HSV infection during pregnancy, the fetus is often spared [84]. HSV DNA can be detected by PCR in a surprising proportion of placentas (roughly 10%) [85, 86]. Immunohistochemical detection of HSV antigen is less common in placentas, but small foci of virus may be detected in the maternal decidua adjacent to the placenta [86].

*3.2. Intrapartum and Postpartum Infection.* The majority (roughly 85%) of neonatal HSV infections are acquired during passage through the birth canal [19, 87]. Only approximately 10% of neonatal HSV infections occur in the postpartum period, generally through contact with virus shed by caregivers, and these are typically caused by HSV-1. Infection of the newborn is thought to occur through mucosal (eyes, mouth) or cutaneous inoculation.

Neonatal HSV disease is classified into three clinical syndromes: localized skin, eye, and mouth (SEM); central nervous system (CNS) involvement with or without SEM; disseminated disease, which involves spread to visceral organs. Disseminated disease may or may not involve the CNS and can lead to hepatitis, pneumonitis, disseminated intravascular coagulation, shock, and multiple organ dysfunction syndrome. SEM disease accounts for approximately 45% of neonatal HSV cases, though these infants often progress to CNS or disseminated disease in the absence of treatment. CNS and disseminated disease represent approximately 30% and 25% of cases, respectively. Either HSV-1 or HSV-2 can cause SEM, CNS, or disseminated disease, although CNS infection with HSV-2 has been associated with greater morbidity [4, 88–90]. Newborns with severe HSV infection frequently present without fever or skin lesions. Delayed diagnosis and initiation of therapy occur often and contribute to poor outcomes [88]. Even with optimal treatment (60 mg/kg/day of intravenous acyclovir for 21 days), mortality is still approximately 6% with CNS disease and >30% with disseminated disease [4]. Furthermore, treatment has had little effect on neurologic morbidity among survivors of CNS disease [91]. Encouragingly, recent studies have shown that suppressive oral antiviral therapy can improve long-term

neurologic outcomes after CNS disease; however, abnormal neurologic outcomes were still reported for 31% of newborns with a history of HSV encephalitis who received suppressive treatment [92]. Thus, additional strategies are needed to better prevent and treat neonatal HSV infection.

## 4. Immune Control of HSV Infection

Despite intense study, our understanding of immunologic control of primary and recurrent HSV infections in humans remains incomplete. Virtually every aspect of immune defense appears to be involved in control of HSV infection, from antimicrobial peptides (AMPs; reviewed in [93]) through the intrinsic antiviral responses of infected cells (reviewed in [94]), innate immune effector cells and cytokines [95, 96], adaptive cellular responses [97, 98], and humoral responses [99]. In immunocompromised humans, defects in multiple arms of the immune response have been described as leading to severe HSV disease, including deficiencies in AMPs [100, 101], various defects in signaling (including signals mediated by TLR3 [102–107], STAT1 [108], tyrosine kinase 2 [109], and NF-$\kappa$B [110]), other mutations affecting lymphocyte function [111], and abnormalities in numbers or function of NK cells [49, 50], plasmacytoid dendritic cells (pDCs) [112, 113], and T cells [97].

Studies in animal models of HSV infection have shed light on the interaction of innate and adaptive immunity in the response to primary infection. The pattern recognition receptors (PRRs) that have been reported to recognize HSV include TLR2, TLR3, TLR9, the RIG-I-like receptors (RLRs) RIG-I and melanoma differentiation-associated gene 5 (MDA5 or interferon (IFN) induced with helicase C domain 1 (IFIH1)), NOD-like receptors (NLRs), interferon-inducible protein 16 (IFI16), the helicase Ku70, DNA-dependent activator of IFN-regulatory factors (ZBP1), and the helicases DEAH box 9, DEAH box 36, and DDX60 (reviewed in [43, 44]). HSV is capable of infecting monocyte-derived dendritic cells (DCs), including Langerhans cells in the skin and vaginal mucosal epithelia, inducing partial maturation but ultimately leading to apoptosis [114]. Migratory submucosal or dermal DCs phagocytose apoptotic debris, including HSV antigen, and migrate to draining lymph nodes [115, 116]. These cells then appear to either transfer antigen to resident DCs within the lymph node for priming of effective $CD8^+$ T cell responses [117] or in some situations will themselves contribute to priming of $CD4^+$ and $CD8^+$ responses [118].

T cell responses against HSV in humans are polyfunctional [119] and directed against a wide array of viral epitopes [120]. Infiltration of HSV-specific T cells into infected tissue initially involves $CD4^+$ T cells, which in mice are required for subsequent $CD8^+$ T cell entry into the mucosa [121]. Local NK cells likely contribute to control of HSV replication early in infection and make some IFN-$\gamma$ in the infected tissue [51], but IFN-$\gamma$ from infiltrating $CD4^+$ T cells and production of CXCR3-dependent chemokines (likely by epithelial cells) are required for $CD8^+$ T cells to efficiently enter the vaginal mucosa [121]. The chemokine gradient required for proper migration of T cells to the site of infection is coordinated in part by regulatory T cells (Tregs) [75].

HSV-specific $CD8^+$ T cells appear to be the central effectors controlling latent HSV infection in neurons. In mice, activated HSV-specific $CD8^+$ T cells are retained in latently infected sensory ganglia, blocking viral reactivation through IFN-$\gamma$ production without killing the neurons [122, 123]. A reduction in these cells can be seen in conditions of stress, leading to viral reactivation [124]. Recurrence of HSV lesions in skin and mucosa after reactivation from latency also activates local NK cell responses and memory $CD4^+$ T cells, followed by infiltration of virus-specific $CD8^+$ T cells, in a manner similar to primary infection [125, 126]. Coordination of cellular responses to HSV reactivation in the skin and mucosa is also largely mediated by DCs, in conjunction with B cells [127, 128]. Memory $CD4^+$ T cells in mice are restimulated to produce IFN-$\gamma$ by local MHC-$II^+$ DCs and B cells [127]. Memory $CD8^+$ T cell responses are also initiated by tissue-resident DCs, without requiring DC migration to draining lymph nodes [128]. In humans, infiltrating virus-specific $CD8^+$ T cells persist at the dermal-epidermal junction for weeks after virus has been cleared, localizing to peripheral nerve endings [56]. Evidence suggests that these cells may be frequently exposed to viral antigen even in the absence of lesions [129], consistent with observations of frequent short bursts of asymptomatic HSV shedding at mucosal surfaces [14]. Modeling studies based on human data suggest that the local immune response is the critical determinant of genital HSV-2 shedding episodes and the development of lesions [57].

In the central nervous system, innate immune signaling through the TLR3 pathway is clearly important in controlling HSV replication during both primary infection and recurrence. Humans with specific defects affecting TLR3 signaling have increased susceptibility to encephalitis, with mutations described in UNC93B [103], TRAF3 [104], TRIF [105], and TLR3 [106, 107]. In mice, TLR2 signaling appears to be important in controlling HSV replication in the brain [130]. Interestingly, however, TLR2 has also been described as contributing to lethality of mice with HSV infection in the central nervous system by dramatically increasing the inflammatory response [131], in a manner regulated by the surface glycoprotein CD200R1 [132]. This concept that CNS inflammation can promote pathogenesis in HSV encephalitis is supported by other murine studies [65–67, 133].

Given that the human immune system directs multiple varied mechanisms at detection and control of HSV infection, it is not surprising that the virus allocates a significant proportion of its genome to overcoming the anti-HSV immune response. HSV modulation of immune responses essentially begins from the time the virus encounters a susceptible cell. Engagement of the HSV entry receptor known as herpes virus entry mediator (HVEM) modifies expression of a number of cellular genes, which may immediately alter the cell environment to promote viral replication [134] or alter mucosal chemokine and cytokine production [135]. These consequences of the HSV-HVEM interaction are thought to be due to transient NF-$\kappa$B activation, which at later times after infection may promote viral gene expression in addition

to modifying expression of cellular targets of NF-$\kappa$B [136]. Subsequently, the viral "virion host shutoff" protein (vhs), which is delivered to the cell within the tegument of the viral particle, promotes degradation of cellular mRNA, inhibiting synthesis of a variety of inflammatory proteins including cytokines and type I IFNs [137]. Additional proteins are expressed relatively early in infection to target different intrinsic antiviral cellular responses, inhibiting various proinflammatory and proapoptotic signaling proteins such as PKR and IRF3, and inhibiting type I IFN signaling pathways by mechanisms such as repression of STAT1 activation [94]. Other innate and adaptive immune responses are also targeted by HSV proteins, including binding of complement by the glycoprotein C (gC) [138, 139], binding of the Fc domain of IgG by the gE/gI complex [140], and interference with TAP-mediated peptide loading onto class I MHC by ICP47 [141–143].

In addition to suppression of cellular production of antiviral proteins, recent work suggests that other cellular processes intended to inhibit viral replication are targeted by HSV. There has been increasing appreciation of the importance of autophagy in resistance to HSV infection [144], particularly in neurons. HSV needs to evade autophagy to cause encephalitis [38], and the virus encodes at least two proteins which target this process. HSV ICP34.5 protein binds and inhibits the cellular autophagy protein Beclin-1 to promote neurovirulence [39], and US11 directly binds and inhibits the double-stranded RNA-dependent kinase PKR, which functions in induction of the autophagic response [40]. Importantly, neurons, in contrast to mucosal cells, require autophagic activity to limit HSV replication but do not respond effectively to stimulation by type I IFN [145].

## 5. Aspects of HSV Immunity Specific to Neonates

Human fetuses and newborn infants are more susceptible to severe infection with a wide variety of different pathogens compared to older children or adults. Fetal and neonatal immune responses have long been recognized to have qualitative differences that change during infancy, and we are beginning to understand the mechanisms that underlie this process (reviewed in [1–3]). The immunology of the young infant is dynamic and complex; for example, some innate responses become more "adult-like" within weeks, while others take a year or more [3, 146–149]. Beyond several weeks of life, acquiring HSV portends virtually none of the severe risks of neonatal infection [150]. In comparison, the risk of progression from tuberculosis infection to active disease remains elevated until approximately 4 years old [149, 151].

Fetal immunology likely represents an evolutionary strategy that contributes to successful parturition through maternofetal tolerance. The fetus and its mother are haploidentical, and thus have the potential for alloreactive responses akin to rejection. Inflammation is generally harmful to the developing fetus, resulting in a number of adverse outcomes including intrauterine growth retardation, premature birth, and spontaneous abortion (reviewed in [152]). The mechanisms that underlie maternofetal tolerance remain incompletely understood but include differential expression of class I HLA molecules [153], altered NK cell activity [154], increased numbers and suppressive activity of regulatory T cells (Tregs) [155–159], myeloid-derived suppressor cells (MDSCs) [160], high levels of adenosine [2] and progesterone [161, 162], and differences in TLR responses [45, 46]. Some or all of these mechanisms may impact immune responses during postnatal life and contribute to the vulnerability of neonates and young infants to infection, as well as to vaccine responses that are generally inferior to older children [1, 2, 46, 61]. Here we focus on those aspects of immune ontogeny with apparent importance for neonatal HSV infection (Table 1).

*5.1. Skin Barrier Function.* Differences in epithelial mechanical integrity and production of AMPs may contribute to increased HSV severity in neonates. The epidermis of the fetus and newborn is thinner than that of an adult, predisposing to disruption by trauma [34]. Skin disruption likely increases the risk of neonatal HSV infection, given the association between invasive monitoring (scalp electrodes) and neonatal HSV infection [163]. Other aspects of skin also develop during the neonatal period, including acidification and production of sebum lipids, and might affect HSV infection or replication. Although in theory maternal- and fetal-derived AMPs may help prevent HSV infection by blocking entry and replication of virus on mucocutaneous epithelial surfaces, there is no evidence that differences in newborn AMP expression or activity contribute to HSV disease. AMPs such as cathelicidin and lysozyme are abundant in amniotic fluid, vernix caseosa, and newborn epithelia [164–167], and levels of some AMPs appear to be elevated in neonates compared to later in life [35–37]. Nevertheless, identifying and augmenting barrier host defenses may have potential to protect against HSV acquisition or reduce disease severity.

*5.2. Autophagy.* It is unclear if differences in autophagy in neonatal central neurons relative to older children and adults affect the severity of HSV infection. However, this would not be surprising given rapid growth and development of the brain during this period and the role of autophagy in neurodevelopment [168, 169]. In addition, autophagy can be induced by signaling through several TLRs associated with control of HSV in the nervous system, including TLR2, TLR3, and TLR9 [41, 42]. As discussed later, there are differences between neonates and adults in the effects of TLR signaling on conventional innate immune responses; similar developmental differences may exist with respect to TLR induction of autophagy.

*5.3. Pattern Recognition Receptor Mediated Responses.* TLR responses change profoundly with age (reviewed in [46]). The expression and function of other PRRs (including RLRs, NLRs, IFI16, etc.) during the neonatal period have not been as well described but are also likely to change during early life [170] and may be important in the pathogenesis of neonatal HSV. The involvement of TLR signaling in protection (TLR3) or pathologic inflammation (TLR2) during HSV CNS disease

Table 1: Immune defenses against HSV with relevance for neonates.

| Immune defense | Role in controlling HSV infection | Immunologic differences in newborns | Comments |
|---|---|---|---|
| Integument | The skin and mucosa provide mechanical and innate antiviral impediments to HSV infection and spread. | Neonates have thin, easily disrupted skin, with differences in pH and sebum production [34]. | Differences in neonatal epithelial anatomy or function have not been formally shown to contribute to susceptibility to HSV infection. Levels of some AMPs appear to be increased during the neonatal period [35–37]. |
| Autophagy | HSV-mediated suppression of autophagy is central to the pathogenesis of CNS infection [38–40]. | Autophagy is mediated by signaling through TLRs, which have age-dependent responses [41, 42]. | Age-dependent differences in autophagy are plausible but poorly understood. |
| PRR responses | PRR signaling in HSV-infected cells induces type 1 IFN production that limits initial spread of infection through and attracts and primes protective Th1-type responses [43, 44]. | Neonates have qualitatively different monocyte and DC TLR responses that result in reduced type 1 IFN and IL-12 production, resulting in weaker Th1-type responses [45–48]. | Age-dependent TLR3 responses to HSV are likely important based on the association between CNS HSV infections and defects in TLR signaling. Age-dependent effects of other TLR or PRR responses are unclear but may also be important for the severity of HSV infection in neonates. |
| NK cells | NK cells are important for control of initial HSV infection prior to development of specific T cell responses [49–51]. | Neonates appear to have impaired NK cell killing of HSV-infected cells [52–54]. | Whether neonatal NK cells have any intrinsic defects or kill less well as a result of impaired activation, for example, decreased IL-12 production by DCs, is unclear [55]. |
| T cell responses | $CD8^+$ T cell responses appear central to control of HSV replication and prevention of recurrence [56, 57]. | Neonatal T cells respond relatively poorly to HSV [58–60]. | Impaired Th1-type responses against HSV in neonates may be due to differences in innate responses by antigen-presenting cells, intrinsic epigenetic factors (e.g., hypermethylation of the IFN-$\gamma$ promoter in $CD4^+$ cells), or perhaps active suppression by suppressor cells [2, 61, 62]. |
| Antibody | HSV neutralizing antibody or ADCC may protect against acquisition of infection [17–21]. | Infants born to women with established HSV infections receive virus-specific transplacental maternal antibody [4]. | Although infants of women with established HSV infection are much less likely to become infected compared to those who acquire primary infection during pregnancy, no definitive proof exists that antibody alone is protective in humans. After infection, antibody responses do not appear to contribute significantly to control of HSV replication. |

suggests the possibility that developmental differences in TLR responses may be involved in the susceptibility of neonates to severe HSV infection. Compared to adults, conventional DCs from cord blood produce significantly less IFN-$\alpha$ and IL-12 upon stimulation with the TLR3 agonist poly(I:C) and show lower expression of CD40 and CD80 [171]. Indeed, IFN-$\alpha$ and IL-12p70 (and consequently IFN-$\gamma$) responses to most TLR agonists, including $PAM_3CSK_4$ (TLR2/6) and CpGA (TLR9), appear to be relatively weak in conventional and plasmacytoid DCs as well as monocytes from cord blood, while IL-1$\beta$, IL-6, IL-23, and especially IL-10 responses are as high or often much higher than in adult PBMC [45]. Experiments using neonatal mice found improved control of HSV-1 infection by expanding the number of DCs with Flt-3 ligand (Flt3-L) treatment, which resulted in increased production of IFN-$\alpha/\beta$ and IL-12 [71]. Consistent with these findings, IFN-$\alpha$ production in cord blood or neonatal mononuclear cells appears to be reduced in response to *in vitro* HSV-1 stimulation [18, 172]. This pattern of a neonatal bias, toward Th2- and Th17-type and away from Th1-type responses, is consistent with impaired control of HSV infection (as well as other intracellular pathogens), and perhaps with increased pathologic inflammation [46]. Interestingly, the ontogeny of individual TLR responses varies. Stimulation of monocytes using TLR3 agonists leads to lower levels of IFN-$\alpha$ when cells isolated from children age 1 or lower are compared with those from adults; however, for TLR9 agonists, comparable responses can be demonstrated within the first few weeks of life [47, 146], that is, the same time period during which infants are susceptible to severe HSV infection.

The mechanistic basis for differences in TLR responses in neonates is not well understood [46], but preliminary evidence suggests that both cell-cell interactions and soluble blood factors may be involved [45, 173]. Decreased MyD88 expression in neonatal monocytes [174, 175] might explain some of these effects given that this adaptor protein is utilized by all TLRs, with the exception of TLR3. Of note, adult MyD88-deficient mice do not control HSV after corneal

inoculation and progress to fatal encephalitis [176]. However, MyD88 expression was reported to be equal in purified neonatal and adult pDCs, while reduced type 1 IFN production in neonatal pDCs appeared to be due to impaired nuclear translocation of IRF7 [177]. Decreased IFN-$\beta$ production may be due in part to altered DNA binding of CREB and IRF3 [178]. Developmental differences in nucleosome remodeling and availability of cytokine promoter sites, for example, IL-12p35 [179, 180], in neonatal monocytes and antigen-presenting cells may be responsible for some of observed cord blood TLR responses, similar to what has been reported for IFN-$\gamma$ in T cells [181]. Increased levels of adenosine, which increases intracellular cAMP [182], as well as other soluble factors [173] in neonatal blood, also appear to be important for suppressing Th1-polarizing responses.

*5.4. Natural Killer Cells.* Impaired killing of HSV-infected cells by cord blood mononuclear cells has long been recognized [52–54]. This could result from impaired activation by cytokines such as type 1 IFNs and IL-12, the production of which is reduced in neonates as mentioned earlier, or may reflect intrinsic differences of neonatal NK cells. NK cells in cord blood may differ with respect to the expression of cell surface markers compared to those from the peripheral blood of adults (reviewed in [55]). For example, some studies have found higher levels of the inhibitory receptor complex of CD94/NKG2A and CD158b/j on cord blood NK cells [183, 184]. However, NK cell IFN-$\gamma$ production in response to mitogen appears to be similar between cord blood and adult peripheral blood [183, 185]. Several studies showed that resting cord blood NK cells are less cytotoxic; however, these cells may actually express higher levels of effector molecules such as perforin and granzyme B [183, 186] and can be induced to be highly cytotoxic using stimulation with various combinations of IL-2, IL-12, IL-15, and IL-18 [183, 187–190]. Thus, any impairment in neonatal NK function may represent extrinsic factors, for example, deficient IL-12 production by DCs. The extent to which any NK cell differences in cord blood are relevant to neonatal HSV infection is unknown, but it merits additional study given that NK cells likely play an important role control of HSV infections in general [49, 50, 191, 192].

*5.5. Adaptive T Cell Responses.* Early studies of neonatal T cell responses to HSV suggested that newborns generate fewer virus-specific cells, which have impaired proliferation to stimulation with virus [58–60]. Furthermore, IFN-$\gamma$ production in response to HSV antigen was significantly lower among neonates and parturient women compared to nonparturient adults, all with recent HSV acquisition [60]. In that study, HSV-specific neonatal IFN-$\gamma$ responses lagged behind those of nonparturient adults until 3–6 weeks after the onset of symptoms. Numerous differences have been described between general responses of neonatal T cells relative to those of adults [2, 61, 62]. Fetal T cells appear to be derived from a distinct lineage compared to adult T cells and are biased toward tolerance [193]. Compared to those from adults, neonatal $CD4^+$ T lymphocytes are more apt to produce Th2- than Th1-type cytokines under the same conditions [194, 195]. Newborn $CD4^+$ T cells may produce lower levels of IFN-$\gamma$ than adult naïve T cells due to hypermethylation at CpG and non-CpG sites within the IFN-$\gamma$ promoter [181, 196]. Interestingly, neonatal $CD8^+$ T cells produce similar levels of IFN-$\gamma$ and have a pattern of IFN-$\gamma$ promoter methylation comparable to that of naïve adult cells [196]. In addition, neonatal cellular responses may be inhibited by the presence of suppressive soluble factors or suppressor cell populations.

Substantial evidence suggests that regulatory T cells (Tregs) play a critical role in maternofetal tolerance [155–159]. Studies in mice suggest that Tregs may also differentially suppress neonatal $CD8^+$ T cell responses to HSV compared to adults [197]. Other suppressor cell populations may be involved in neonatal immunity. MDSCs are heterogeneous populations of immature granulocytes or monocytes that suppress T cell responses and are important in tumor immunology (reviewed in [198–200]). MDSCs appear to prevent inflammation *in utero* based on studies in mice [160]. Although they are present in peripheral blood in very small numbers in healthy adults, MDSCs are found in high frequencies in pregnant women and cord blood and wane during infancy (Helen Horton, personal communication). MDSCs have been reported to preferentially induce Th-2 responses and impair NK and DC responses (reviewed in [201–203]), all of which are characteristic of neonatal immune responses. However, the extent to which Tregs, MDSC, or other suppressor cells contribute to susceptibility to HSV or other infections during postnatal life requires additional research.

*5.6. Antibody.* As discussed earlier, virus-specific antibody might prevent HSV acquisition via neutralization or contribute to control of infection through neutralization or ADCC. A large number of observational studies suggest that, though not completely protective, maternal anti-HSV antibody can reduce the risk of neonatal HSV acquisition [17–21]. This is also supported by studies in mouse models showing protection against neonatal HSV infection by virus-specific maternal antibodies [204–206]. High titers of antibody to HSV among infected newborns at presentation have been suggested to result in less severe disease in some studies [18, 207] but not others [208, 209]. Infants typically develop virus-specific IgM within weeks of HSV infection; however, there is no evidence that these responses contribute to control of infection, recurrences, or outcome. This is consistent with the apparent lack of association between humoral immunodeficiencies and severe HSV infections.

## 6. Possible Interventions Targeting Host Defenses to Prevent Neonatal HSV

*6.1. Vaccination.* Vaccination is considered the intervention with the greatest potential for preventing neonatal HSV [4]. Since acquisition of primary infection during pregnancy confers the highest risk for neonatal HSV disease [163, 210], a prophylactic vaccine would ideally confer high levels of protection against genital HSV in pregnant women. Despite extensive efforts, there are currently no licensed vaccines to

either prevent HSV acquisition or minimize transmission in humans [63, 211]. Clinical trials of candidate prophylactic vaccines against HSV-2 have demonstrated limited clinical activity [212–214]; however, these studies have uncovered important information about host immunity to HSV. Randomized trials of a subunit vaccine comprised of the HSV-2 surface glycoproteins gB and gD revealed that serum neutralizing antibody levels did not correlate with protection from HSV infection in humans [213], suggesting that additional responses, perhaps mucosal or cellular responses, are also needed to confer sterilizing immunity [215]. Even in the absence of complete protection against HSV infection, a prophylactic vaccine that modified the course of infection, by limiting maternal viral reactivation and genital shedding, could still reduce neonatal disease. Such a vaccine would likely require induction of cellular immune responses to HSV. Indeed, a prior study of therapeutic vaccination of individuals with latent genital HSV-2 infection suggested that recurrences might be diminished in some individuals in a manner that did not correlate with antibody production, supporting the concept that cellular immunity is critical to protecting the mucosa from HSV replication [216]. From this standpoint, recent studies elucidating the mechanism of infiltration of HSV-specific $CD8^+$ T-cells into genital mucosa [121] may provide insight into novel vaccination strategies, such as the "prime-pull" strategy suggested by Shin and Iwasaki [217].

Alternative approaches to improve vaccine responses have aimed to enhance immunogenicity. Among those showing preclinical promise in animal models are novel delivery systems such as liposomes [218], modified recombinant bacteria expressing HSV antigens [219], and incorporating the use of novel adjuvants [220] or DNA vaccines [221, 222]. One possible limitation of these approaches is their reliance on a single or a limited set of viral antigens (typically gD, often with 1-2 additional targets) to provide protection against a virus with a genome that encodes more than 80 proteins. Broader antiviral responses could be generated with attenuated or replication-defective viral vectors [215, 223, 224], which in some instances have demonstrated protection in animal models [225–228]. However, it is not clear whether some of these alterations of the virus may remove important targets of human immunity. Other modifications to the viral genome, such as inserting a dominant-negative mutant gene [229] or inserting costimulatory genes [230, 231], may carry the risk of gene transfer to a wild-type virus through heterologous recombination [232]. Novel delivery systems or adjuvants need to be produced in a cost-effective manner, require careful evaluation for safety in humans, and may promote unacceptable inflammatory responses [233].

*6.2. Other Immunologic Strategies to Prevent HSV Infection.* Biological products based on immune proteins, such as cationic AMPs, have been proposed as potential candidates to protect against HSV infection [93, 234, 235]. These molecules might be formulated as microbicides, for example, to prevent maternal acquisition of HSV, or transmission to the infant during birth [64].

## 7. Possible Interventions to Modulate the Immune Response to Neonatal HSV Infection

Alternative approaches to modify host immunity for treatment of neonatal HSV infection have been suggested (Table 2). These strategies have hypothetical benefits that merit study but should not be considered for clinical use until safety and efficacy have been established.

*7.1. Suppression of Local Inflammatory Response in the CNS.* As discussed earlier, data from animal models suggest that deleterious inflammatory responses may play an important role in the pathogenesis of HSV encephalitis [65–67]. Numerous cases of HSV encephalitis treatment using adjunctive corticosteroids with good outcomes, mostly in adults, have been reported [68–70]; however, given the risk of increased viral replication and cytotoxic effects, this approach is controversial [236, 237]. In order to develop targeted immunomodulatory therapies for neonatal HSV infection, a better understanding is needed of the relative contributions and temporal dynamics of the specific inflammatory pathways that mediate control of viral replication and immune-mediated CNS damage [238].

*7.2. Targeting Autophagy.* Development of novel antivirals has been proposed to target HSV proteins that inhibit autophagy [76], and early studies suggest that agents that induce autophagy can inhibit HSV replication [77, 78].

*7.3. Promotion of Th1-Type Responses.* Strategies can also be envisioned that promote Th1-type responses during the neonatal period through novel adjuvants like TLR agonists [47, 48], growth factors such as Flt3-L [71], or other agents that target antigen-presenting cells. If suppressor cell populations are confirmed to impair neonatal immune responses, interventions to oppose the effects of these cells might result in Th1-type responses to HSV more similar to those produced by adults. In models of other diseases, such as HIV infection and melanoma, therapies have been proposed to reverse Treg activity and enhance protective T cell responses, for example, with recombinant IL-7 or blockade of negative costimulatory receptors CTLA-4 and PD-1 [72, 73]. Similarly, studies in solid tumor patients have shown that the suppressive activity of MDSC can be reversed by 25-hydroxyvitamin $D_3$, all-trans-retinoic acid, and other therapies [74]. Any of these immunomodulatory strategies might be expected to improve neonatal responses not just to HSV, but also other neonatal pathogens and vaccines, as well as to potentially prevent atopic diseases [48]. It should be stressed again that any benefit of these interventions for neonatal HSV infection is currently entirely theoretical, and their use for any indication requires extensive study to assure safety in newborns.

Table 2: Potential interventions targeting host defenses against neonatal HSV infection.

| Potential intervention | Comments |
|---|---|
| Maternal vaccination | No effective HSV vaccine is yet available. Neonatal HSV infection could be prevented by a vaccine that either conferred sterilizing immunity to women prior to pregnancy and/or by modifying infection in women to reduce viral replication and shedding in the genital mucosa [63]. |
| Antimicrobial peptides | AMPs formulated as a vaginal microbicide might prevent HSV infection during pregnancy and/or reduce intrapartum transmission [64]. |
| Immunosuppressive therapy for CNS infection | Some component of the immune response to HSV encephalitis may result in pathologic inflammation and contribute to poor outcomes [65–67]. Despite case reports of good outcomes using adjunctive corticosteroids in adults or neonates with HSV CNS infection [68–70], no controlled studies have been performed and this or other immunosuppressive treatments cannot currently be recommended given the risks of increased viral replication and cytotoxic effects. |
| Immunomodulation of neonatal Th2/Th17 bias | Th1-type responses might be promoted during the neonatal period with novel adjuvants such as imazoquinolines [47, 48], growth factors such as Flt3-L [71], or other agents that target antigen-presenting cells. These strategies might conceivably be used therapeutically during infection or to prime all neonates to respond to infection and vaccinations [48]. |
| Inhibition of suppressor cell function | Tregs, MDSCs, or other suppressor cell populations might contribute to impaired T cell responses during early infancy. Modulation of these cells' activity might improve immunity to HSV infection, such as what has been proposed for HIV and cancer [72–74]. Inhibition of suppressor cell function during HSV infection might also result in uncontrolled inflammation and worse outcomes [75]. |
| Induction of autophagy | Novel antivirals have been proposed to target HSV virulence factors that inhibit autophagy [76], and early studies suggest that agents that induce autophagy can inhibit HSV replication. Nelfinavir and pentagalloylglucose both induce autophagy and inhibit HSV replication *in vitro* [77, 78]. |

## 8. Conclusions and Directions for Future Research

An effective prophylactic HSV vaccine represents an ideal way to prevent neonatal HSV infection. In the absence of such a vaccine, early recognition and aggressive antiviral treatment of neonatal HSV infection remain the mainstays of care. The development of new interventions for neonatal HSV discussed earlier requires a better understanding of the mechanistic basis of immune control of HSV infection in general and how neonatal responses to HSV are ineffective by comparison. Specifically, more studies are required to understand the basis of differential TLR and other pattern recognition receptor responses in early life and their effects on neonatal HSV infection. Differences in T cell responses to HSV between neonates and older children or adults also merit more study, as do the relative contributions of impaired priming, inherent differences in T cell signaling, and/or active suppression on poor cellular control of HSV infection during the newborn period. The role of Tregs, MDSC, and other suppressor cell populations in immune control of HSV infection is also of great interest. More complete knowledge of immune ontogeny could lead to interventions that might be routinely given to all newborns to improve immune responses not just to HSV, but to a wide range of other infectious pathogens and vaccines as well.

Just as important as understanding the immunology of how neonates differ from older children and adults, however, is to determine what benefits if any there are during early postnatal life that come from the apparent persistence of *in utero* tolerance. If immune response patterns in early infancy simply represent a transition between fetal and adult-type immune responses that requires time but serves no function, it may be safe and advantageous to expedite this process. It is possible, however, that the ontogeny of immune system during early postnatal life is evolutionarily adaptive. It has been hypothesized that without relative tolerance immediately postpartum, rapid colonization of newborns with myriad microorganisms and non-self-antigens might lead to overwhelming inflammation [2]. Other possibilities, which are not mutually exclusive, include the possibility that relative neonatal tolerance protects against autoimmunity and allergies [170, 239]. Neonatal HSV infection represents both an important clinical problem and a fascinating example of age-dependent immunity. Through a greater understanding of the dynamic interplay between the virus and host, there are opportunities to rationally develop safe and effective therapies to prevent or treat neonatal HSV infection.

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