Factors Affecting the Immunity to Respiratory Syncytial Virus: From Epigenetics to Microbiome

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Respiratory syncytial virus (RSV) is an omnipresent virus that infects virtually all children by 2 years of age (1). It is also the most common cause of hospitalization in children under 2 years of age, causing severe lower respiratory tract infection, bronchiolitis, and pneumonia, and is associated with an increased risk of developing childhood asthma and recurrent wheezing (1, 2). Severe RSV infections often exhibit an inefficient activation of innate immunity and skewing of the acquired immune response toward a Th2 and/or Th17 phenotype leading to airway mucus overproduction, with both an excessive Th2 response and with IL-17 highly upregulated in infants (1, 3–6).

Considerable effort has been expended to develop an effective vaccine against RSV infection without success. The prophylactic use of a neutralizing monoclonal antibody against the RSV F envelope protein (palivizumab) prevents progression of RSV severity in the high-risk patient with RSV infection (7). It is also used as a therapy to control the infection in patients who present with RSV-related bronchiolitis (7, 8). However, this treatment is expensive, and most infants who develop severe infection do not exhibit risk factors, necessitating a better understanding of what drives severe disease in order to develop new therapeutics or effective vaccines.

In this review, we cover several aspects that contribute to the immune response to RSV, including the development of the immune system and the lung environment, the role of epigenetics...
in this development, and the contribution of the microbiome to long-term pulmonary health. These factors are summarized in Figure 1.

THE NEONATAL IMMUNE SYSTEM AND RSV

The age at which a child is infected with RSV plays a critical role in disease outcome. Infants who are infected before 6 months of age are at higher risk for developing bronchiolitis that requires hospitalization, even when compared with bronchiolitis caused by other viral infections (9). One of the primary reasons for this is that the neonatal immune system has a number of functional differences when compared with the immune response of adults.

Early in life, immune protection is mediated primarily by the innate immune system, as adaptive immunity does not begin to develop until after birth and continues developing through early childhood (10). Furthermore, the immune system is skewed away from a pro-inflammatory state, as this can lead to spontaneous abortion and damage to the developing tissue, especially the lungs (11). Thus, neonatal immune cells exhibit low levels of pro-inflammatory cytokines including the type I interferons (IFN), IFN-α and IFN-β, as well as low levels of IL-12 and TNF-α, but higher levels of anti-inflammatory cytokines such as IL-10 and TGF-β (12–14).

Because the neonatal system is reliant on innate immune recognition, signaling through Toll-like receptors (TLRs) is relatively well developed in neonates. These pattern recognition receptors are a critical component of the innate sensing of pathogens in infants. However, although TLR sensing and signaling in infants occurs on a similar level to that in adult cells, the immune mediators that result from these signaling pathways are impaired in infants. While type I IFN levels rise rapidly in the first month of life, IL-12, which is important for driving a Th1 response, is one of the last cytokines to reach adult-like levels (13, 14). This
increase in pro-inflammatory cytokines is mirrored by a decrease in anti-inflammatory IL-10 following TLR signaling (13). IL-6 and IL-23 are also enhanced in TLR-stimulated neonatal cells compared to adults (13, 14). As these cytokines are potent inducers of IL-17, neonates have an increase in Th17 cells (15). Because increased IL-17 is often associated with enhanced RSV-induced pathology, the prevalence of this cytokine in infants may contribute to enhanced disease (3, 4, 6). An additional level of TLR regulation in neonates is the high level of adenosine found in the plasma of umbilical cord blood (16). Adenosine is a metabolite that is released in response to inflammatory events and acts as a feedback mechanism to decrease TLR-dependent Th1 inflammation (17–19). This combination of both cell-intrinsic factors such as decreased cytokine production and exogenous factors such as plasma adenosine limits the function of TLRs in the neonates.

Dendritic cells (DCs) express a number of TLRs that are essential for dictating T cells responses based on the ability of DCs to present antigen to T cells causing their activation, and subsequent cytokine production. Myeloid DCs (mDCs) are the primary source of the IL-12 needed to drive a Th1 response. As mentioned above, IL-12 levels are reduced in neonates compared to adults. mDCs are also a significant source of other pro-inflammatory cytokines including IFN-β, which is robust in adult cells but impaired in neonatal DCs (20, 21). Neonatal and adult mDCs also exhibit a number of other differences including lower expression on neonatal cells of MHC-II for antigen presentation and CD80/86 for costimulation of T cells (21, 22). Activation through TLR signaling or cytokine addition fails to upregulate these surface molecules to a level seen in adult mDCs (21). Plasmacytoid DCs (pDCs) are another subset of DCs involved in directing inflammatory responses. While in adults, the number of mDCs is higher than pDCs, neonates have approximately three times the amount of pDCs as mDCs (23). However, even with this increased predominance, pDCs from neonates fail to adequately mature upon stimulation, including decreased production of type I IFNs that are critical for the antiviral response (12, 24).

Neutrophils are another critical innate cell type, which function independently of antigen recognition. While neutrophil infiltration into the lungs in response to RSV may contribute to tissue damage, these cells also help limit the spread of the virus and participate in the induction of the adaptive immune response (25–28). Infants have a higher number of neutrophils in the blood than adults, but these cells have decreased function (29). Neutrophils can control viral infection through phagocytosis and release of cytotoxic granules for killing the infected cells; both of these features are impaired in neonatal neutrophils (29, 30). During RSV infection, neutrophils can phagocytose RSV-infected cells as well as protect epithelial cells from infection, providing a protective role against viral replication (25, 31, 32). Although damaging effects of neutrophils are thought to contribute to a decline in lung function following infection, they are an essential part of the innate immune response to RSV (28).

Natural killer (NK) cells are one of the more prominent cells types in the blood of neonates, with levels exceeding those of adult blood. However, like many other cell types, the function of these cells is drastically reduced early in life. NK cells bind to target cells, such as virally infected cells, and release cytotoxic granules to kill the infected cells. The cytotoxic ability of neonatal NK cells is decreased compared to adults, as cells from infants have fewer cytotoxic granules as well as a decreased capacity for degranulation to release their contents (33, 34). Whether these cells have dysfunctional migration is unknown; however, infants with severe RSV infection have been noted to have low numbers of NK cells, underscoring the need for these cells in a productive response to the infection (35). NK cells also produce IFN-γ, and neonatal NK cells produce less of this Th1 cytokine than adult cells (36). However, unlike other cell types, these defects are not intrinsic to neonatal NK cells but are instead due to decreased signaling and activation from pro-inflammatory cytokines made by other immune cells. Exogenous addition of IL-12 to neonatal NK cells restores their cytotoxic effects and ability to produce IFN-γ (36–38). As mentioned above, IL-12 production from DCs is delayed in neonates, thereby resulting in an environment that is less permissive for NK cell activation, as well as Th1 cell differentiation.

Neonatal CD4+ T cells are skewed away from a pro-inflammatory Th1 response and instead favor a Th2 or Th17 response, both of which have been implicated in severe RSV infection (4, 39). Like NK cells, this is at least in part due to extrinsic factors such as decreased DC-derived IL-12 in the environment, as these cells can produce IFN-γ when transferred into an adult environment (40). However, there are T cell-intrinsic factors that limit Th1 responses as well, including a number of epigenetic factors that will be described later in this review. Additionally, many of the circulating T cells in neonates are known as recent thymic emigrants, which do not proliferate as well as other circulating T cell subsets and produce lower levels of IFN-γ (41). Finally, neonates have increased numbers of regulatory T cells (Tregs), which limit inflammatory responses early in life (42–44). As previously noted, this is important for preventing tissue damage. In addition, maternal immune cells can cross the placenta and reside in developing fetal lymph nodes. As some of these cells can recognize proteins derived from paternal genes, Tregs are a critical mechanism for suppressing this maternal immunity and preventing fetal rejection (45).

The decrease in CD4+ T cells in neonates translates to altered B cell function early in life. As these T helper cells are needed to drive antibody production from B cells, neonates have delayed antibody responses compared to adults, including antibody responses to RSV (46, 47). Neonates also have a different distribution of IgG isotypes, with lower levels of Th1-associated IgG2 (48). Antibody responses in neonates are also of lower titer, lower affinity, and persist for less time, but begin to reach adult-like function after 6 months of age (49–51). In general, the primary antibody protection in infants comes from maternal antibody transfer, both in utero and through breast milk following birth. This latter aspect has led to the support of developing a vaccine for RSV that would target mothers in order to transfer maternal protective antibodies to infants to provide early life protection from severe infections (52–54).

**Preterm Birth and RSV Risk**

As previously mentioned, infants under 6 months of age are at a higher risk of severe RSV infection requiring hospitalization
for RSV-induced bronchiolitis. This is especially true of infants who are born prematurely (<37 weeks gestation) (55, 56). There are number of reasons why premature birth results in increased risk for severe RSV infection. The differences in the immune response between infants and adults are more pronounced in preterm neonates. As mentioned above, maternal antibodies are a significant source of immune protection in neonates and are able to cross the placenta during pregnancy, and the majority of this occurs during the third trimester (57). Babies born to mothers with high levels of RSV neutralizing antibody are protected from severe diseases. However, this protection is to an extent lacking in preterm infants (58). Thus, treating preterm infants with palivizumab has become more standard for these infants born within RSV season, which occurs during the winter months (58).

As noted earlier, neutrophils are critical regulators of innate immunity and are especially important in early life when the adaptive immune response has not fully developed. However, neutrophils in preterm neonates exhibit reduced function compared with those from term infants. It has long been known that neutrophils from preterm neonates have decreased migratory ability compared with the cells from term neonates (59, 60). Neutrophils from these infants release lower levels of bactericidal/permeability-increasing protein than cells from term infants, which may result in increased susceptibility to infection (61). Recently, it has been shown that neutrophils from preterm infants have decreased pathogen recognition and antimicrobial ability as well (62). One study found that nasal washes from preterm infants have fewer leukocytes and lower levels of IL-8 than washes from term infants, which is a key cytokine in the recruitment of neutrophils, indicating a role for neutrophils in RSV infection of preterm neonates (63).

Numerous other innate immune defenses are also deficient in preterm neonates compared to term neonates. A small study of infected infants found that preterm infants infected with RSV had fewer pDCs in the bronchoalveolar lavage fluid than those born at term (64). In addition to decreased cell number in preterm infants, monocyte activity is also decreased. A recent study found decreased inflammatory gene expression in monocytes of preterm cord blood compared with both term cord blood and adult peripheral blood (65). Upon exposure to RSV, lower IL-6 production was noted in monocytes from the cord blood of preterm infants compared to term and adult blood (66). The overall reduction in the innate immune cell responsiveness to RSV infection may be intrinsic and therefore difficult to overcome by vaccination, especially if muted adjuvant reactivity is also a lingering issue (66).

In addition to immunologic deficiencies in preterm neonates compared to those born at term, complications also arise due to differences in lung development. The initial capacity for oxygen exchange occurs late in development when alveolar septal formation begins at approximately 34 weeks of gestation (67, 68). This increases the number of alveoli in the distal airways, thereby increasing the surface area available for oxygen exchange. As this process continues into the postnatal period, premature birth before the process is fully underway can have serious consequences. The primary pathology that occurs in the lungs from premature birth is bronchopulmonary dysplasia (BPD), which results in a decrease in the number of alveoli (69). RSV infection of infants with BPD results in a much higher rate of hospitalization than for RSV-infected infants without BPD, even when comparing infants born at similar gestational ages (70). Treatment of these patients with appropriate antiviral therapy or palivizumab, although complex, would greatly reduce the hospitalization rates due to RSV infection (70).

**EPIGENETIC MODIFICATIONS AFFECTING THE IMMUNE RESPONSE TO RSV**

Many of the differences in immune function between neonates and adults are due to epigenetic modification of genes that control inflammation. Epigenetics refers to mechanisms that alter gene expression without changes to the sequence of underlying DNA, thereby controlling many aspects of development. The three primary types of epigenetic regulation are histone modifications, DNA methylation, and microRNA expression. Histone modifications can regulate gene expression by altering residues on the histone tails, such as methylation, acetylation, and phosphorylation. In general, histone modifications "fine tune" the gene expression by altering chromatin structure. This change in structure can wind the chromatin more tightly to prevent access of transcription factors and repress gene transcription. Alternatively, the change in chromatin structure can result in increased DNA accessibility to transcription factor binding, therefore promoting gene activation. These modifications are referred to as "activation marks" or "inhibitory marks." For example, methylation of lysine (K) 27 of histone (H) 3 (i.e., H3K27) is associated with repression of gene transcription, whereas H3K4 methylation is permissive for active gene transcription (71–73). This process is orchestrated by methyltransferases that catalyze the addition of methyl groups and demethylases that remove them. Other modifications such as acetylation and phosphorylation have similar mechanisms of action. On the other hand, DNA methylation results in the inhibition of gene transcription, resulting in genes being turned “off” (74–76). This primarily occurs on cytosine residues in regions rich in cytosine and guanidine, known as CpG islands, in which methylation of the cytosine residues results in suppression of gene transcription (77). Finally, microRNAs are small non-coding RNAs that bind to messenger RNA. miRNA recognition of target mRNA is not always 100% complementary, so a single miRNA can target multiple mRNAs (78). miRNA interaction with mRNA results in either mRNA degradation (in the case of complementary recognition) or translational repression (in the presence of base pair mismatches), but both with the final result of inhibiting protein synthesis (79, 80). Together, these mechanisms are able to alter gene expression on multiple levels, as summarized in Figure 2.

**Epigenetic Regulation of Immune Cell Development**

Several recent studies suggest that epigenetic modulation is important in dictating immune cell phenotype and function and
allows the immune environment and/or the external environment to influence the outcome of the immune response. For example, IL-4, IL-5, and IL-13, which are produced by Th2 cells, are silenced in Th1 cells through histone modification (81). Conversely, Th17 cells have increased levels of the activating mark H3K4 methylation at the Th17 promoter (82). In DCs, decreased H3K4 methylation at the IL-12 promoter has been measured following severe sepsis, thereby altering subsequent immune responses and leading to higher susceptibility to subsequent infections (83). The epigenetic alteration of immune cells allows a more stable phenotype to develop in response to the environment, however sometimes in negative contexts (83).

In the neonatal immune system, further epigenetic regulation is present and prepares the immune cells for subsequent responses. As discussed above, neonatal immune cells are poised toward Th2 effector function. There are a number of epigenetic mechanisms behind this Th2 bias. Many studies have identified differential DNA methylation patterns in human neonatal cells compared to that of adults. In differentiated adult Th2 cells, the Th2 locus is hypomethylated, leading to a more permissive transcriptional state of Th2 cytokines (84, 85). In neonatal naïve CD4+ T cells, a similar hypomethylation is observed, indicating that these cells are poised for a Th2 response even before activation (86). Hypomethylation of the IL13 distal promoter has also been observed in naïve human neonatal CD4+ T cells (87). Increased methylation of Th2 genes begins in the first year of life and continues out to 5 years of age (88, 89). Conversely, naïve human neonatal CD4+ T cells have increased Cpg methylation at the IFNG promoter compared with adult cells, which correlates with decreased IFN-γ production (90, 91). Furthermore, hypermethylation has been observed at numerous Th1-associated genes in neonatal mononuclear cells compared with adults, indicating increased regulation of Th1 responses in neonates (92). These phenotypes must be actively altered to allow a more balanced response to occur, including moving the responses to environmental and infectious agents toward a more appropriate, nonpathogenic response (92).

MicroRNAs contribute another level of regulation of immune development early in life. Many miRNAs have been found to be differentially regulated in human cord blood samples compared to adult bone marrow or peripheral blood samples (93, 94). For example, monocytes isolated from cord blood express more miR-146a following LPS stimulation compared to cells from adult peripheral blood (95). As miR-146a is a negative regulator of TLR4 and increased expression of this miRNA results in a decrease in TLR4 responsiveness in neonatal monocytes, this prevents a robust pro-inflammatory response to bacterial infection (96). T cells are also differentially regulated by miRNAs in neonates. In neonatal CD8+ T cells, two miRNAs, miR-29 and miR-130, drive cells to become short-lived effector cells instead of memory effector cells, thereby altering the immunological memory to an antigen (97). Similarly, cord blood CD4+ T cells express higher levels of miR-181a and miR-184 compared to adult peripheral blood cells, which decrease maturation and activation of these cells (98, 99).

Fewer studies have identified changes in histone modifications in neonatal immune cells, although some work has been done in monocytes. In particular, altered nucleosome remodeling in neonatal monocytes has been linked with defective DC development and impaired IL-12 production (100, 101). More recently, an in-depth analysis of H3K4 methylation on neonatal monocytes was performed (65). Increased methylation of H3K4 is associated with increased gene transcription, and cord blood monocytes have dramatically lower levels of H3K4 methylation compared with adult cells. Furthermore, it was noted that H3K4 methylation is nearly absent in preterm neonates and is even lower than cord blood from term infants, indicating that DNA methylation is continuously increasing during development and that monocytes...
from premature infants are not “poised” for an appropriate innate immune response (65).

Epigenetic Regulation by Environmental Exposures
There are a number of epigenetically regulated factors that may affect the severity of RSV infection in infants. Environmental exposure both in utero and following birth results in alterations of the lung environment that may be detrimental to developing the appropriate response to RSV infection. One of the best-studied exposures is that of tobacco smoke. Infants who are exposed to tobacco smoke in utero are more likely to be admitted to the intensive care unit with bronchiolitis (102). This risk is further increased when these infants continue to be exposed to tobacco smoke following birth (102). A number of studies have shown that in utero smoke exposure results in detrimental epigenetic changes. Prenatal exposure has been shown to alter the DNA methylation status of a number of genes into childhood (103). In particular, differential methylation of genes involved in T cell development has been observed in infants exposed to tobacco smoke prenatally compared with those that were not (104). A recent study has also identified miRNAs that are altered in the lungs of mice exposed to prenatal tobacco smoke, including miRNAs that regulate immune pathways (105).

Maternal diet is known to alter the epigenetic profile in utero. In particular, women are encouraged to take a folic acid supplement during the first trimester of pregnancy to prevent neural tube defects. However, folate also acts as a methyl donor, and children born to women who begin folic acid supplementation 6 months before pregnancy and continue supplementation throughout pregnancy have increased methylation at CpG islands in cord blood DNA (106). These children have an increased incidence of wheezing during childhood, as well as increased risk of hospitalization with respiratory infection early in life (107, 108). These early studies set up new paradigms to focus on the role of epigenetic changes that have long-term effects on the development of lung pathology.

Epigenetic Modifications in Response to RSV
A number of studies have shown that inflammatory stimuli can alter histone methylation of immune cells, including influenza and sepsis (83, 109). We have previously found that RSV infection of DCs leads to alterations in histone methylation by the H3K4 demethylase KDM5B, resulting in decreased pro-inflammatory cytokine production and a subsequent increase of Th2 cytokines from T cells (110). We have also found that methylation of H3K4 in regulatory T cells by the histone methyltransferase SMYD3 is necessary to control inflammation in the lungs following RSV infection (111). Another group recently identified that IFN-γ stimulation of epithelial cells resulted in changes in H3K9 methylation status of the RIG-1 promoter, thereby upregulating RIG-1 (112). As RIG-1 is an important component of the immune response to RSV, this resulted in decreasing viral load (112, 113). Other histone modifications induced by RSV infection include histone acetylation. Infection of bronchial epithelial cells results in increased histone deacetylase 2 (HDAC2) that appears to alter innate immune responses; inhibition of HDAC2 results in increased production of type I IFN and decreased viral replication both in cultured cells and following RSV infection of mice (114).

Respiratory syncytial virus infection has also been shown to alter DNA methylation status, as shown by a number of recent studies. In humans, cultured bronchial epithelial cells infected with RSV upregulate Nodal, a member of the TGF-β superfamily, resulting in increased Th2 and Th17 skewing of T cells (115). A study in the cord blood of children found that those who had increased methylation in the enhancer region of the perforin-1 gene had increased risk of developing lower viral respiratory tract infection, including an increased risk of RSV infection (116). Perforin-1 is an essential cytotoxic protein in CD8+ T cells and NK cells in the control of viral infection. A follow-up study found that children at 3–4 years of age who had been hospitalized with severe RSV-induced bronchiolitis as infants had decreased methylation of the perforin-1 enhancer, indicating a role for RSV in altering the immune response even years after infection (117).

Studies in mouse models suggest that RSV can alter miRNA expression in the airway and affect how the tissue responds to future stimuli, especially in the development of post-viral allergic asthma. Infection of allergic mice with RSV results in the development of steroid-insensitive allergic airway disease, and this event is driven by the upregulation of a miRNA that inhibits steroid sensitivity (118). Another model using pneumonia virus of mice (PVM, a rodent-specific RSV homolog) found that neonatal infection changed the expression of a set of miRNAs that altered the way the mice responded to allergen challenge as adults (119). The regulation of how these miRNAs are expressed and what cell populations are expressing the specific miRNAs is not clear, but this regulation likely dictates how they alter the subsequent allergic responses (119). Together, these studies demonstrate the many ways in which the immune system is regulated during RSV infection and the potential development of allergic asthma.

MICROBIOME, RSV, AND ASTHMA
As previously described, a number of studies have linked early severe RSV infection and the consequent RSV-bronchiolitis immunopathology to the development of chronic allergic diseases like asthma in the later years of life (120, 121). We have discussed some factors that contribute to the development of severe RSV disease such as prematurity (immature immune system), congenital conditions like bronchopulmonary dysplasia, and age (from premature infants to 6 months old). There are many additional known factors that predispose to develop severe RSV disease, including Cesarean (C)-section delivery and breastfeeding for less than 1 month, which are factors that are also known to affect the composition of the microbiome and to contribute to the development of allergic disease (122–124). The microbiome assembles during the first year of life and critically sets the characteristics of the adult microbiome, as well as contributes to healthy immune responses and metabolic processes in infants (124–126).

Our lab and others have shown that microbiome composition can impact the development of RSV pathogenesis and
allergic asthma in mice previously exposed to enriched micro-
bial suspension or probiotic prior to RSV infection or allergen
challenge (127, 128). In this section, we will discuss the impact
of the infant microbiome in the development of allergies and
severe lower respiratory tract infection (LRTI).

**Infant Microbiome and the Development
of the Immune System**

The human microbiome has been shown to be involved in sev-
eral essential processes in humans, from organ development to
modulation and fine tuning of the immune system. The micro-
biome is formed by microbial communities, collectively termed as
microbiota. Their composition is in constant change during life,
with variations of the microbiota in a number of environments,
such as the lung, gut, or skin. Several factors affect the diversity
of the microbial communities: the use of pharmaceuticals,
nourishment, microbial exposure, and infection. Changes in the
microbial diversity can impact the general homeostasis of the
organism (122–126).

**Impact of Prenatal Microbiome:
Respiratory Virus and Allergies**

Historically, the uterus has been thought to be a sterile envi-
ronment, although studies have questioned whether there is
microbial exposure during the prenatal period. Microbes have
been described in the placenta, amniotic fluid, fetal membrane,
umbilical cord blood, and meconium (129, 130). The possibil-
ity of the impact of this microbial community to the develop-
ment of the immune system, metabolite production, and fetus
health, in general, is an active area of investigation for many
groups (129, 131–134). For example, in a study where pregnant
mothers received probiotic supplements, a microbial alteration
was detected in infant meconium compared to controls (130).
Others have found that changes in microbiome during pregnancy
are linked to different outcomes in the offspring from behavior
alterations to allergy predisposition (130, 135).

Atopic dermatitis is one of the first and most studied diseases
in infants used to predict their allergy susceptibility and the early
manifestation of atopic dermatitis–like eczema has been associated
with an increased risk of asthma (136, 137). A double-binding
study showed that administration of probiotics to mothers during
the last trimester of pregnancy and during lactation increased the
immune-protective potential for the breast milk, by increasing
TGF-b2, which is a known immunoregulatory factor, in the milk
compared with placebo-receiving mothers. In this case, the infants
from probiotic treated mothers had a significantly reduced risk of
developing eczema during the first years of life compared with the
children from placebo–treated mothers (130, 132).

Increased microbial exposure has been shown to modify the
microbiome (127, 131). In a longitudinal study, a cohort of
infants was followed from birth to 2 years of age and IgE was
measured in blood samples. Infants born to mothers who owned
pets (parental indoor pet exposure) had a lower titers of total
systemic IgE from 6 months old than the children lacking pre-
natal pet exposure and the difference was significantly stronger
at 2 years of age (134). In a double-blind trial, mothers from
families with allergic disease received probiotic supplements in
the last trimester of pregnancy. After birth, their babies con-
tinued with supplementation until 1 year old, and these babies
had decreased allergic incidence compared to control groups.
Specifically, these infants had less eczema associated with
IgE-associated at 2 years of age and reduced risk of developing
later respiratory allergic disease (136). Another longitudinal
study found that infants who were at risk of developing asthma
had a transient disruption of the gut microbiome during the
first 100 days of life, with significant decreases of *Lachnospira*,
*Veillonella*, *Faecalibacterium*, and *Rothia* genera (138). The
reduction of these bacterial taxa also impacted the levels of
acetate in the feces, as well as dysregulation of liver metabolites.
Germ-free mice inoculated with these four bacteria showed
decreased airway inflammation demonstrating the importance
and impact of the early microbiome composition in preventing
the development of asthma and possibly other allergic diseases
in children (138). Together, the microbiome during pregnancy
has been proposed as a critical component for the overall health
of the infant. A healthy microbiome during pregnancy may
modify microbial communities in the mother’s gut, vagina,
and uterus and would transfer the healthy microbiome in gut,
lung, and skin to the fetus/infant in utero and during vaginal
delivery. Evidence suggests that this is correlated with a healthy
microbiome in the infant.

**Mother to Offspring: Microbiome Alteration**

It has been widely described that the mode of delivery strongly
affects the microbiome species found in the neonates, specifi-
cally that C-section delivery increases the risk of several health
conditions in the newborn including increases in the likelihood
of respiratory distress even at term (139, 140). In addition,
C-section deliveries are associated with an increased risk of
developing asthma and gastrointestinal diseases, among others
(141). Industrialized countries have experienced a rise in the
rate of Cesarean delivery in the 20 last years, correlated with
an increase of autoimmune diseases and also allergic disease
including asthma, eczema, atopic dermatitis, and food allergies
(142, 143). The implication of the microbiome modification by
C-section and the impact in the early and adult life of the off-
spring to develop asthma is currently an area under investigation.

Using 16S rRNA sequencing to define the bacterial microbiota
in stool specimens of a birth cohort collected during the first year
of life, one group evaluated the associations between pre- and post-
natal environmental and sociocultural factors and the composi-
tion of the early life gut microbiome. This study found substantial
age-related taxonomic variation in the microbiota (144). At birth,
the primary bacterial communities were *Bifidobacteriaceae* or
*Enterobacteriaceae* taxa, while infants (<1-year olds) were typi-
cally dominated by either *Bifidobacteriaceae* or *Lachnospiraceae*,
the latter of which represents a typical dominant family in adult
gut microbiomes (144). The impact of delivery mode in the com-
position of the infant microbiome has been previously described,
with infants delivered by C-section presenting significant dif-
f erent diversity in the gut microbiome compared with vaginally
delivered infants (145). Besides compositional variation by mode of delivery overall (vaginal vs. C-section), significant differences were observed whether the C-section was planned or unplanned, indicating that even early labor may affect the microbial composition of the infant (139).

In a population-based national register cohort study, the effects of acute or elective C-section vs. vaginal delivery in children from 0 to 23 months of age were assessed. Hospitalization for RSV disease, with adjustment for external factors such as birthrate and maternal smoking during pregnancy, showed a positive association between delivery by C-section and increased risk of hospitalization for RSV infection, with this effect continuing out to 2 years of age (123). Together, these results suggest a correlation between RSV hospitalization and microbiome diversity modification by mode of delivery, although more research is needed in this area.

Breastfeeding: Microbiome, Respiratory Virus, and Allergies

The microbiome and immune system are actively modulated by specific factors within breast milk, including IgA that confers passive immunity. Breast milk also includes several metabolites that can impact metabolism, development, and maturation of the immune system in the neonates (146, 147). As we described above, the neonatal immune system is not entirely mature during the first months of life, and thus the interaction with the different organisms, foreign proteins and chemicals will set the basis for a successful interaction between the innate and adaptive immune systems. The infant’s interaction with components of the commensal bacteria population helps to stimulate the maturation of the immune system. A critical component of the immune development driven by the microbiome is immune suppression at the gut mucosal surface, which induces oral tolerance to food antigens (145). Breast milk is known to contribute commensal bacteria to the infant gut microbiome, and several studies have shown that breast milk contains approximately $10^3$–$10^4$ colony forming units per milliliter, thus being an excellent source of probiotics (147).

A number of studies have investigated the link between breastfeeding and the development of respiratory disease. In a prospective cohort study, newborns were followed up for 1 year to monitor hospitalization for bronchiolitis (148). These infants were classified as “never breastfed” and “ever breastfed,” with the second group further divided into those “exclusively breastfed” and “breastfed associated with milk formula.” The risk of hospitalization at 1 year of age for bronchiolitis was significantly higher in the “never breastfed” group. Infants who were exclusively breastfed or breastfed associated with formula milk demonstrated a similar, reduced risk of bronchiolitis-related hospitalization, thus confirming the protective effect of maternal milk in the development of severe bronchiolitis (148).

In another study, infants who tested positive for RSV were divided into full, partial, or never breastfed as above, and were followed for 10 days after the initial diagnosis to analyze the incidence and duration of hospitalization and the frequency of requiring oxygen therapy. While there were no differences in the hospitalization rate among the three groups, those infants who were never breastfed had an increase in the duration of hospital stay and required oxygen therapy at a greater rate than the breastfed groups (149). This study agrees with another cohort of infants (<1-year olds) hospitalized for bronchiolitis, which found that the lack of breastfeeding correlated with higher rates of bronchiolitis (150).

We describe above that the prenatal microbiome composition impacts the development of allergies in the offspring and that probiotic supplementation infants received from their breastfeeding mothers decreased the risk of atopic dermatitis (146), indicating that the microbiome composition of the mother during breastfeeding also impacts the immune system of the infants. It is known that human milk composition can be modulated by the maternal environment and also that breast milk is unique between mothers (146). These data suggest that probiotic supplementation by the mother during breast feeding could be a best strategy aimed at decreasing further the incidence of allergy development and LRTI (RSV) in breastfed infants.

Microbiome Modification in Infants: Supplementation and Microbial Exposure

In addition to probiotic supplementation of pregnant women, supplementation of children has also been studied. These studies show a similar effect to those observed in mothers on probiotic supplements during both pregnancy and breastfeeding, reinforcing the idea of the gut microbiome shaping the immune response to asthma and LRTI. The lung microbiome has also been studied. A shift in the microbial composition of the lung during respiratory diseases suggests a link to the host immune response to viral infection (151). An experimental study in mice that were exposed nasally to two different strains of Lactobacillus rhamnosus showed that the intranasal administration of these commensal bacteria modulated the TLR3/RIG-I antiviral respiratory immune response, where both strains demonstrated increased resistance of infant mice to RSV infection compared to controls (152). Another study in which young mice (3 weeks old) were orally supplemented with Lactobacillus rhamnosus CRL1505 before a RSV challenge showed improved resistance against RSV infection with decreased immunopathology, along with altered expression of IFN-γ and IL-10 in bronchoalveolar lavage, suggesting that the modulation of the immune response by probiotics early in life could favor protective immunity against RSV (153).

In humans, a randomized study with preterm infants was grouped to receive oral probiotics (Lactobacillus Rhamnosus GG) or placebo, between days 3 and 60 of life. These infants were followed to assess the effects on viral respiratory tract infection. The results showed a significant decrease in the detection of rhinovirus in nasal swabs in the children that were treated with probiotic compared with the placebo group, suggesting that the supplementation of probiotics in premature infants may prevent rhinovirus infection, although there were no significant differences in the detection of RSV (154). Prenatal characteristics were described but not analyzed (154), and as mentioned above, the prenatal microbiome regime likely modified the immune response of the infants positively (130, 132). These results suggest
that the supplementation in infants after birth may not be as an effective as prenatal intervention.

Household microbial exposure during the first years of life can shape the microbiome of children, and it has been suggested that the increased microbial exposure during the prenatal stage and the early years of life affect maturation of the immune system and help to reduce the risk for the development of allergic diseases (155). The protective effect of allergic diseases by microbial interaction with farm animals and pets during early life has been studied by a number of groups (155, 156). In a cross-sectional survey in rural areas in Europe, children from farming families who were exposed younger than 1 year to farm animals had significantly decreased allergy sensitization with lower frequencies of asthma, hay fever, and atopic sensitization (155).

In another study demonstrating the importance of microbial exposure in early life, a birth cohort study of healthy, full-term infants were followed until the age of 6–7 years. These children had a significantly decreased risk of atopy when exposed during the first year of life to two or more dogs or cats, suggesting that local household microbial exposure in the early years of life confers protection against allergic disease development (156). Following these studies, the same group determined whether the presence of pets in the household increased the microbial communities and if this microbial material conferred protection (126). They examined the diversity of bacterial and fungal communities in dust collected from the homes with pets and observed significant enrichment of bacterial communities in the dust samples from houses with pets compared to the dust from houses without pets. A vast majority of the enriched bacteria found were also detected in the human gut microbiome, suggesting that the exposure to enriched microbial communities conferred a protective effect on the development of allergies (126, 156). Thus, it appears that multiple factors during pregnancy and early infancy impact the development of the microbiome, which can shape immune function throughout childhood.

**MICROBIAL METABOLITES AND THEIR ROLE IN RSV AND ASTHMA**

The gut microbiota is necessary for proper food digestion, and a number of metabolites are a result of digestion by these bacteria. Several studies support the concept that airway immune status is impacted by the metabolite profile produced by the gastrointestinal microbiota (128, 157, 158). The metabolites from intestinal microbiota are the result of the microbial-host interactions, which are a complex system dependent on the food intake of the host, the external environment, the use of antibiotics, and the other microbial genera occupying a person’s microbiome. Together, these interactions generate a host of metabolites that can impact the immune response systemically and locally in organs of the host, including the lung and gut (128, 157, 159).

Using animal models, the effect of the dietary fermentable fiber content has been studied in the composition of the lung and gut microbiota (157). In one study, mice were raised on a diet with standard, low or high fiber, followed by intranasal exposure to house mite extract. The mice that had low fiber diet showed increased inflammatory cell infiltration and increased Th2 cytokines in the lung, with elevated total IgE serum levels compared with the control, standard diet. The authors also observed that DC isolated from the lung of the low fiber diet mice exhibited a more active phenotype, as determined by increased CD40 and CD80 (157). Furthermore, the mice that were fed high fiber diets demonstrated reduced inflammatory cell infiltration and decreased Th2 cytokines in the lung, as well decreased levels of total IgE. The isolated lung DCs were also less activated, with reduced expression of CD40 and CD80. Finally, the authors observed that increasing the fiber in the diet modified the composition of the microbiota, with a difference in the ratio of *Firmicutes* to *Bacteroidetes*. The gut microbiota degraded the fiber and increased the concentration of systemic short-chain fatty acids (SCFAs). Increased circulating levels of SCFAs were observed in mice fed a high fiber diet, resulting in protection from allergic airway inflammation. Conversely, mice fed a low fiber diet had decreased circulating SCFAs, correlating to increased allergic disease in the lungs (157). Supplementing mice with the SCFA propionate altered hematopoiesis and the phenotype of the DCs. The authors concluded that fermentable dietary fiber and SCFAs could modify the immunological environment in the airway and influence the severity of allergic inflammation (157). In a human study, bacteria were grown from the intestinal microbiota of infants who were at high risk of atopic diseases, followed by gas-liquid chromatography to detect bacterial cellular fatty acids (158). The authors found that the bacterial cellular fatty acid profile in fecal samples was significantly different between atopic and healthy infants, and that the atopic infants had increased *Clostridium* cells in their feces compared to non-atopic, healthy infants, concluding that increased *Clostridium* numbers were associated with childhood allergic sensitization and that differences in neonatal gut microbiome and bacterial fatty acids precede the development of atopy in infants (158). These data also suggest the key role that the microbiome and their metabolites play in the development and maturation of the immune system.

We have previously shown that oral supplementation of mice with *Lactobacillus johnsonii* resulted in significantly reduced airway allergic sensitization and RSV-induced pulmonary immunopathology (127). The gut microbiota was modified considerably in the supplemented mice, but *L. johnsonii* was not detected in the airways, and furthermore, only viable *L. johnsonii* was able to generate protection (127). Following this study, we tested the metabolic potential of *L. johnsonii* for its impact on the lung immune response against RSV, and we found that *L. johnsonii* supplementation of the mice diet modified the systemic metabolic profile. In plasma samples analyzed by liquid chromatography/mass spectrometry, we were able to detect more than 50 different metabolites altered at baseline in supplemented mice (128). Following RSV infection, the plasma samples from *L. johnsonii* supplemented animals exhibited substantial metabolic reprogramming in response to RSV infection, involving significant increases in a broad range of lipid-, bile-, amino acid, and peptide-derived metabolites (128).
We also observed that RSV-infected bone-marrow derived DCs from *L. johnsonii* supplemented mice had altered cytokine secretion, reduced expression of co-stimulatory molecules, and modified CD4+ T cell cytokine production. In this study, we outlined a potential mechanism for immune alteration by manipulating the microbiome and facilitating the availability of a complex and broad profile of microbial and mammalian-derived immunomodulatory metabolites that collectively may play a regulatory role (128). This result supports the idea that the metabolic potential of the gut microbiome is substantial and extends beyond fatty acid production, and may extend to immune regulation at tissues other than the gut.

In a US birth cohort of neonates, stool samples were analyzed to identify differences in microbiota-composition and associated metabolites. The authors observed that the microbiome composition of the infants that developed multi-sensitized atopy at the age of 2 years, present significant different compositions of microbial communities than those who did not. The group of children who developed atopy showed lower relative abundance of specific bacteria (*Bifidobacterium*, *Akkermansia*, and *Faecalibacterium*) and higher relative abundance of fungi (*Candida* and *Rhodotorula*) (160). Furthermore, the authors observed a distinct set of fecal metabolites, which were tested to generate pro-inflammatory responses using sterile fecal water from the samples of the infants with higher risk of developing allergies. When adding this sterile fecal water to the growth medium of adult human peripheral T cells, the authors observed an increased proportion of CD4+ IL-4+ cells and reduced numbers of regulatory T cells (160). Interestingly, one of the metabolites identified in the fecal samples of the high-risk infants was 12,13-DiHOME, which can suppress Treg cells. Addition of 12,13-DiHOME recapitulated the effect of the fecal water on T cell culture. This suggests that the gut microbiome in infants can modify the metabolic profile and that those metabolites can potentially impact the immune response to cause the development of allergies in later life (160). This study correlates with another study in which bronchoalveolar lavage fluid (BALF) was collected from adult pollen-allergic patients with mild asthma (age 22–42 years) and healthy non-allergic people, in basal conditions or under inhaled-pollen challenged (161). 87 lipid mediators were screened, and a significant difference in lipid metabolites was observed between allergic and healthy patients, including increases in both 12,13-DiHOME and 9,10-DiHOME in the BALF of allergic patients (161). These data highlight the importance of metabolites in lung allergic disease through modulation of the immune response, and the impact of the microbiome on the metabolic profile of the host. These pioneering studies suggest a young field ripe for further scientific investigation bringing together the microbiome and its metabolites and exploring their relationship to a healthy immune response.

**CONCLUDING REMARKS**

Severe RSV infection followed by the development of allergies and asthma is determined by the interaction of environmental and inherited factors (162, 163). Nevertheless, genetics cannot explain the continued increased in allergies and asthma, and any change in population genetics would require multiple generations to occur (162). On the other hand, epigenetics could be induced more rapidly and can be generated by environmental changes. Importantly epigenetic modifications can be passed down from parents to offspring or can result from *in utero* modification (162). As we describe above, epigenetic changes can modify the immune phenotype, with alterations inherited from the mother are fivefold more significant than the paternal factors (140, 164).

The ability of epigenetic modifications to impact gene expression to regulate or dysregulate the homeostasis of the organisms has been widely studied from cancer to modulation of the immune system (165). The concept of modulation of the immune response through epigenetic modification caused by environmental factors, diet, microbiome, and metabolites may have clinical therapeutic implications for human health (165). Future studies will now focus on how the microbiome metabolite profiles impact immune responses in the long term through epigenetic alteration of not only ongoing immune responses but also progenitor cell populations. These changes would have long acting effects on future immune responses. Whether these changes can be reversed will be a significant challenge for future therapeutic progress (165).

Many known factors that predispose to develop severe RSV disease are linked, from the immune system and lung development through microbiome composition (Figure 1). In this review, we summarize the role of diverse factors that can impact the immune response during RSV infection. The further pursuit of the interactions among the environment, the microbiome, and its metabolites, and their impact on epigenetics in the context of severe RSV infection has the potential to reveal new therapeutics to alter the development of this disease and the predisposition to asthma. Ideally, immune development would be altered favorably early in childhood prior to the initiation of pathogenic immune phenotypes including the development of severe disease caused by RSV infection, and this is likely to be a future focus of many studies. The complex interactions among the numerous factors reviewed herein will likely also have far reaching effects on other aspects and types of disease.

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WF, NL, and CP conceived of and wrote the manuscript.

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